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Overwintering energetics of Lepidoptera: the effects of winter warming and thermal variability.

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A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Biology

OVERWINTERING ENERGETICS OF LEPIDOPTERA: THE EFFECTS OF WINTER WARMING AND THERMAL VARIABILITY
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Abstract and Keywords

Winter temperatures are changing rapidly, and although winter warming reduces cold stress for overwintering ectotherms, temperature-mediated increases in metabolic rate can decrease fitness in dormant insects by increasing consumption of energy reserves. Increases in thermal variability also increase energetic demands, due to non-linear thermal response curves. My objective was to quantify the negative effects of winter warming and increases in thermal variability on a range of Lepidopteran species. As overwintering insects rely on lipid catabolism, accurate lipid measurement is central to my dissertation; so I first compared four methods of lipid quantification; concluding thin layer chromatography was the only method sufficiently accurate and robust to variation in lipid composition. I then examined the physiological and life-history costs of winter warming in *Erynnis propertius* [EP], *Papilio glaucus* [PG], *P. troilus* [PT], and *Hyphantria cunea* [HC]. A simple increase in temperature caused lipid depletion in EP, but PT, PG and HC were insensitive to winter warming. In HC, this insensitivity was mediated by a plastic suppression of metabolism and a decrease in development time in the warmer winter. HC from their northern range edge had increased thermal sensitivity at the end of winter, as predicted by metabolic cold adaptation theory. In EP, I also investigated the impact of daily thermal variability on overwintering energetics, demonstrating a facultative and obligate suppression of thermal sensitivity in response to high daily thermal variability, which partially compensated for the increased energetic demands of the more variable environment. Modelling energy use with meteorological data demonstrated that phenology changes had disproportionate influence on energetics in variable environments; thus timing of entry into winter dormancy will strongly influence ectotherm fitness in temperate environments. Metabolic suppression in EP and HC are the first demonstrations of metabolic compensation in overwintering insects. Finally, I outline a framework to predict insect vulnerability to winter warming. Winter warming and increases in thermal variability may negatively impact the fitness of some overwintering insects, but diverse physiological mechanisms compensate for increased energetic demands over winter.

Keywords: Temperature, energy, triacylglyceride, lipid, insect, phenotypic plasticity, local adaptation, thermal sensitivity, metabolic compensation, metabolic cold adaptation, development time, phenology.

Co-Authorship Statement

Chapter 1 will form part of a review on the effects of climate change on overwintering insects, with Brent J. Sinclair (BJS), who contributed to the conception of many of the ideas and provided editorial comments on the writing.

Chapter 2 was published in similar form in the Journal of Insect Physiology (reprint permission in Appendix 1). I was the first author, with co-authors Raymond H. Thomas (RHT), Heath A. MacMillan (HAM), Katie E. Marshall (KEM) and BJS. RHT provided expert technical assistance with the TLC-FID; KEM provided advice on the laboratory assays and statistical analyses; HAM provided advice on the laboratory assays and helped prepare the figures, BJS provided advice on the laboratory assays, contributed to experimental design, and helped write the manuscript.

Chapter 3 is currently under review at Climate Research with Jessica J. Hellmann (JJH) and BJS, who had creative input into the conception of the work, contributed to developing the experimental design, and helped write the manuscript.

Chapter 4 is currently in preparation for submission to Functional Ecology, with Wesley D. Chick, who collected and cared for the insects, and BJS, who contributed to the conception of the study, experimental design and choice of methods, gave advice on data analysis, and helped write the manuscript.

Chapter 5 is under review in similar form at PLoS ONE, with myself as the first author and KEM, HAM, Jason D.K. Dzurisin (JDKD), JJH, and BJS as co-authors. KEM, JJH and BJS contributed to experimental design; HAM, KEM, JDKD and BJS helped perform the experiments; KEM performed the non-linear modeling of metabolic rate - temperature relationships, KEM, JDKD and HAM helped prepare the figures; KEM, JJH and BJS helped write the manuscript.

Chapter 6 will (with Chapter 1) form part of a review on the effects of climate change on overwintering insects, with BJS, who contributed to the conception of many of the ideas and provided editorial comments on the writing.

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List of Abbreviations

CE: Cholesterol ester

CHOL: Cholesterol

CO: Columbus

DAG: Diacylglyceride

DEE: Diethyl ether

DM: Dry mass

E: Energy

EP: *Erynnis propertius*

FFA: Free fatty acid

FOL: Folch reagent

FM: Fresh mass

GC: Gas chromatography

GLY: glycerol

HC: *Hyphantria cunea*

HPLC: High-performance liquid chromatography

LFDM: Lipid-free dry mass

MAG: Monoacylglyceride

OR: Oregon

OT: Ottawa

PG: *Papilio glaucus*

PT: *Papilio troilus*

RER: respiratory exchange ratio

TAG: Triacylglyceride

TAR: Triarachin

TLC-FID: Thin-layer chromatography coupled to a flame-ionisation detector

TPT: Tripalmitin

TOL: Triolein

VI: Vancouver Island

VCO_2: Rate of CO_2 emission

Chapter 1: The effects of temperature on overwintering energetics of insects

1.1 Overview of dissertation

Temperature is one of the most important abiotic factors influencing the physiology, ecology, and evolution of ectothermic organisms. Anthropogenic climate change is driving the most rapid changes in thermal conditions in human history, which are already having documented effects on biota. Insects comprise a large fraction of biodiversity and are extremely sensitive to climate change. To predict insect population dynamics in a warming world, it is essential to understand the effects of temperature changes on ectotherms, particularly in the winter, when temperatures are changing fastest and population bottlenecks can occur. In my dissertation, I explore the physiological and ecological consequences of changes in the thermal environment of overwintering Lepidoptera, as models for holometabolous insects in general. My examples throughout are biased towards Lepidoptera, with examples from other taxa where necessary. Lepidoptera are large insects (Lease and Wolf 2011) that have above-average adult dispersal ability compared to insects in general (Peterson and Denno 1998); thus generalizations from Lepidoptera to insects in general must be interpreted with care. The species I used were not chosen to make phylogenetically-based comparisons – such a goal is beyond the scope of this work. Each study system was chosen for its utility in answering a particular question, and also to represent species with a range of life-history traits.

I take an integrative approach, measuring responses at several levels of biological organisation. I particularly focus on the impact of changes in the mean and variance of temperatures over winter on energetics, with consideration of the roles of local adaptation and phenotypic plasticity in mediating these effects. As background for this work, I will briefly review the effects of changing thermal conditions on insect physiology, chiefly through the lens of energetics, and summarise the main ecological outcomes of climate change to date. I will end this introduction by outlining the structure of my dissertation.

1.2 The role of insects in ecosystems

Herbivorous insects dominate terrestrial biodiversity and play key roles in ecosystems both directly as pollinators, predators, parasites, and detritivores; and indirectly through the effects of herbivory on plant physiology, activity and population dynamics (Weisser and Siemann 2004). Within the class Insecta, Lepidoptera is among the most-studied orders due to their economic importance as pests, pollinators, and silk producers. Their visibility and long history of collections means that good historic range and phenology data are available in many areas, allowing the analysis of long-term population trends (Thomas et al. 2004, Altermatt 2010, Chen et al. 2011). Insect population dynamics can drive ecosystem carbon flux (Clark et al. 2010), and irruptive species can have very large impacts; for example, spruce budworm (*Choristoneura fumiferana*, Lepidoptera: Tortricidae) in North America regulate boreal forest dynamics with their 30-40 year outbreak cycles (reviewed in Gray 2008); and the autumnal moth (*Epirrata autumnata*; Lepidoptera: Geometridae) causes extensive damage and mortality to mountain birch forests in Fennoscandia (Jepsen et al. 2008). Decreases in abundance of pollinating insects have been causally linked to declines in the plants they pollinate (Beismeijer et al. 2006), and declines in insect populations are linked to parallel declines in aerial insectivores (Nebel et al. 2010). Thus, understanding insect population dynamics, and factors controlling these dynamics, is of great importance for understanding ecosystem function.

1.3 Predicted impacts of climate change on insects, and underlying physiological mechanisms

Over the past twenty years, global mean temperatures have risen by c. 0.5 °C, and are projected to continue to rise at an unprecedented rate (IPCC 2007a). As climate warms, isotherms are shifting polewards and upwards at a rate of approximately 17 km and 1 m, respectively, per decade (Chen et al. 2011). Evidence from the historical record and model predictions demonstrates that rates and magnitudes of change are greater in autumn and winter than in summer, and at night as opposed to during the day (IPCC 2007a, Bonsal and Kochtubjada 2009). These increases in minimum temperatures are leading to

a general decrease in annual and daily thermal variability (Easterling et al. 1997), but the frequency of extreme events such as storms, heat waves, floods and droughts (IPCC 2007b) is increasing and these events are already impacting natural systems (Easterling et al. 2000). In North America, the primary outcomes of climate change have been a decrease in snow cover, and an increase in the length of the growing season (IPCC 2007b, Jeong et al. 2010). The timing of snowmelt has shifted, resulting in increased winter runoff and decreased summer water availability (IPCC 2007b).

Temperature is one of the primary factors regulating insect physiology, as the majority of Lepidoptera are poikilotherms in most life stages: their body temperature is not kept constant within narrow limits but can range widely over tens of degrees depending on their physical environment and activity metabolism (Heinrich 1995). As body temperature influences rates of biochemical reactions, a positive exponential relationship exists between temperature and metabolic rate (Gillooly et al. 2001), and thus all aspects of growth and development. Development occurs only above a species- and life-stage-specific developmental threshold (Brier et al. 1999), thus if temperatures rise above that threshold earlier in the year, growth and development will be initiated earlier, resulting in an advancement of spring phenology. Shifts in phenology have been an ubiquitous response to climate change, not only in insects but in multiple taxa across marine, freshwater and terrestrial environments (Thackeray et al. 2010), and in some cases these phenological shifts have been causally linked to anthropogenic warming (e.g. Kearney et al. 2010). In insects, changes in phenology may occur by alterations to environmental sensing mechanisms, particularly photoperiodism (Bradshaw and Holzapfel 2008). The vast majority of spring and summer events have advanced (i.e. become earlier) in insects (Thackeray et al. 2010, Walther et al. 2002), while autumn phenology, albeit less well-studied, seems to have been delayed (i.e. become later; Bradshaw and Holzapfel 2001, Gomi et al. 2007).

The delay in autumn phenology could be explained by an increase in voltinism (generations per year). Higher temperatures during the growing season will result in faster rates of growth and development, and extensions of the growing season can mean host

plants are available longer. These factors can combine to provide species with the opportunity to complete an extra generation, resulting in individuals of the new generation being present later in the autumn (explaining the apparent delay in phenology). Increases in voltinism have been demonstrated in European butterflies and moths as a response to recent climate change (Altermatt 2010). Increases in voltinism can benefit populations in three ways: 1) by increasing the population growth rate, because the intrinsic rate of population increase is inversely related to generation time (Fenchel 1974, Chevin et al. 2010); 2) by increasing the speed of adaptation, via more opportunities for genetic recombination; and 3) by shortening the period of dormancy experienced by temperate insects, due to the new generation developing later into the autumn. In temperate climates, the last generation of the year must also reach the appropriate life stage for overwintering before the onset of adverse temperatures; thus there is a risk that, in cool years, the new generation may be unsuccessful (Gomi 2000). This cost maintains transition areas of mixed voltinism. In a warm year, individuals that develop fast and produce another generation before winter are more fit, whereas in cool years, individuals that develop slowly and enter dormancy earlier are more fit (Gomi 2000). If voltinism does not increase, such as would happen in species that lack genetic variation in voltinism, advanced spring phenology and warmer growing seasons are likely to lead to an advancement of autumn phenology, as the overwintering stage will be reached earlier.

Phenological shifts are significant because of the potential for the development of trophic mismatches that could cause serious disruption to ecosystem function (Walther 2010, Forrest and Miller-Rushing 2010). If organisms that depend on one another (e.g. an insect herbivore and its host plant) have different phenological shifts in response to climate change, an asynchrony can develop. This phenomenon has been seen in *Operophtera brumata* (Lepidoptera: Geometridae), whose emergence time has advanced more in response to climate change than has bud burst of its host, leading to the risk of starvation for larvae that hatch before buds open (van Asch et al. 2007). Rates of phenological change have differed among trophic levels. For example, within a single ecosystem, advances in tree budburst have been much slower than advances in caterpillar abundance. Similarly, moderate advances have been documented in passerine birds, but no advances

have been seen in sparrow hawks (Walther 2010). In fact, for the majority of interactions for which sufficient data exist to address the question of altered synchrony, phenological synchrony of interacting species has decreased (Visser and Both 2005). These increasing asynchronies within ecosystems are likely to disrupt ecosystem stability.

Geographic range shifts are the best documented consequences of recent climate change in insect populations (Parmesan and Yohe 2003). Range shifts can be facilitated by the enhancement of peripheral populations by warming, which then provide a source of colonisers for newly suitable habitats at higher latitudes or elevations (Thomas et al. 2001). This paradigm assumes that individuals in peripheral populations have higher fitness under warmer conditions, which is not always the case (Pelini et al. 2009). Higher fitness may result from a longer, warmer growing season (Jeong et al. 2010), with an associated increase in energy availability and possibly voltinism (most relevant if energy limitation is setting the northern range boundary; Chown and Gaston 1999); or from a release from winter cold (if limits to cold-tolerance are setting the northern range boundary; e.g. Crozier 2004). Conversely, range contraction at the trailing edge may be caused by local extinction of marginal populations, due to limits of high temperature tolerance being exceeded (Thomas et al. 2001). Shifts in isotherms have been matched by poleward and elevational range shifts in the majority of insect species for which data are available (Chen et al. 2011, Parmesan et al. 1999), some as a direct consequence of winter warming (Jepsen et al. 2008, Crozier 2003, Crozier 2004, Battisti et al. 2005, Battisti et al. 2006). Importantly, range shifts bring species into contact that have not previously coexisted, changing biotic interactions such as predation, competition, and mutualisms, and potentially disassembling community structure and disrupting ecosytem function (Thuiller 2004). In addition to temperature, range shifts may be constrained by a number of biotic factors such as mobility, resource specialisation, species interactions, and climatic variables such as precipitation; and abiotic factors such as habitat loss and fragmentation, and topography. For example, as mobility and generation times differ between insect herbivores and their host plants, range shifts of herbivorous insects may be constrained by the absence of suitable host plant outside the current range, although host plant switching has been demonstrated in response to climate change (Thomas et al.

2001). If species cannot shift their ranges to track their optimal temperatures, they are faced with two options: adapt *in situ* or suffer population declines and eventual extinction.

There is a great deal of interspecific variability in insect responses to climate change. Life-history and demographic traits such as dispersal, resource specialisation, overwintering stage and geographic distribution mediate the effects of climate change. British butterflies with low dispersal and specialized habitat have experienced greater population declines than have generalist species occupying the same habitats, leading to the conclusion that future communities will be dominated by mobile and widespread habitat generalists (Warren et al. 2001). Advances in phenology have been more pronounced in species of butterflies that require very specific resources, and those that overwinter as adults as opposed to larvae or pupae (Diamond et al. 2011). Geographic distribution affects vulnerability as local climates vary in their proximity to organisms' thermal limits: despite a smaller relative degree of predicted warming, tropical organisms are predicted to be more at risk of negative consequences of climate change than their temperate congeners, due to the proximity of their operative temperatures to upper thermal limits (Walther et al. 2002, Deutsch et al. 2008). Conversely, species whose ranges are currently limited by winter cold temperatures will likely benefit from climate warming (Jepsen et al. 2008, Crozier 2004).

This interspecific variation has resulted in a dichotomy in the effects of climate change on species abundance. Some insect species have benefitted from climate warming, and outbreaks of pest species in particular have increased (Gray 2008, Jepsen et al. 2008, Logan et al. 2003), and should continue to increase (IPCC 2007b). However, the majority of non-pest insect taxa for which data are available have exhibited population reductions during the past few decades (Thomas et al. 2004, Beismeijer et al. 2006, Conrad et al. 2006, van Dyck et al. 2009), and insect biodiversity appears to be in general decline. Although I have confined my discussion here to insects, the major classes of response to climate change (range shifts, population fluctuations, and phenological shifts) have been documented in the majority of studied taxa across marine, terrestrial, and freshwater environments (Chen et al. 2011, Parmesan and Yohe 2003, Root et al. 2003, Thackeray et

al. 2010); however, there is evidence that insects are particularly sensitive, because they have experienced faster rates of decline than vascular plants or birds over the same time period (Thomas et al. 2004, Conrad et al. 2006).

Many of the previously-mentioned studies have attributed changes to warming during the growing season, despite the greater magnitude of changes in winter. The impact of changes in temperature during the overwintering period have not been well characterized, and in the light of climate change predictions of winter warming in coming years, together with existing evidence that winter warming can have important effects on insects, it is essential to develop an understanding of the impacts of changing thermal environments during the winter. Because life history is so closely tied to overwintering, I will first give an overview of some aspects of insect life histories, particularly as they pertain to energetics.

1.4 Thermal control of lepidopteran life cycles

Holometabolous insects like Lepidoptera have four life stages - egg, larva, pupa and adult, and their rate of passage through these life stages is controlled predominantly by temperature. Eggs hatch after accumulating sufficient thermal units above their developmental threshold. Larvae grow and moult through a succession of instars, at a rate determined jointly by their body temperatures, and their thermal sensitivity of growth and development, until they pupate or moult into an adult (Nijhout 1975, Davidowitz and Nijhout 2004). During the summer growing season, the total amount of energy used to complete a set developmental stage remains constant regardless of temperature (Gray 1994), as increased metabolic demand (growth) is matched by decreased duration of each life-stage (development), although different thermal sensitivities of growth and development can result in size differences among individuals reared at different temperatures (Kingsolver et al. 2009, Forster et al. 2011).

For holometabolous insects, transitions between life stages are programmed and irreversible, and the life stages can differ markedly in their ecology (Boggs 2009). Larvae and adults of Lepidoptera typically have very different diets; most lepidopteran larvae

feed on plants that contain both carbohydrates and diverse nitrogenous compounds, while adults feed only on nectar, which has abundant dilute carbohydrates but relatively few sources of amino acids (Boggs 2009). Thus essential amino acids from larvally-derived resources are necessary for reproduction, and must be conserved to adulthood (O'Brien et al. 2002). Indeed, in some extreme cases adults do not feed at all (e.g., many moths; Boggs 2009), and reproductive and somatic resources must be supplied wholly from larval energy reserves. The duration of the life cycle varies among species, ranging from semivoltine species (multiple years are required to complete a generation), through univoltine (one generation per year) to multivoltine (many generations per year) species.

The insect life cycle may be continuous, or may be interrupted by a period of dormancy which occurs at a species specific life-stage (Koštál 2006). Periods of dormancy (diapause or quiescence) may occur during any life-stage (though for any given species that stage usually remains constant), may be hibernal or aestival, and may occur either in response to (quiescence) or in preparation for (diapause) adverse environmental conditions, usually cold or aridity (Koštál 2006). Here, I will consider only hibernal dormancy. These periods of dormancy can start well in advance of the winter solstice, and can extend beyond the vernal equinox; thus throughout this dissertation, I will refer to the period of dormancy as the overwintering period.

1.5 **Physiology of diapause**

Although diapause is characterized by the cessation of activity, it is not a state of suspended animation but a dynamic succession of physiological events that proceeds through a series of stages: induction, preparation, initiation, maintenance, termination, and post-diapause quiescence (Koštál 2006). In facultative diapause, common among insects, induction occurs in response to photoperiod declining below a genotype-specific critical threshold during a sensitive life stage (Bradshaw and Holzapfel 2008), and once the diapause program has been initiated, it will continue regardless of environmental conditions. Less commonly, diapause may be programmed ontogenetically, and in these species with an obligate diapause, it will be expressed during each life cycle regardless of the environmental conditions. The preparation phase may involve behaviours such as

migration to appropriate microhabitats, aggregation, or additional feeding to build energy reserves for the winter (Koštál 2006). The initiation phase begins when direct development ceases, and sometimes involves molting into a characteristic form, pupation, or formation of a cocoon (Koštál 2006). During the initiation phase, cold hardiness may increase, and metabolic rate becomes increasingly suppressed. The maintenance phase is characterized by continuing suppression of metabolic rate, enhanced cold-hardiness, and refractoriness to permissive temperatures until a chilling or time threshold has been crossed (Koštál 2006). During this phase of continued metabolic suppression, most insects catabolise predominantly lipid, with species-specific contributions from carbohydrate and amino acids such as proline (Hahn and Denlinger 2011). The animal's responsiveness to environmental stimuli gradually increases throughout the maintenance period. A negatively-temperature dependent removal of an inhibitory substance has been proposed to explain the gradual increase in responsiveness to stimuli; a model which accurately predicts the complicated dynamics of diapause termination in gypsy moth eggs (*Lymantria dispar*, Lepidoptera, Lymantriidae; Gray et al. 2001). In support of this model, warm winter temperatures slow the development of diapause and delay the onset of pupation for other insects (e.g. Goettel and Philogene 1980, Bosch and Kemp 2004). Conversely, warm winter conditions can also speed development (Irwin et al. 2001, Williams et al. 2003), perhaps by promoting increased secretion of a developmental promoter (Denlinger et al. 2005).The termination phase of diapause may overlap or be concordant with the maintenance phase, and is characterized by the decrease of diapause intensity to a minimal level. By the end of the termination phase, direct development is possible, if environmental conditions are permissive (Koštál 2006). However, if environmental conditions remain unfavourable, insects may remain in a state of post-diapause quiescence, whereby development and metabolism are suppressed exogenously until temperatures increase in the spring.

The mechanisms underlying metabolic rate suppression during diapause are difficult to separate from those underlying diapause itself, as the two always occur in concert. The endogenous developmental arrest and cessation of activity are mediated hormonally, and governed by a diversity of regulatory mechanisms across species and even sexes, which

may involve a developmental inhibitor (e.g. ecdysteroids, sorbitol or heat shock proteins), or the lack of a developmental stimulator (e.g. prothoracicotropic hormone, juvenile hormone, or cyclic AMP; Denlinger et al. 2005). Although the cessation of energetically expensive activities, such as growth and development, account for some of the metabolic suppression observed in diapausing insects, intrinsic mechanisms for attaining such a hypometabolic state are likely also at play (Storey and Storey 2004). Degradation of mitochondria has been demonstrated in concert with a decrease in oxidative metabolism in a diapausing caterpillar (Kukal et al. 1989), but a causal link was not established. In vertebrates, hypometabolism occurs due to a global suppression of protein synthesis (apart from molecular chaperones and antioxidants that protect and stabilise macromolecular structures), gene transcription, and ATP-dependant ion pumps (e.g. McMullen and Storey 2008; McMullen et al. 2010), which is frequently mediated by reversible protein phosphorylation of metabolic enzymes and/or ion pumps (Storey and Storey 2004), but these mechanisms have not yet been explored in diapausing insects. An additional mechanism for global control of metabolism is gaining increasing attention: recent work suggests that, at least in vertebrates, transitions into hypometabolic states may be coordinated by microRNAs; short, non-coding RNAs that induce mRNA degradation or decrease transcription rate (e.g. Morin et al. 2008; Biggar et al. 2009). Increased levels of microRNAs can arrest cell growth and are associated with metabolic suppression in overwintering wood frogs (reviewed in Biggar and Storey 2011), and distinct diapause-related microRNA expression profiles have been described in the nematode, *Caenorhabditis elegans* (Karp et al. 2011). MicroRNAs are ubiquitous and abundant in insect species examined so far (Jia et al. 2010), and expression patterns of microRNAs change over development in *B. mori* (Huang et al. 2010). Nothing is known about the role of microRNAs in insect diapause, but they deserve attention due to their potential to coordinate the cascade of signalling events leading to the global suppression of metabolism. In short, although much is known about the physiological, hormonal, and molecular bases of diapause, little is known about the biochemical and molecular mechanisms governing metabolic suppression in diapausing insects.

1.6 Energetic aspects of insect overwintering

The overwintering period is a strong determinant of fitness in temperate insects through both lethal and sub-lethal effects (Leather et al. 1995, Bergland et al. 2005), and can cause a population bottleneck (Roy and Sparks 2003). The challenges of winter fall under two (interacting) main categories; those associated with cold temperatures (reviewed by Sinclair et al. 2003), and those associated with energy limitation. The intuitive outcome of winter warming is a release from cold-induced mortality, and indeed that is an important factor mediating the effects of climate change on some insects (Jepsen et al. 2008, Crozier 2004, Battisti et al. 2005). However, due to long periods of dormancy with no opportunity to feed, winter can also be a time of energy shortage, which will become more pronounced as winters warm.

Insects usually do not feed while overwintering, and must rely on stored energy reserves not only to survive the winter, but in many cases to also fuel subsequent metamorphosis and reproduction (Leather et al. 1995). Energy reserves generally decline during dormancy and energetic depletion during winter can cause mortality (Hahn and Denlinger 2011). Energy reserves are positively correlated with adult size in Lepidoptera (Lease and Wolf 2011), and increased size leads to higher fecundity (Oberhauser 1997, Berger et al. 2008), overwintering survival (Bosch and Kemp 2004), and starvation resistance (Stockhoff 1991, Chippendale et al. 1996). Thus, depletion of energy reserves can reduce insect fitness through decreases in both survival (Bosch and Kemp 2004, Koštál et al. 2011), and reproductive capacity (Irwin and Lee 2000, Irwin and Lee2003).

During winter diapause, development becomes decoupled from metabolism such that, although increases in temperature still cause an increase in metabolic rate (Gray 1994, Irwin and Lee 2002; Chapters 4-5 of this dissertation), this increase no longer fuels development (as no development can occur while insects are in the early stages of diapause). Therefore any increase in temperature over winter will increase the depletion of stored energy reserves, and may simultaneously increase development time (due to a slower progression of diapause; Gray et al. 2001), compounding energy drain. This energy drain caused by winter warming can lead to increased mortality (Williams et al.

2003, Mercader and Scriber 2008, Koštál et al. 2011, Sorvari et al. 2011), or reduced fecundity (Williams et al. 2003, Irwin and Lee 2003).

Energetic drain in response to winter warming is not universal, however, and even closely-related species may show differential responses to winter warming. For example, a cold-adapted species of rose-galling wasp, *Diplolepis spinosa* (Hymenoptera: Cynipidae) had decreased survival and fecundity at high overwintering temperatures, while a congener (*Diplolepis variabilis*) from a generally warmer habitat was not affected by warm winter temperatures (Williams et al. 2003). The cold-adapted *Papilio canadensis* (Lepidoptera: Papilionidae) showed decreased survival and increased mass loss in response to warming in the autumn, and increased mass loss in response to warmer overwintering temperatures (Mercader and Scriber 2008). *P. glaucus*, a closely-related warm-adapted species, showed no negative effects of warming at any time in the overwintering period (Mercader and Scriber 2008). Thus adaptation to warm overwintering conditions seems possible, perhaps through increased reserve storage or metabolic compensation (Williams et al. 2003, Mercader and Scriber 2008). The lack of a comprehensive framework to address likely vulnerabilities to winter warming based on species traits means that more empirical data on the effects of winter warming on species with a range of life-history and demographic traits are required.

1.7 Local adaptation and phenotypic plasticity

Three mechanisms enable a population to persist when its environment changes: 1) range shifts to track its preferred environment; 2) genetic adaptation to the new environment; or 3) phenotypic plasticity. Increasing habitat fragmentation and anthropogenic barriers to dispersal means that population persistence may increasingly depend on local adaptation and phenotypic plasticity to produce phenotypes that can persist in the new thermal environment. I will briefly review the theories of local adaptation and phenotypic plasticity, how each may be detected and what potential influence they have, and how equilibrium between the two processes is maintained.

1.7.1 **Local Adaptation**

The outcome of Darwinian natural selection is most apparent at higher taxonomic levels, particularly in adaptive radiations of species (Schluter 2000), but it also occurs on a smaller scale, in the adaptive divergence among populations of a single species. Heterogeneous thermal environments result in time- and location- specific selection pressures, which can lead to genetic differentiation among populations and thus increased fitness of each population in the natal environment, compared to non-adapted genotypes. Local adaptation may occur through the genetic adaptation of physiological, biochemical or behavioural traits (Hochachka and Somero 2002, Angilletta 2009), which combine to determine fitness (an index of the propensity of the average individual with a given genotype to pass on their genes to the next generation; Maynard-Smith 1989). Thus, fitness encompasses both viability and fecundity.

Local adaptation may be assessed by reciprocal-transplant studies, wherein individuals are transplanted among environments and fitness is assessed. Alternatively, if local adaptation to only one abiotic variable is of interest, individuals from different populations may be exposed to conditions designed to replicate population-specific levels of that variable in the lab, and fitness (or correlates thereof) is assessed (a reciprocal common garden experiment). If local adaptation has a cost (i.e. it reduces fitness in other environments), local adaptation will manifest as a genotype by environment (G × E) interaction, such that the fitness of a genotype depends on its environment, and there is a home-site advantage (Kawecki and Ebert 2004, Hereford 2009). To detect local adaptation, the key comparison is between natal vs. non-natal genotypes, whereby within each environment, the natal genotype is more fit than the non-natal genotype. This definition avoids confounding local adaptation with environmental quality (Kawecki and Ebert 2004).

Local adaptation is widespread, and has substantial implications for population responses to climate change. Latitudinal clines in heat tolerance and critical photoperiod for diapause induction have developed in natural populations of insects as a result of local adaptation (Bradshaw and Holzapfel 2008). Among published reciprocal transplant

studies, local adaptation is very common (estimated frequency of 0.71), and the magnitude of the fitness benefits is large: native genotypes perform 45% better compared to non-native populations in a given environment (Hereford 2009). Thus it seems that local adaptation of populations to their environments is more the rule than the exception, and may in some cases make poleward range expansions less likely, as locally adapted peripheral populations may not be enhanced by warming (Pelini et al. 2009). Local adaptation of overwintering energy metabolism (Williams et al. 2003, Mercader and Scriber 2008; Pelini et al. 2009) would also counter the enhancement of peripheral populations under climate change. Of course, if local adaptation can be reversed at a rate greater than or equal to the rate at which climate changes, it will not be an impediment to range expansions under climate change. Although insects have shown rapid evolutionary change in response to climate change, it has not been sufficient to prevent population extinctions (Parmesan 2006). Since high levels of gene flow generally swamp local adaptation and homogenise populations (Kirkpatrick and Barton 1997), highly locally-adapted species will also tend to be more genetically isolated, potentially reducing the pool of standing genetic variation from which new mutations or combinations of genes may arise. This could render them unable to match, in terms of evolutionary adaptation, the fast rates of change being imposed by recent climate change (IPCC 2007a); introducing a lag which will be more pronounced in species with longer generation times (as occurs in more poleward populations). Although empirical data on this issue are lacking, there is certainly the potential for local adaptation to preclude simple species range shifts in response to climate change.

1.7.2 **Phenotypic plasticity**

An alternative mechanism for minimising the negative effects of environmental fluctuations without genetic change is phenotypic plasticity. Phenotypic plasticity refers to the expression of different phenotypes by a single genotype, depending on the environment (Miner et al. 2005). Phenotypic plasticity can occur over a range of timescales; from developmental acclimation to rapid hardening responses (Hochachka and Somero 2002). However, for the purposes of this review, I will confine my discussion to acclimation (in the laboratory) and acclimatization (in the field) responses, which are

reversible changes to a physiological trait which occur over days to weeks, in response to, or in preparation for, changing environmental conditions (Angilletta et al. 2006, Chown and Terblanche 2007).

In the reciprocal-transplant or common garden design, plasticity manifests as a significant effect of environment, whereby the initial trait value is assumed to be the value expressed by each genotype at home, and any change expressed in the reciprocal environment is assumed to be due to plasticity (note that to assess plasticity, only one population is required and the environments do not have to represent the natal environments of any population). The adaptive nature of changes in trait values must be estimated through fitness or putative correlates thereof. However, a complication arises in that the null hypothesis for change in phenotypic traits in response to environmental change is often not zero, but must instead be based on knowledge of the acute trait response to environmental change (Schulte et al. 2011). For example, if growth and development rates increase with temperature, the null hypothesis would be that a set developmental stage will be reached earlier under warmer temperatures. Alternative null hypotheses must be taken into account when interpreting plastic responses to temperature, as no change in a trait value may represent a plastic response (such as a reduction in the thermal sensitivity of development rate) which functions to maintain homeostasis (Schulte et al. 2011). Genetic differences in the degree of plasticity manifest themselves in a G × E interaction, but in this case the key comparison is not between natal vs. non-natal populations in one environment, but instead between each genotype "at home" vs. "away". This genetic modification of plasticity, in fact, represents local adaptation of plasticity itself.

1.7.3 **Equilibrium between local adaptation and phenotypic plasticity**

While locally-adapted species perform well in their natal environment, phenotypically-plastic species can (theoretically) perform well in any environment. Despite this apparent benefit, plasticity is far from universal (Hereford 2009). Equilibrium between locally-adapted and plastic strategies may be maintained by costs of plasticity (Chevin et al. 2010, Auld et al. 2010), i.e. a plastic phenotype has reduced fitness compared to locally

adapted specialists in any given environment. This may result from costs of being plastic *per se*, i.e. maintenance costs of the sensory or regulatory systems that sense and respond to the environment, or production costs of expressing a new phenotype; or it may be a consequence of expressing the wrong phenotype in a given environment, due to time lags in mounting the phenotypic response or inaccurate sensing of environmental conditions (Auld et al. 2010). Although costs to plasticity are intuitive, and theoretically predicted to strongly impact the evolution of plasticity, empirical evidence for specific costs of plasticity is scarce (Chevin et al. 2010, Auld et al. 2010). The optimal degree of plasticity will also be determined by environmental heterogeneity (provided the time-scale of thermal flucuations allows matching of the plastic response to the environment) and migration (with higher levels favouring increased plasticity; Auld et al. 2010, Sultan and Spencer 2002).

Phenotypic plasticity can reduce the strength of selection on a trait, but can also facilitate adaptation by permitting populations to persist for long enough to undergo evolutionary change (Forrest and Miller-Rushing 2010, Chevin et al. 2010). As the climate warms, locally-adapted species may experience declines as they become less fit, while phenotypically plastic species may be favoured, and may come to dominate communities (e.g. Warren et al. 2001). Local adaptation and phenotypic plasticity are thus important modifiers of insect responses to temperature, and must be incorporated into any models predicting the responses of insects to climate change. It is also important to note that the raw material available to evolution is variation in individual traits, and thus a whole organism strategy may consist of a combination of plasticity in some traits, and local adaptation in others. It has been suggested that focusing on the fitness consequences of changes in individual traits is the most direct route to understanding the evolution of plasticity, to separate out the effects of the environment on overall quality of individuals from possible adaptive changes in individual traits (Woods and Harrison 2002). A mechanistic focus on individual traits should thus be combined with some tests of the adaptive values of those traits (Woods and Harrison 2002). I additionally propose that consideration of interacting selective pressures on different traits, life-stages, and genetic backgrounds, will illustrate the trade-offs that occur during adaptation.

1.8 Evolution of thermal sensitivity

1.8.1 Thermal performance curves

A thermal performance curve is a function describing the temperature-dependence of some aspect of an ectotherm's performance (Huey and Stevenson 1979). A typical ectotherm's metabolic rate accelerates from a minimum life-supporting metabolic rate (Makarieva et al. 2006) to an inflection point well below the thermal optimum then decelerates until the assymptote is reached at the thermal optimum, after which it declines precipitously towards zero, until death from heat stress occurs. Due to common biochemical mechanisms underlying metabolic rate and other performance measures, the same asymmetrical shape is found for whole-organism thermal performance curves (Huey and Stevenson 1979). The slope, or derivative, of any section of this curve describes the thermal sensitivity of the organism in question over that range of temperatures (Angilletta 2006).

1.8.2 Thermal specialist - generalist trade-offs

Thermal sensitivity modulates the impact of the thermal environment on an organism's physiological function, and is, at least in some cases, evolutionarily labile (Angilletta et al. 2002). At one end of the spectrum, stenothermal organisms (thermal specialists) are extremely sensitive to temperature, and thus have a relatively narrow curve with a high peak, while eurythermal organisms (thermal generalists) can tolerate a wide range of temperatures. Inter- and intra-specific differences in thermal sensitivity may result from qualitative (e.g. Kawall et al. 2002) or quantitative (e.g. Schulte et al. 2000) differences of enzymes in key metabolic pathways, or from modulations to the membrane environment in which many metabolic enzymes reside (Hochachka and Somero 2002). The concept of a trade-off between performance breadth and maximal performance is a common assumption of evolutionary theory (reviewed by Angilletta 2009), in that specialists are assumed to have better fitness at their optimal temperature than generalists at the same optimal temperature. Mathematically, this trade-off is imposed by holding the area under the performance curve constant. This parallels the costs of plasticity discussed in Section 1.8.2, and again this trade-off is not empirically well-supported (reviewed by Angilletta et

al. 2002). Models with strong evolutionary trade-offs between performance breadth and optimal performance incorporated predict that environmental heterogeneity among, but not within, generations will promote increased performance breadth (e.g. Lynch and Gabriel 1987, Gilchrist 1995). At the other extreme, if there were no trade-offs between maximal performance and performance breadth, one would expect to see a gradual increase in performance at all temperatures until equal to performance at the thermal optimum; i.e. thermal sensitivity was zero. This is not generally the case, as most ectotherms exhibit some degree of thermal sensitivity, even in relatively insensitive life stages (e.g. Gray 1994, Irwin and Lee 2002; Chapters 3-5 of this dissertation).

As the environment changes, the mean thermal optima within a population will begin to lag behind mean environmental temperatures. The probability of population extinction will thus be determined jointly by the degree of lag between the thermal optimum and mean environmental temperature which a population can tolerate, and the speed at which the mean thermal optimum of a population evolves (Lynch and Lande 1993, Chevin et al. 2010, Kingsolver 2009). A wide performance breadth (i.e. decreased thermal sensitivity) can increase the tolerance of deviation from the thermal optimum, and is therefore theoretically predicted to lessen the strength of selection on thermal optima, potentially slowing the speed of evolution in thermal physiology and preventing organisms from tracking changing environmental conditions, imposing an addition constraint on the evolution of thermal sensitivity (Huey and Kingsolver 1993). Moderate performance breadths are thus predicted to maximize fitness in variable environments (Chevin et al. 2010, Huey and Kingsolver 1993). I will therefore assume that weak-moderate evolutionary trade-offs constrain the evolution of thermal sensitivity; in which case environmental heterogeneity both within and among generations is predicted to decrease thermal sensitivity (Gilchrist 1995, Angilletta et al. 2002, Lynch and Gabriel 1987).

The nonlinear relationship between temperature and metabolic rate produces inequalities at fluctuating compared to constant temperatures, which could drive a decrease in thermal sensitivity in organisms exposed to variable compared to relatively stable temperatures. Jensen's inequality states that for an accelerating function (e.g. Figure 1A), increasing

variability (Figure 1B) will increase the mean of the response variable (Figure 1C), while for a decelerating function, the converse is true (Ruel and Ayres 1999). The effect of thermal variability will therefore depend on both the position of the temperatures on the performance curve of the individual, and on the magnitude of curvature of the rate-temperature function over those temperatures (Pazstor et al. 2000; Figure 1D). Fluctuating temperatures can modify rates of growth, development, performance, and population increase of insects compared to constant temperatures of the same mean (e.g. Folguera et al. 2008, Ragland and Kingsolver 2008, Kingsolver et al. 2009, Folguera et al. 2009, Paaijmans et al. 2010, Estay et al. 2011, Folguera et al. 2011). These effects can theoretically drive decreases in thermal sensitivity in organisms that experience variable compared to stable thermal environments (Figure 1D), although empirical data show mixed support for population divergence in thermal sensitivity, with some studies finding population differentiation (e.g. Ragland and Kingsolver 2008), while others find no divergence in thermal sensitivity among populations (e.g. Kingsolver et al. 2009).

The timescale of variability is an important determinant of the types of responses available to organisms. Temperature changes over geological, climatic, seasonal and daily cycles. The timescale of variability affects the type of responses which are available to organisms, due to different mechanistic bases of responses over different timescales (Schulte et al. 2011). Evolutionary or acclimatory responses take place over periods of months to days, and are thus available as responses to thermal change over time-sclaes greater than the generation time of the organism in question. However, daily thermal cycles pose a special type of challenge. Although rapid cold-hardening responses (Lee et al. 1987) modify cold or heat tolerance over a daily time scale (Worland and Convey 2001, Kelty 2007), shifts in thermal optima have not been demonstrated on a daily scale. Thus, modification of thermal sensitivity represents the only avenue for (non-thermoregulating, ectothermic) organisms to modulate the impact of daily thermal cycles on their physiology. Daily thermal cycles are therefore a powerful potential inducer of decreases in thermal sensitivity, although increased daily thermal amplitude has not been found to alter thermal sensitivity in larvae of *Pieris rapae* (Kingsolver et al. 2009) or tadpoles of *Limnodynastes peronii* (Niehaus et al. 2011). However, both of these studies

were performed on growing, feeding animals, for whom energy is not limiting, thus the animals may not have been under pressure to reduce metabolic expenditure by lowering thermal sensitivity. Overwintering insects are particularly vulnerable to energetic drain caused by increased metabolic rate over winter (Chapter 1.7), and thus are likely to be strongly selected to reduce thermal sensitivity in response to increased daily thermal variability. A reduction of thermal sensitivity is commonly associated with diapause (Chapter 1.6), and thus overwintering insects represent an excellent system in which to test the hypothesis of a decrease in thermal sensitivity in response to daily thermal cycles.

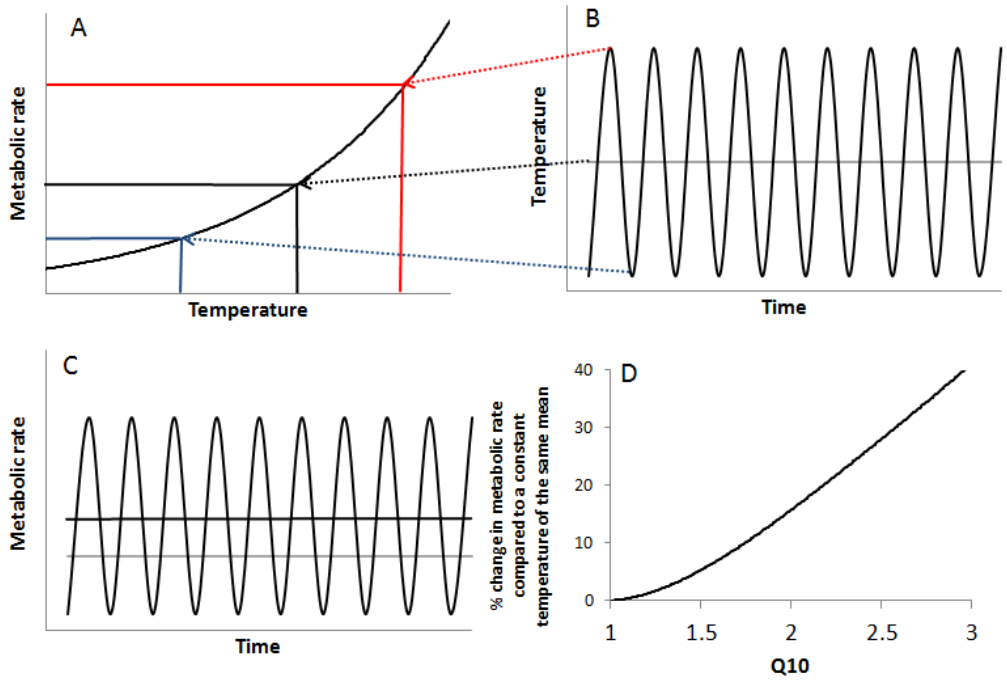

Figure 1.1 - The effects of Jensen's inequality: the mechanism by which thermal variability increases metabolic rate. (A) The accelerating portion of a hypothetical metabolic rate - temperature curve. (B) shows a constant and a fluctuating temperature regime, each with the same mean. The high (red arrow and lines) and low (blue arrow and lines) points of the fluctuating thermal regime are marked on the metabolic rate- temperature curve in (A), illustrating that although the high and low points are equidistant from the constant temperature (black arrow and lines) on the x-axis, the acceleration of the curve means that metabolic rate increases disproportionately at high temperatures (Jensen's inequality), compared to the reduction during low temperatures. (C) Jensen's inequality explains why the mean value of metabolic rate at fluctuating temperatures (horizontal black line) is higher than the mean value of metabolic rate at constant temperatures (horizontal grey line). (D) When thermal variability is held constant at that of the fluctuating regime in (B), decreased thermal sensitivity (here, Q_{10}), lessens the increase in metabolic rate caused by thermal variability (i.e. the difference between the two horizontal lines in [C]).

1.9 Ecophysiological models

A rapidly changing climate is bringing a sense of urgency to efforts to predict future species distributions. There are two main modelling approaches to predicting future species distributions: bioclimatic envelope models and mechanistic (or eco-physiological) models. Bioclimatic envelope models use a correlative approach, wherein historic species distribution records and climatic data for those locations are combined to infer abiotic correlates of the realised niche of a species (Chevin et al. 2010), which are then overlain onto simulated future climates to predict range shifts and extinctions resulting from climate change (Jackson et al. 2009). Although these models have been successfully applied in a wide variety of contexts, including understanding invasions, predicting glacial refugia, and identifying conservation hotspots, there are growing concerns about their validity in a dynamic context such as that provided by climate change (Buckley et al. 2010). This concern arises from an underlying assumption of such models: that the processes setting range limits will remain fixed in space and time. This will likely be violated in two ways as climate changes: correlations between environmental variables will likely not remain constant (IPCC 2007a, Bonsal and Kochtubjada 2009), and species are likely to show evolutionary adaptation which may modify their responses to future climates (Bradshaw and Holzapfel 2001). In addition, correlative models are only feasible for species for which there are long-term distribution records.

Eco-physiological models present a promising alternative to correlative models (Kearney and Porter 2009). Eco-physiological models use data on physiological tolerances, combined with climate or microclimate data, to predict where species will occur in the future (Buckley et al. 2010, Crozier and Dwyer 2006). A main strength of these models is their ability to accommodate evolutionary adaptation and changes in phenotypic plasticity into their predictions of species persistence. Ecophysiological models can predict species ranges with similar accuracy to correlative models in species for which both types of models can be parameterised, although species interactions such as competition cannot be accounted for by these models which tend to thus over-predict species distributions (Buckley et al. 2010). A further drawback to these models is the necessity for detailed

information on both the physiology of the focal species and the microclimate conditions experienced by that species. To that end, it is important to gather data on the physiological responses of species to changes in their thermal environment, and the intra-specific variability of those responses, and to incorporate ecologically-relevant microclimate temperatures into models where possible (Buckley et al. 2010).

1.10 **Summary**

Insects are drivers of ecosystem function and are highly sensitive to temperature, thus they are an important mediator of climate change effects on global ecosystems. Insect populations have exhibited marked responses to recent climate change, including range shifts, phenological shifts, and changes in population abundance; which herald disruptions to the synchrony and composition of ecosystems. The overwintering period is a stressful and energy-demanding portion of a temperate insect's lifecycle, and the predicted rise of winter temperatures could place increased energetic demands on overwintering insects that may reduce fitness and increase the likelihood of population declines. Local adaptation and phenotypic plasticity are potential mechanisms by which overwintering insects could mitigate the negative impacts of winter warming (through metabolic suppression or a decrease in thermal sensitivity), yet the extent to which these processes occur in insect populations is largely unknown. A clear understanding of the effects of changes in the thermal overwintering environment on insect physiology, with an emphasis on the mechanisms, is thus essential to predict future responses to climate change. It is also vital to assess intra-specific variation in overwintering energetics, and the differential responses of populations to thermal change.

1.11 **Objectives and hypotheses**

My first objective wass to find an accurate and reasonably high-throughput method for quantifying storage lipid in insects, as the accurate quantification of lipids is central to my thesis. I then quantified the negative effects of winter warming on a range of species of Lepidoptera with diverse life-history characteristics. Although lethal and sub-lethal effects of winter warming on insects have been documented (Irwin and Lee 2003,

Williams et al. 2003, Bosch and Kemp 2004, Mercader and Scriber 2008, Sorvari et al. 2011, Koštál et al. 2011), it is not known how universal these negative effects may be, nor what physiological mechanisms insects may use to combat these negative effects. I therefore investigated the physiological and life-history changes that occur as a response to winter warming in two populations of *Hyphantria cunea*, a highly successful pest species, whose distribution spans a wide range of climatic zones. I hypothesised that *H. cunea* has multiple mechanisms for dealing with changes in the thermal environment, which may include metabolic suppression in response to increased temperatures over winter. Using two populations allowed me to investigate the extent to which local adaptation and phenotypic plasticity in physiological and life-history traits can buffer organisms from changes in their thermal environment over winter, in the light of theoretical predictions based on life-history and demographic species traits. Finally, I investigated the impact of daily thermal variability on the overwintering energetics of *Erynnis propertius*, with the hypothesis that increased daily thermal variability will elicit a decrease in thermal sensitivity of metabolic rate, which will compensate for the energetic demands of a variable thermal environment. I further hypothesised that populations originating from that environment may be locally adapted to high daily thermal variability, which could manifest in an obligate suppression of thermal sensitivity.

1.12 **Structure of dissertation**

Chapter 2 consists of a validation of methods to measure storage lipids in insects. I then present data on the effects of winter warming on three butterfly species; *Papilio troilus*, *P. glaucus*, and *Erynnis propertius* (Chapter 3), before moving on to an investigation of local adaptation and plasticity of overwintering energy use in response to winter warming in *H. cunea* in Chapter 4. Chapter 5 centres on the impacts of daily thermal variability on the thermal sensitivity of metabolism in *E. propertius*. I end by synthesising the results of these studies into a framework of the impact of temperature changes on the overwintering energetics of insects (Chapter 6).

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Chapter 2: Triacylglyceride measurement in small quantities of homogenised insect tissue: comparisons and caveats.

I validate a thin-layer chromatography technique for accuracy in quantifying triacylglycerides in small quantities of homogenised insect tissue, and compare three common high-throughput methods of lipid quantification against thin-layer chromatography, in order to determine if any of the high-throughput methods are suitable for testing the hypothesis that winter warming or increases in thermal variability will cause a depletion of storage lipids in overwintering Lepidoptera. This paper was published in similar form in the Journal of Insect Physiology.

2.1 Introduction

Triacylglycerides (TAGs) are the dominant energy reserve in most insects (Bell and Coleman, 1980), and the storage, mobilization, and metabolism of fat stores in insects have been the subject of substantial investigation (Fast, 1964; Gilby, 1965; Fast, 1970; Downer and Matthews, 1976, Beenakkers et al., 1985, Arrese and Soulages, 2010). Lipid stores are key fitness determinants in overwintering insects, especially those relying on lipid catabolism to meet metabolic demands and to fuel metamorphosis and reproduction in the spring (Hahn and Denlinger, 2011), and are widely used as indirect measures of insect performance and fitness (e.g. Otronen, 1995; Ojeda-Avila et al., 2003; Hahn, 2005; Huho et al., 2007; Bosch et al., 2010).

Triacylglycerides are comprised of a trihydric alcohol glycerol esterified with three long chain fatty acids (acyl chains). Hydrocarbon chain length, number and position of double bonds, and relative molecular mass of the acyl chains will all influence the polarity and chemical reactivity of TAG molecules (Nikolova-Damyanova, 1999), which can lead to systematic biases in some methods of quantification. The presence of other neutral lipids with similar chemical properties but different functional significance (e.g. mono- and di-acylglycerides) makes the development of a specific TAG assay difficult, particularly when multiple tissue types are present in a sample.

Small body size and a diffuse fat storage organ (the fat body) also complicate lipid analysis in insects and make techniques such as condition indices or fat scoring (Krementz and Pendelton, 1990), magnetic resonance imaging (McGuire and Guglielmo, 2010), or dual energy x-ray absorptiometry (Stevenson and van Tets, 2008) less accessible than they are to researchers working on larger animals such as birds or mammals (but see Bosch et al., 2010). In addition, storage lipids are not the only analyte of interest in many studies, and in order to measure protein, RNA, DNA, or other metabolites (as will be required in this dissertation) it may be necessary to homogenise and aliquot small tissue samples, prohibiting the dissection of specific tissues. Chromatographic methods such as gas chromatography (GC), high performance liquid chromatograpthy (HPLC), or thin-layer chromatography (TLC) can be used for the quantification of insect lipids in mixed tissue samples (e.g. Cvačka et al., 2006), but these methods are not usually high-throughput and may be qualitative or semi-quantitative depending on the detector. Current high-throughput methods used for the quantification of storage lipids in insects include gravimetric determination after organic solvent extraction (e.g. Pullin, 1987; Blows and Hoffmann 1993; Ojeda-Avila et al., 2003; Colinet et al., 2007; Sinclair et al., 2011), glycerol-linked enzyme assays (e.g. Clark and Wang, 1994; Gefen et al., 2006; Pospisilik et al., 2010; Marshall and Sinclair, 2010; Marshall and Sinclair, 2011), or chemical methods such as the (sulfo-phospho-) vanillin assay (e.g. Otronen, 1995; Timmerman and Briegel, 1999; Lorenz, 2003; Hahn, 2005; Huho et al., 2007).

2.1.1 **Chromatography**

Analytical chromatography, including GC, HPLC and TLC, can be used to quantify TAGs (D'Alonzo et al., 1982; Homan and Anderson, 1998; Fried, 2003). It involves separating substances based on their polarity by passing the substance of interest, dissolved in a mobile phase (solvent or gas), through a stationary phase (Fried, 2003) to which the components will adsorb differentially depending on their hydrophobicity. The resulting classes of each molecule are then quantified using one of a range of detectors (Scott, 1996). As the various lipid classes contained in a biological sample may be separated and thus quantified separately, these techniques are highly specific.

Chromatographic techniques are often used in a qualitative or semi-quantitative manner, with gravimetric methods used to quantify total TAG content before performing subsequent analyses on the extracted lipid (e.g. Chirumilla et al., 2010). HPLC can be used to quantify TAGs but requires the use of multiple methods such as reversed phase and silver ion HPLC and a range of detectors (e.g. Cvačka et al., 2006), which makes it inaccessible for high-throughput analysis. GC is the most commonly used chromatographic technique in the analysis of oils and fats. It offers superb resolution and reliability, and is often coupled to a flame ionisation detector (FID) which is one of the most accurate quantitative detectors currently available (Scott, 1996). However, the high molecular weights of TAGs mean that the temperatures required to elute them from GC columns typically approach the limits of thermal stability of the stationary phase and the column itself, and at these temperatures there is a risk of losses due to pyrolysis (Christie, 1989). Thus GC is usually performed on fatty acid methyl esters rather than on intact TAGs (but see Yocum et al., 2011), making it difficult to quantify TAGs without an additonal step to separate TAGs from other acylglycerols and free fatty acids. TLC is a powerful and simple method for separating lipid classes, though the perceived weakness of this method is in the quantitation aspect (Shantha and Napolitano, 1998). This can be overcome by combining TLC with densitometric analysis (e.g. Nikolova-Damyanova, 1999; Al-Anzi and Zinn, 2010; Santos et al., 2011) or flame ionisation detection (TLC-FID) (Mukherjee, 2003), although to my knowledge the latter has not been performed in insects. TLC-FID gives highly repeatable and accurate measurements of TAGs, requires only one solvent system, and utilises a highly accurate quantitative detector (Mukherjee, 2003). Compared to GC, it is fast and inexpensive, and gives comparable results in the analysis of acylglycerides (Shantha and Napolitano, 1998). Chromatography is relatively low-throughput, and requires expertise and specialised equipment. These restrictions can be prohibitive and a host of alternative methods are used to quantify storage lipids without prior separation.

2.1.2 **Gravimetric measurement of lipid**

In gravimetric methods, the animal is weighed to give fresh mass (FM), dried to give dry mass (DM) from which water content can be calculated, then the lipids are extracted by a

solvent (sometimes in conjunction with a Soxhlet apparatus that recycles heated solvent through a thimble containing the tissue) and the tissue dried and reweighed to give lipid-free dry mass (LFDM). Lipid can be calculated from the difference between DM and LFDM. Alternatively, the lipid extract may be weighed directly or used for further chromatographic or chemical analysis. Folch reagent (2:1 v/v chloroform: methanol; Folch et al., 1957) has been widely used for gravimetric lipid quantification in insects (e.g. Pullin, 1987; Ziegler, 1991; Ziegler and Ibrahim, 2001; Colinet et al., 2007; Sinclair et al., 2011). Extraction with Folch reagent removes glycerol, carbohydrates, amino acids, and phospholipids, as well as the non-polar neutral lipids (Newman et al., 1972), thus lipid mass will be overestimated by this method. Non-polar solvents such as chloroform (e.g. Hahn, 2005), petroleum ether; a mixture of light hydrocarbons formed from petroleum (e.g. Djawdan et al., 1997; Ojeda-Avila et al., 2003; Lease and Wolf, 2011), or diethyl ether (Blows and Hoffmann, 1993; Ballard et al., 2008) can be used to extract neutral lipids. Chloroform also extracts small amounts of non-lipid material (c. 6%), whereas petroleum ether and diethyl ether extract only neutral lipids (Dobush et al., 1985).

2.1.3 **Vanillin Assay**

The vanillin assay (Zöllner and Kirsh, 1962) is widely used to estimate insect storage lipids (e.g. Mwangi and Goldsworthy, 1977; Lorenz, 2003; Hahn, 2005; Huho et al., 2007; Geister et al., 2008). In this assay, TAGs are hydrolysed by sulfuric acid producing glycerol and fatty acids, whose double bonds react with the sulfuric acid to form an alkenyl cation (which is the chromogen; Johnson et al., 1977). Any molecule containing an unsaturated double bond or a hydroxyl group will react to form the chromogen, thus cholesterol and cholesterol derivatives, aliphatic alcohols, and fatty acids and fatty acid derivatives that have an unsaturated double bond, will all contribute to forming chromogens (Johnson et al. 1977). The chromogen then reacts with the vanillin reagent (an aromatic hydrocarbon) to form a chromophore with maximum absorbance at 530 nm (Johnson et al., 1977). This absorbance is compared to a standard calibration curve, often cholesterol (e.g. Lorenz, 2003, Geister et al., 2008) or extracted insect fat (e.g. Otronen, 1995). The choice of standard is important, as saturated fatty acids do not form a

chromogen and are not detected. In addition the reactivity of polyunsaturated fatty acids decreases with increasing unsaturation (Knight et al., 1972). Since insect TAGs have a high proportion of saturated and polyunsaturated fatty acids (Fast, 1970), I expect that a standard with 100% reactivity on a molecular basis (i.e. every molecule reacts to form the chromogen), such as monounsaturated TAGs or cholesterol, will under-estimate lipid reserves.

2.1.4 **Enzymatic Assays**

Enzyme assays are specific, high-throughput, generally avoid the use of strong acids or toxic solvents, and are commercially available as kits, e.g. Serum Triglyceride Determination Kit, Sigma-Aldrich, St Louis, MO, USA; Infinity triglyceride assay, Thermo Electron, Arlington, TX, USA; StanBio Triglyceride Liquicolor Kit, StanBio, Boerne, TX, USA; Genzyme Triglyceride-SL, BioPacific Diagonostics Inc., Bellevue, WA, USA; Triglycerides - GPO reagent set, Pointe Scientific, Canton, MI, USA; and many other similar assays. These assays involve the hydrolysis of TAGs to glycerol and free fatty acids by a lipase of bacterial origin. The liberated glycerol is broken down in a series of reactions catalysed by glycerol kinase and glycerol phosphate oxidase (hence these are often referred to as GPO assays) (Fossati and Prencipe, 1982; Kohlmeler, 1986). The end products include hydrogen peroxide, which (in a peroxidase-catalysed reaction) produces a quinoneimine dye that absorbs maximally at 500 - 540 nm.

Enzymatic assays have been widely used to estimate storage lipids in *Drosophila* spp. (e.g. Montooth et al., 2003; Gefen et al., 2006; Merritt et al., 2006; Pospisilik et al., 2010; Marshall and Sinclair, 2010) and a few other insects (e.g. Abd Al-Aziz, 2011; Marshall and Sinclair, 2011). As the products of glycerol breakdown are measured, free glycerol will also increase the absorbance. This can be a potential source of error in the analysis of overwintering insects since they may accumulate glycerol at molar concentrations as a cryoprotectant (Lee, 2010). Therefore free glycerol must be measured in parallel aliquots, or before the addition of the lipase to the samples. Recently, the ability of enzymatic assays to accurately quantify insect storage lipids has been questioned for two reasons: 1) The apparent inability of the lipase in two of the commercially available kits to fully

hydrolyse TAGs; and 2) *Drosophila* eye pigments absorb at similar wavelengths to the quinoneimine dye (Al-Anzi and Zinn, 2010). The latter problem can be circumvented by removing the heads of flies prior to homogenisation (Al-Anzi and Zinn, 2010), and will not apply to species that lack such eye pigments. The inefficiency of the lipase may be caused by insolubility of storage TAGs, as the assay was designed for use in serum, on lipoprotein complexes (Al-Anzi and Zinn, 2010). Alternatively it may be caused by incompatibility with the buffer in which the reaction occurs.

2.1.5 **Objectives**

Accurate measurement of storage lipids is of central importance to my dissertation. In addition, a high throughput method for measuring lipid in small tissue samples of whole insect homogenate would be desirable to facilitate screening of large numbers of samples. Gravimetric, chemical and enzymatic methods are all potentially high-throughput, but emerging concerns about the quantitative accuracy of each of these methods require further exploration. Here I test TLC-FID as a quantitative method to accurately and reliably measure TAG content in small insect tissue samples with low lipid content, and compare TLC-FID to the three commonly used lipid assays (gravimetric, vanillin, and enzymatic). A good high-throughput method would give absolute values that are not significantly different from my gold standard (for which I propose TLC-FID), and would rank samples equivalently to TLC-FID. Modifications of each method were investigated in an effort to improve efficiency and accuracy, paying particular attention to the impact of standards or solvent choice for quantification.

2.2 **Materials and Methods**

2.2.1 **Study species**

Twelve *Erynnis propertius* larvae (Lepidoptera: Hesperiidae) were reared on freshly cut oak leaves from eggs collected from butterflies that were caught from Garry Oak Savannahs on Vancouver Island, British Columbia, Canada (48.38°N, 123.25°W) and in Medford, Oregon, USA (42.23°N, 122.52°W; Pelini et al., 2009). Larvae were reared on a 12L:12D photoperiod under daily thermal cycles adjusted in two week increments to

reflect mean minima and maxima at the collection sites, based on long-term climate data (1997-2006) from Rogue Valley Medford-International or Victoria International airports (The Weather Underground, Inc.) until they entered diapause, after which food was removed and the caterpillars spent the winter in darkness. The larvae do not have red eye pigments, which could interfere with the detection of the chromagen in the enzymatic assay. Larvae were frozen in liquid nitrogen at the end of winter (late March) and stored at -80°C until analysis. By the end of winter, larvae are expected to have depleted lipid reserves, and are thus suitable to test the sensitivity of different methods of lipid quantification in samples containing relatively low TAG concentrations.

The larvae were weighed (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; repeatability = 0.1 μg) to determine fresh mass (FM) then completely homogenized with a ceramic pestle in a metal mortar cooled with liquid nitrogen. The resultant powder was transferred frozen and lyophilized at ~13 Pa for 24 h (Lyocentre lyophiliser, Virtis, Warminster, PA, USA), then reweighed to determine dry mass (DM). Samples were then stored at -80°C (for up to two weeks) before extraction of lipids. This method also yielded subsamples appropriate for RNA extraction and analysis of other metabolites (e.g. amino acids) via chromatographical-linked mass spectrometry.

2.2.2 **Reagents**

Chemicals (chloroform, methanol, benzene, isopropanol, dichloromethane, diethyl ether, formic acid 98% v/v, concentrated sulfuric acid, phosphoric acid 88% v/v, vanillin colour reagent, Tween 20, SDS, Triton X-100) and standards (tripalmitin, triolein, trilinolenate, triararchin, cholesterol, glyceryl 1,3 distearate, 1-stearoyl-rac-glycerol, stearic acid) were obtained from Sigma-Aldrich (St Louis, MO, USA) and were analytical, chromatography or otherwise highest available purity. Cholesterol palmitate (reagent grade) was obtained from Avanti Polar Lipids (Alabaster, AL, USA). Enzyme assays were performed using the Serum Triglyceride Determination Kit (TR0100; Sigma-Aldrich). Chromarods (Type SIII, 5u Silica) for TLC-FID were obtained from Shell USA (Fredericksburg, VA, USA). Vanillin colour reagent consisted of 2 $g \cdot L^{-1}$ vanillin in 70% orthophosphoric acid aqueous

solution. Microcentrifuge tubes were 1.7 mL clear microtubes (Axygen, Union City, CA, USA).

Using plastic pipettes and microcentrifuge tubes to transfer organic solvents during the extraction process significantly increased absorbance of a blank in the vanillin assay (two-tailed unpaired t-test; $t_4 = 38.3$, $p < 0.001$), thus all standard preparation was performed in borosilicate glass vials with Polytetrafluoroethylene (PTFE)-lined caps (VWR, Mississauga, ON, Canada) using glass Hamilton syringes (Reno, NV, USA), to prevent plastics reacting with organic solvents. Stock solutions were prepared of tripalmitin, triolein, trilinolenate, triarachin and cholesterol (Table 2.1), then sequentially diluted to obtain concentrations from 0.5 - 2.5 $\mu mol \cdot mL^{-1}$. A 2.7 $\mu mol \cdot mL^{-1}$ mixture of tripalmitin: triolein: trilinolenate in a 41: 36: 23 (saturated: monounsaturated: polyunsaturated) mass ratio was prepared to match the fatty acid composition of *E. propertius* (Custom Mixed TAG, Table 2.1), measured as described in Section 2.2. Mixtures of the TAG standards (with concentrations of 1 $\mu mol \cdot mL^{-1}$) were made by mixing equal quantities of each 1 $\mu mol \cdot mL^{-1}$ standard in chloroform (Table 2.1), to test the ability of TLC-FID to accurately quantify mixed TAGs similar to those present in biological samples.

2.2.3 **Fatty Acid Composition Analysis**

The saturation and chain length of the fatty acid side chains comprising TAGs can strongly influence some assays due to their effects on chemical reactivity (Knight et al., 1972) and polarity (Raclot, 1997). Thus the fatty acid composition of the neutral lipids of *E. propertius* was quantified using GC-FID (Guglielmo et al., 2002; MacMillan et al., 2009). Briefly, 3 mg tissue from 10 individuals was weighed and homogenised in a glass homogeniser with 4 mL chloroform/methanol 1:1 v/v. Samples were centrifuged (2000 × *g*,15 min), filtered (Grade 1 cellulose filter, Whatman International, Maidstone, Kent, UK) into clean test tubes and 5 mL chloroform/methanol 2:1 v/v was added to the pellet. The solution was then refiltered into the same tubes and 3 mL of 0.25% KCl added before incubation (70 °C, 10 min). The upper, aqueous layer was discarded, and the organic layer dried under a stream of nitrogen at 70 °C. The organic layer was resuspended in

pure chloroform and neutral lipids isolated by elution with chloroform: isopropanol 2:1 v/v through 1 mL solid-phase extraction columns (Supelclean LC-NH_2 SPE tubes; Supelco; Bellefonte, PA, USA) mounted on a Supelco Visiprep solid-phase extraction vacuum manifold. The solvent was dried under a stream of nitrogen at 70 °C then transesterified by incubation in 1M acetyl chloride (90 °C, 2 h) prior to washing with methanol to remove residual HCl. The solvent was again evaporated under nitrogen at 70 °C, then reconstituted in 1 mL dichloromethane and transferred to GC glass vials. Quantitative analysis of fatty acid methyl esters present in the samples was carried out on an Agilent 6890N gas chromatograph coupled with a flame ionization detector (GC-FID) (Agilent Technologies; Santa Clara, CA, USA) using a DB-23 high resolution column (30 m x 250 µm ID x 0.25 µm film thickness). The samples (1 µL) were injected in splitless mode and separated under the following temperature profile: injection at 80 °C (held for 2 min), temperature ramped to 180 °C (5 °C·min^{-1}), then to 200 °C (1.5 °C·min^{-1}), and to 240 °C (10 °C·min^{-1}) and held for 3 min. Peaks were identified by comparison of retention times to standard mixtures of fatty acid methyl esters (Supelco 37 component mix, Supelco PUFA # 3, Supelco F.A.M.E. mix C8-C24). Area of each fatty acid as a percentage of total area of all peaks was calculated for individual fatty acids, and summed to give total percentages of saturated, mono- and poly-unsaturated fatty acids.

2.2.4 **Lipid Extraction**

Lipids were extracted from tissue using a Folch extraction (Folch et al., 1957), with some modifications (Guglielmo et al., 2002). The method of extraction influences total lipid yield (Folch et al., 1957; Bligh and Dyer, 1959; Lorenz, 2003), however issues relating to extraction efficiency are beyond the scope of this study. The Folch method of lipid extraction results in the loss of less than 1% of lipids (Folch et al., 1957), thus I consider it to be a suitable method for this comparison.

Two aliquots of ground, lyophilised tissue from 2.2.1 (1 mg for TLC-FID; and 3 mg for enzymatic and vanillin assays) from each sample (n=12) were weighed. Tissue was then extracted in glass test tubes with 2.5 mL chloroform/methanol 2:1 v/v with 0.1% butylated hydroxytoluene (BHT) to prevent auto-oxidation of unsaturated fatty acids. For

TLC samples only, 30 µL of 2.6 µmol·mL^{-1} cholesterol was added to each sample at the beginning of the lipid extraction as an internal standard. Cholesterol was chosen because preliminary TLC-FID analyses without internal standard did not detect cholesterol in *E. propertius*. Samples were centrifuged (2000 × *g*, 15 min) then 1 mL KCl (0.25%) added and the solution incubated (70 °C, 10 min). The lower organic layer (containing neutral and polar lipids) was carefully removed using a Pasteur pipette, transferred to a fresh vial, and dried under N_2 before being reconstituted in 2 mL (in the case of the 5 samples destined for spiking) or 1 mL (other samples) of chloroform: methanol 2:1 v/v with 0.01% BHT. Samples were stored at -20 °C for up to one week until lipid content was analysed by TLC-FID, vanillin, and enzymatic assay.

2.2.5 **Quantitative thin layer chromatography (TLC-FID)**

Separation and quantification of lipid classes was done according to the modified methods of De Schrijver and Vermeulen (1991) using an Iatroscan MK-6 TLC-FID Analyzer (Shell-USA). Aliquots of extracted samples and standards dissolved in chloroform were used for the analysis. Prior to sample application, chromarods were blank scanned repeatedly to burn off impurities and activate the rods. A 1.5 µL glass syringe (Pressure Lok, VICI Precision Sampling; Houston, TX, USA) was used to spot 1.5 µL of standard mix (Table 2.1) on the first rod followed by samples (in triplicate) on the subsequent rods. The rods were developed in a TLC chamber using benzene: chloroform: formic acid (70:30:0.5 v/v/v) to separate the neutral lipids present in the samples. The chromarods were developed until the solvent front reached the 100 line mark on the rod holder. Rods were removed and dried (75 °C, 5 min) in a rod dryer (TK8, Iatron Inc, Tokyo, Japan) and then analyzed at the following settings: flow rate of 2 L·min^{-1} for atmospheric air, 160 ml·min^{-1} for hydrogen, and scanning speed of 3.0 cm·s^{-1}. Lipid components were identified by comparing their retention time with that of reference standards (Standard Mix, Table 2.1). Identified lipid components were quantified using standard curves prepared from the standard mixtures (0.5 - 2.5 µmol·mL^{-1}) after standardisation to the internal standard peak. Standard curves were also constructed for tripalmitin, triolein, trilinolenate and triararchin (Table 2.1) to rule out a differential response to the solvent or detector according to chain length or saturation. Five mixed

TAG standards (TAG Mix 1-5; Table 2.1) were run in triplicate to test the effects of standards used on accuracy of quantification of mixed TAG samples of differing composition and number of components.

Table 2.1- Composition of standards used for lipid analyses.

Standard	Assay	Composition
Standard Mix*	TLC-FID	Cholesterol palmitate (CE), triararchin (TAG), stearic acid (FFA), glyceryl 1,3 distearate (DAG), cholesterol, 1-stearoyl-rac-glycerol (MAG).
Tripalmitin (TPT)	TLC-FID	Glycerol backbone, three 16:0 acyl chains
Triolein (TOL)	TLC-FID, vanillin	Glycerol backbone, three 18:1 *n-9* acyl chains
Trilinolenate (TLL)	TLC-FID	Glycerol backbone, three 18:3 *n-3* acyl chains
Triararchin (TAR)	TLC-FID	Glycerol backbone, three 20:0 acyl chains
Custom Mixed TAG	TLC-FID, vanillin, enzymatic	TPT: TOL: TLL (41: 36: 23 w/w/w)
TAG Mix 1*	TLC-FID	50 µL of TAR, x 3
TAG Mix 2*	TLC-FID	50 µL each of TPT and TAR
TAG Mix 3*	TLC-FID	50 µL each of TPT, TOL and TLL
TAG Mix 4*	TLC-FID	50 µL each of TPT, TOL and TAR
TAG Mix 5*	TLC-FID	50 µL each of TPT, TOL, TLL and TAR
Cholesterol	Vanillin	Cholesterol
Glycerol	Enzymatic	Glycerol

*All components had a concentration of 1 $mg \cdot mL^{-1}$

2.2.6 **Gravimetric lipid determination**

Two aliquots (~3 mg DM each) of ground, lyophilised tissue from each individual (n=12) were weighed and placed into separate glass vials, then 1 mL of either chloroform: methanol 2:1 v/v or diethyl ether was added. Petroleum ether and diethyl ether give identical lipid recoveries (Dobush et al., 1985), and I chose diethyl ether as the exact composition is known and stable. The solvent was changed daily (total of 3 changes) using a Hamilton syringe with a 22 gauge needle, by drawing the solvent from top of the vial and leaving a small amount of liquid in the vial to prevent the loss of any tissue. The solution was discarded, and the final volume left to evaporate, then the samples were oven dried at 60 °C for 24 hours and the tissue reweighed to determine LFDM. No further mass loss occurred after three changes (CMW, unpublished data).

2.2.7 **Vanillin Assay**

Aliquots of 200 µL from extracted lipid samples were placed in test tubes. Spiked samples used to determine recovery efficiency were prepared by adding 30 µL of 1 $\mu mol \cdot mL^{-1}$ of the custom mixed TAG standard (Table 2.1) or 30 µL of chloroform to replicates (n=5). Samples were then dried under nitrogen at 70 °C. TAGs were measured using the vanillin assay (Zöllner and Kirsh, 1962), with the final spectrophotometric measurement performed in a 96-well plate rather than cuvettes, to increase throughput. Briefly, 200 µL of concentrated sulphuric acid was added to 200 µL samples in capped test tubes and incubated (95 °C, 10 min). The samples were cooled at room temperature after which 5 mL of vanillin colour reagent was added and tubes vortexed and left in the dark for 45 minutes. Aliquots of 200 µL of the solution were transferred in triplicate into acid-proof polypropylene 96-well plates (Brandtech Scientific, Essex, CT, USA), and the absorbance read at 530 nm on a PowerWave x340 plate reader (Bio-tek Instruments Inc.; Winooski, VT, USA). Concentrations of TAG in each sample were calculated against a custom mixed TAG, cholesterol, or triolein standard curve (Table 2.1). For triolein or the custom mixed TAG standard, a 1:1 molar ratio (standard to sample reactivity) was assumed, but for cholesterol the ratio was 1:3 (cholesterol: sample), as each TAG molecule is expected to react three times: once for each fatty acid (assuming all fatty

acids are monounsaturated); while each molecule of cholesterol will only react once (the hydroxyl group) (Knight et al., 1972).

2.2.8 **Enzymatic Assay**

Aliquots of 200 µL from each sample of extracted lipids (Section 2.2.4) were transferred to microcentrifuge tubes. Each spiked sample received 30 µL of 1 $\mu mol \cdot mL^{-1}$ custom mixed TAG standard (Table 2.1) in chloroform, or 30 µL chloroform, then dried under nitrogen at 70 °C. TAG standards were made by transferring aliquots of 1 $\mu mol \cdot mL^{-1}$ custom mixed TAG (Table 2.1, for final concentrations 0.05-0.4 $\mu mol \cdot mL^{-1}$) into microcentrifuge tubes which were then dried under nitrogen at 70 °C. Triglyceride concentration in samples and standards was measured using a GPO assay (Serum Triglyceride kit, Sigma-Aldrich) with minor modifications from Gefen et al. (2006). Briefly, 250 µL of 0.05% Tween 20 was added to each sample, and the mixture incubated (90 °C, 1 min) and cooled to room temperature twice. The heating step improved the recovery of a TAG standard (CMW, unpublished data). Ten microlitres of each sample was added in triplicate to a 96-well plate followed by 100 µL Free Glycerol Reagent and initial absorbance read at 540 nm. Twenty-five microlitres of Triglyceride Reagent was then added to each well and absorbance read at 540 nm at 5 min intervals until no further change in absorbance was observed (c. 45 min). Glycerol concentrations were calculated from a glycerol standard (concentration range 0.005 – 0.04 $\mu mol \cdot mL^{-1}$), while glycerol produced from TAGs was calculated by subtracting the concentration obtained from the initial absorbance readings from that of the final reading. A 1:1 molar ratio of TAG to glycerol was assumed.

Strong detergent concentrations (1% SDS) compared to more dilute concentrations (0.1%) of the same detergent caused reduced recovery of a triolein standard using a GPO assay (Rodriguez-Suarez and Peinado-Onsurbe, 2005). I thus modified the detergents used to rule out interaction between the enzyme and Tween 20, or incomplete solubilisation of TAGs. No detergent (samples dissolved in distilled water only), SDS 0.1% and Triton X-100 0.1% and 5% were used in place of Tween 20 0.05%, but lipid recovery was not improved (results not shown).

2.2.9 **Statistical Analyses**

All analyses were performed in R (v 2.10.1, R Core Development Team, 2009). Values presented are mean ± SEM. Standard curves for TLC-FID and the vanillin assay were compared among different standards using Type II ANCOVA with concentration as a covariate. In the case of the vanillin assay, cholesterol molar concentrations were first divided by three to give "triolein equivalents", based on the differential reactivity of cholesterol vs. triolein molecules. One-sample t-tests were performed to determine whether the measured lipid concentration of mixed standards by TLC-FID differed from their calculated concentration. The correlation between lipid concentration and caterpillar FM was tested using Pearson's product-moment correlation. Model II Major axis (MA) linear regressions (using the "lmodel2" package) were performed on lipid values from each enzyme assay against values from TLC-FID to parameterise the relationship between TLC-FID and the gravimetric, vanillin, and enzymatic assays. A Model II regression was performed because both of the variables in the regression were measured with some error, and a MA model was chosen as the variables are in the same units and the error variances were approximately equal (Legendre and Legendre, 1998). Pairwise Spearman's rank correlations (using the "lspearman" package) were performed between TLC-FID and each method of lipid quantification to determine whether the methods ranked the lipid content of the samples correctly. A Type II ANOVA was performed on total lipid returned by each method, followed by Tukey's HSD *post hoc* tests to determine which methods gave similar results. Concentrations were calculated on a molar basis and converted to mass assuming an average TAG M_r of 0.912 x10^3, based on the fatty acid composition of *E. propertius* (as measured in this study).

2.3 **Results and Discussion**

2.3.1 **Fatty Acid Composition**

This is the first report of the fatty acid composition of *Erynnis propertius*. Larvae had a fatty acid composition similar to other Lepidoptera (Fast, 1970); with the fatty acids 16:0 (palmitic acid), 18:1 *n*-9 (oleic acid), and 18:3 *n*-3 (linolenic acid) predominating, with smaller amounts of 16:1 *n*-7 (palmitoleic acid), 18:0 (stearic acid), 18:2 *n*-6 (linoleic

acid), and 20:1 *n*-9 (eicosenoic acid) (Table 2.2). The ratio of saturated: monounsaturated: polyunsaturated fatty acids was 41: 36: 23, and thus the TAGs tripalmitin (16:0), triolein (18:1), and trilinolenate (18:3), reflecting the most abundant fatty acids in *E. propertius*, were combined in that ratio to make the custom mixed TAG standard for the TLC-FID, enzyme, and vanillin assays (Table 2.1).

2.3.2 **Validation of TLC-FID**

The benzene: chloroform: formic acid solvent system separated all of the neutral lipid classes in my standard mix (Table 2.1, Figure 2.1A). Initial analysis revealed none of the individuals I analysed had any detectable cholesterol, cholesterol ester, free fatty acid, diacylglycerol, or monoacylglycerol (Figure 2.1B), thus cholesterol was used as an internal standard. The only repeatable peaks seen in any chromatograms belonged to TAG and cholesterol (internal standard; Figure 2.1C).

Table 2.2 - Fatty acid composition (± SEM) of neutral lipids of overwintering *Erynnis propertius* larvae, determined using GC-FID

Fatty acid	Common name	% of total fatty acids
16:0	Palmitic acid	29.0 ± 1.5
16:1 *n*-7	Palmitoleic acid	9.9 ± 2.0
18:0	Stearic acid	12.2 ±1.7
18:1 *n*-9	Oleic acid	23.6 ± 0.5
18:2 *n*-6	Linoleic acid	3.4 ± 0.4
18:3 *n*-3	Linolenic acid	19.2 ± 2.2
20:1 *n*-9	Eicosenoic acid	2.8 ± 0.4

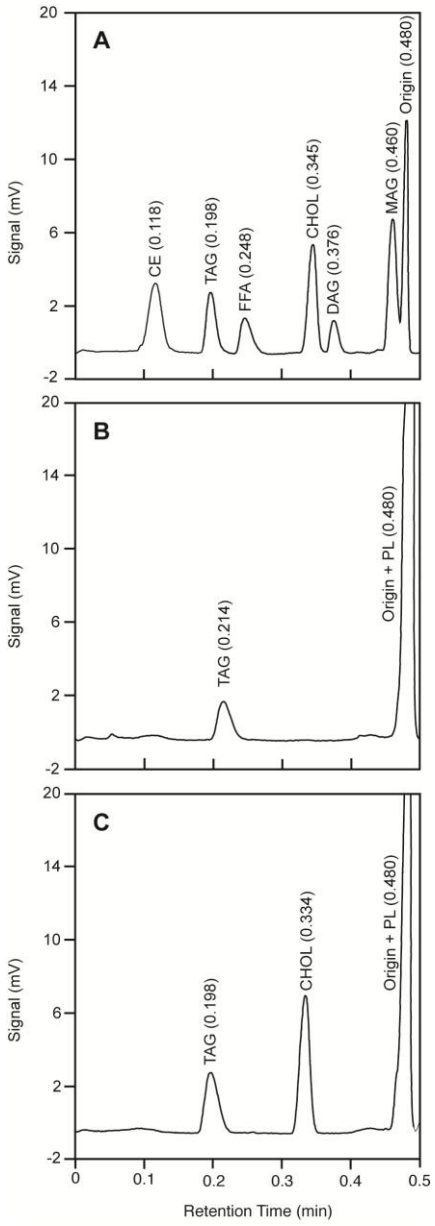

Figure 2.1 - Chromatograms from TLC-FID using the solvent system benzene: chloroform: formic acid (70:30:0.5 v/v/v). (A) The separation of neutral lipid components. CE = cholesterol ester; TAG = triacylglycerol; FFA = free fatty acid; CHOL = cholesterol; DAG = diacylglycerol; MAG = monoacylglycerol (see Table 2.1 for details of standards); (B) an *E. propertius* sample without internal standard showing the absence of all neutral lipid components except TAG (PL=phospholipids); and (C) an *E. propertius* sample with cholesterol added as internal standard showing the separation of the peaks. Slight differences in retention times result from inter-rod variability in rates of migration of solvent, and slight differences in development time among runs.

There were no significant differences among the slopes of the standard curves for any of the single fatty acid TAG standards as determined by TLC-FID ($F_{1,3}$=2.54, p=0.065; Figure 2.2A), confirming that this method is not sensitive to fatty acid chain length or saturation. There was a significant interaction between standard and concentration when comparing the single fatty acid standards with the mixed standard ($F_{1,79}$=10.67, p=0.002, Figure 2.2A and B), indicating that the slope of the concentration curve for a custom mixed TAG standard was lower than the slopes of the single fatty acid standards; potentially causing an underestimate of mixed TAGs (as the study organism will undoubtedly have) if measured with a single fatty acid standard. To confirm the effect of using a single fatty acid versus a mixed standard, I measured the peak area generated by mixed standards with a range of components (TAG Mix 1-5, Table 2.1) and converted this to concentration using standard curves obtained from either triararchin (single fatty acid) or the custom mixed TAG standard curve (Table 2.1). Mixed standards underestimated TAG concentration relative to the pure standard by 14.6 ± 1.3% (Figure 2.3). This seems to be because the TAGs elute at slightly different times due to polarity differences (from earliest [least polar] to latest: 18:1 << 20:0 < 18:3 << 16:0), which spreads out the peak (Figures 2.1B and C) and causes an underestimate of the area. Using the custom mixed TAG standard (Table 2.1) to estimate lipid concentration reduced this error to 2.0 ± 1.4%, and the measured concentrations of each mixed standard (TAG Mix 2-5, Table 2.1) were in no case significantly different than the true concentration (one sample t-test; 0.6>p>0.2; Figure 2.3), illustrating that TLC-FID can accurately quantify mixed TAG standards with varying fatty acid chain length and degrees of unsaturation, providing a mixed TAG standard is used. Extra lipid in spiked samples (after subtraction of total lipid in the matched, unspiked sample) accounted for 98% of the spiked TAG (Table 2.3), confirming that TLC-FID can accurately measure a mixed TAG with sample present.

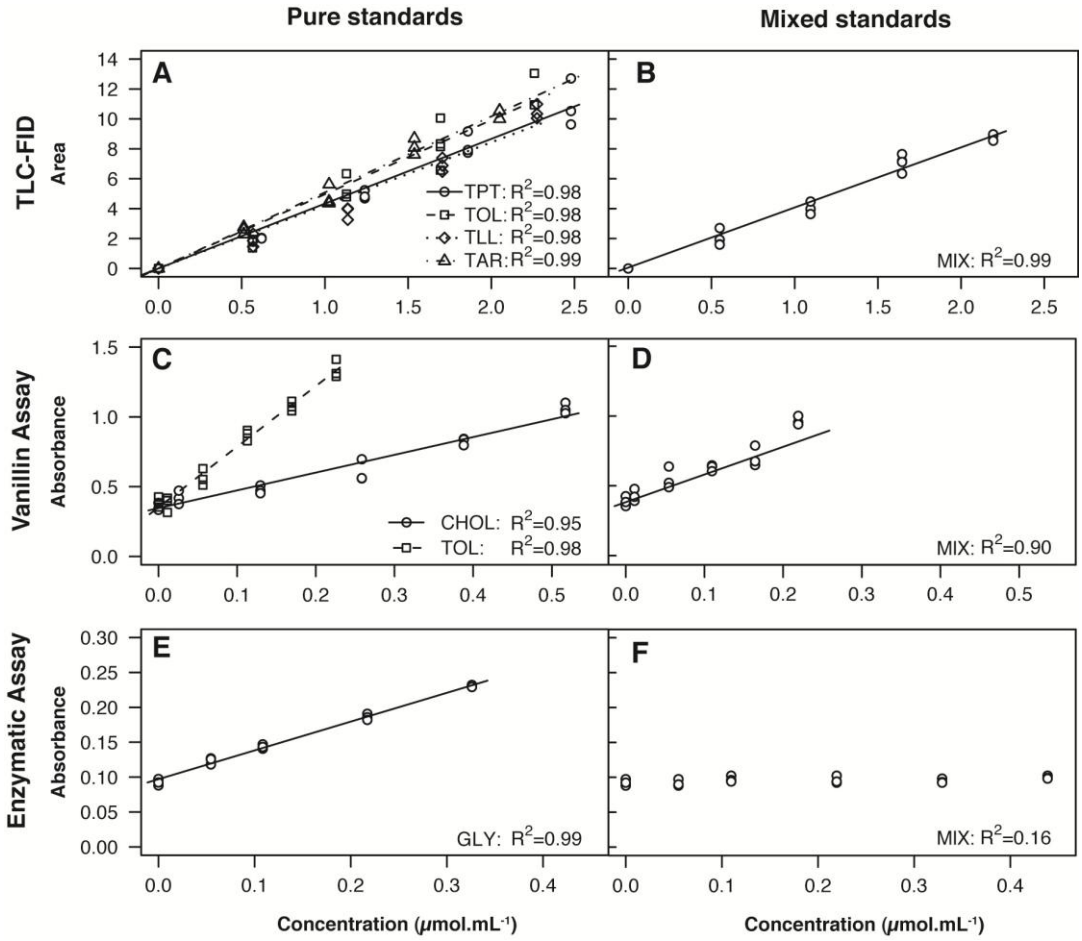

Figure 2.2 - Standard curves for TLC-FID (A-B), vanillin (C-D), and enzymatic assays (E-F) showing the linear regression line (where significant) and correlation coefficient. Standards are indicated in legend; TPT= tripalmitin, TOL= triolein, TLL = trilinolein, TAR = triararchin, CHOL = cholesterol, GLY = glycerol, MIX = custom mixed TAG standard; see Table 2.1 for details.

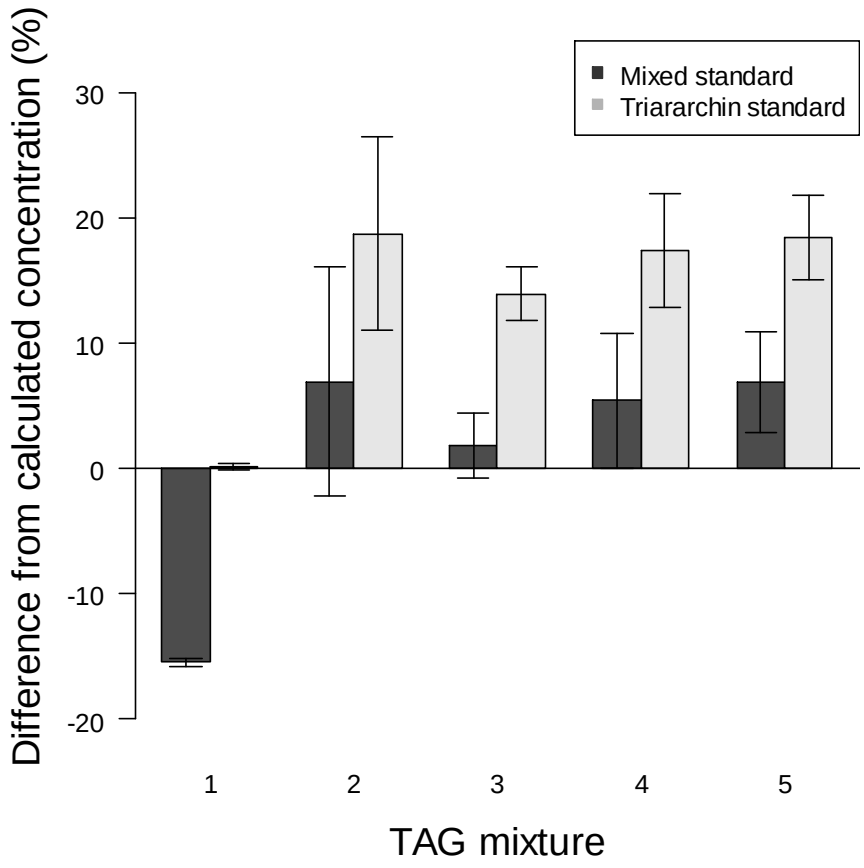

Figure 2.3 - Effect of standard composition on accuracy of TLC-FID estimation of lipid in pure (TAG mixture 1) or mixed (TAG mixtures 2-5) standards of different TAG composition. A concentration curve for a pure TAG standard (triarachin) accurately predicts the composition of a pure standard mix, but overestimates the concentrations of mixed standards. A mixed TAG standard rectifies the discrepancy and results in values not significantly different from calculated values for mixed TAG standards (see text for details). TAG mixtures 1-5 increase in numbers of components as follows: 1=triararchin; 2=tripalmitin and triolein; 3=tripalmitin, triolein and trilinolein; 4=tripalmitin, triolein and triararchin; 5=tripalmitin, triolein, trilinolein and triararchin; see Table 2.1 for details. All standard mixtures had a concentration of 1 $mg \cdot mL^{-1}$.

Table 2.3 - Recovery of spiked TAG for each method of lipid quantification (TLC-FID, enzymatic, and vanillin). Data are mean percent return (± SEM) of spiked TAG standard on a per mass basis. Returns using the Custom Mixed TAG standard in the enzymatic assay are not presented as there was no correlation between absorbance and concentration (Figure 2.2F).

Method	Standard	Recovery of TAG spike (%)
TLC-FID	Custom Mixed TAG	98.9 ± 4.6
Vanillin	Custom Mixed TAG	97.3 ± 11.2
Vanillin	Triolein	70.8 ± 8.2
Vanillin	Cholesterol	84.2 ± 9.7
Enzymatic	Glycerol	45.7 ± 6.1

Lipid loss during the extraction procedure was minimal (97.2 ± 4.7 % internal standard recovered). Caterpillars had an average fresh mass of 135.8 ± 7.8 mg and a water content of 74.8 ± 1.3 % FM, and there was no relationship between lipid concentration and fresh mass of whole caterpillars ($r=0.366$, $p=0.243$). Mean lipid content for larval Lepidoptera is 14.4 % (Lease and Wolf, 2011), therefore the observed mean lipid content of larval *E. propertius* in this study (8.5 % DM, Table 2.4) is low but consistent with expectations given that these individuals were at the end of the overwintering period and were likely to have depleted storage lipids (Hahn and Denlinger, 2011). Based on the accurate return of four mixed TAG standards, the complete recovery of spiked TAG added to samples, and biologically reasonable TAG concentrations of samples, I conclude that TLC-FID reliably and accurately measures TAGs in small biological samples regardless of the chain length and unsaturation of the monomeric fatty acids. Thus I consider TLC-FID (with a mixed TAG standard) to be a suitable standard against which to evaluate the other commonly used methods of lipid quantification.

Table 2.4 - TAG content (mean ± SEM) of *Erynnis propertius* larvae measured by TLC-FID, vanillin, enzymatic, or gravimetric assay. P-values are from ANOVA followed by Tukey's HSD testing for differences between each method and TLC-FID. Standards (or solvents in the case of gravimetric assays) are indicated in the Standard/Solvent column (see Table 2.1 for details of standards). Data using the Custom Mixed TAG standard in the enzymatic assay are not presented as there was no correlation between absorbance and concentration (Figure 2.2F). Bold text indicates methods giving estimates that are statistically indistinguishable from TLC-FID (α=0.05).

Assay	Standard/ Solvent	TAG ($mg \cdot mg^{-1}$ DM)	Total lipid (mg)	p-value (Tukey's HSD)
TLC-FID	Custom Mixed TAG	0.085 ± 0.007	2.93 ± 0.35	NA
Vanillin	**Custom Mixed TAG**	**0.105 ± 0.010**	**3.55 ± 0.40**	**0.862**
Vanillin	**Triolein**	**0.096 ± 0.007**	**3.24 ± 0.33**	**0.991**
Vanillin	**Cholesterol**	**0.111 ± 0.008**	**3.71 ± 0.38**	**0.683**
Enzymatic	Glycerol	0.015 ± 0.003	0.49 ± 0.11	< 0.001
Gravimetric	**Diethyl ether**	**0.128 ± 0.017**	**4.35 ± 0.73**	**0.115**
Gravimetric	Folch	0.585 ± 0.041	18.01 ± 1.56	<0.001

2.3.3 **Evaluation of Gravimetric assays**

Folch extraction (chloroform: methanol 2:1 v/v) ranked samples similarly to TLC-FID according to non-parametric tests, though the regression of Folch extraction lipid concentration on TLC-FID lipid concentration was marginally non-significant (Table 2.5, Figure 2.4A). However, absolute lipid concentrations from Folch extraction were significantly overestimated (more than 6 times higher than TLC-FID; Table 2.4). This indicates that Folch extraction removed substantial amounts of non-TAG components, and that these components represent a constant proportion of total lipid. Phospholipids comprise approximately 18 % of total lipid (Fast, 1970), and it has been previously found at least 17.5 % of the mass removed from goose tissue by a Folch extraction is non-lipid, including amino acids and carbohydrates (Dobush et al., 1985). This explains part of my overestimation. Although not present in my samples, glycerol is an important cryoprotectant in many overwintering insects (Lee, 2010), comprising as much as 20 % DM (e.g. Marshall and Sinclair, 2011), and its presence (or that of other polyol cryprotectants such as sorbitol or innositol) would further inflate lipid values determined by Folch extraction. Thus, Folch extraction may be adequate as an index; but if fat content estimated by a Folch extraction is attributed solely to storage lipid, the error will be unacceptably large. This supports previous suggestions (e.g. Dobush et al., 1985) that Folch extraction is not a suitable method for body composition analyses.

Diethyl ether solvent extraction gave lipid values statistically indistinguishable from TLC-FID (Table 2.4). Mean lipid concentration estimated by this method (14.1 ± 1.9 % DM) lies very close to the mean for larval Lepidoptera reported by Lease and Wolf 2011 (14.4 ± 1.9 %) after extraction with petroleum ether. However, I found no relationship between lipid concentration from the diethyl ether extraction and TLC-FID using regression or non-parametric tests (Figure 2.4A and Table 2.5). As the rank order of sample lipid content was not consistent with TLC-FID (and the trend was towards higher concentrations with diethyl ether) I propose that the solvent was extracting a small but variable amount of non-TAG lipid from the samples. Thus I suggest that diethyl ether

extraction may give reasonable lipid concentrations, but if small treatment effects are anticipated this would not be a suitable method.

Table 2.5 - Comparison of lipid concentration of *Erynnis propertius* larvae measured by vanillin, enzymatic, or gravimetric assays against concentrations measured by TLC-FID. Coefficients are from Model II major axis (MA) regression and Spearman's rank correlation. Standards (or solvents in the case of gravimetric assays) are indicated in the Standard/Solvent column (see Table 2.1 for details of standards). Bold text indicates a significant correlation with TLC-FID ($\alpha<0.05$).

Assay	Standard/ Solvent	MA regression			Spearman's rank correlation	
		Slope	R^2	p-value	rho	p-value
Vanillin	**Custom Mixed TAG**	**1.61**	**0.518**	**0.008**	0.552	0.067
Vanillin	**Triolein**	**1.06**	**0.508**	**0.009**	0.552	0.067
Vanillin	**Cholesterol**	**1.34**	**0.510**	**0.009**	0.552	0.067
Enzymatic	Glycerol	0.13	0.047	0.499	-.098	0.766
Gravimetric	Diethyl ether	6.96	0.101	0.313	0.210	0.513
Gravimetric	**Folch**	4.76	0.269	0.099	**0.627**	**0.044**

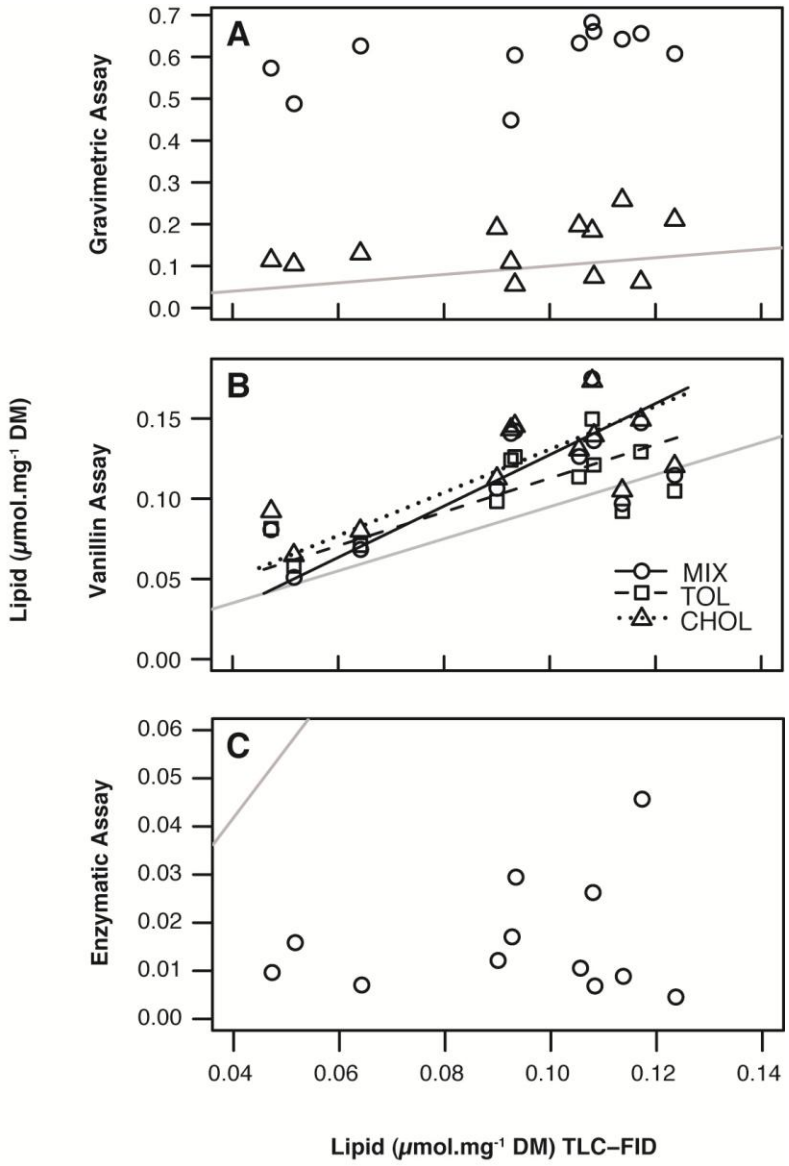

Figure 2.4 - A comparison of the performance of gravimetric, vanillin, and enzyme lipid assays in measuring storage TAGs of *Erynnis propertius*, each validated against lipid measurements from TLC-FID. The grey lines are lines of identity. Regression lines in black indicate a significant correlation between methods (see Table 2.5) as estimated by a Model II Major axis regression. Standard/solvents are indicated in legend; FOL = Folch reagent, MIX = custom mixed TAG standard, DEE = diethyl ether, TOL= triolein, CHOL = cholesterol, GLY = glycerol; see Table 2.1 for details.

2.3.4 **Evaluation of Vanillin assay**

Using the vanillin assay, triolein and cholesterol standards both yielded highly repeatable standard curves with $R^2 > 0.98$ (Figure 2.2C). The difference in the slopes of the regressions of absorbance on concentration for cholesterol and triolein (1.29 and 4.34 respectively; Figure 2.2C) likely reflects the differential reactivity of the two molecules, with each molecule of triolein (or any monounsaturated fatty acid) reacting three times (one per fatty acid double bond), while cholesterol only reacts once. Cholesterol molar concentrations were thus divided by three to give "triolein equivalents". A significant interaction between standard and concentration indicated that the differences among these slopes were significant ($F_{1,48}$=36.88, $p<0.001$), with Tukey's HSD indicating that all three standards were significantly different from each other (triolein and cholesterol $p=0.004$, all others $p<0.001$). The slopes of the standard curves in "triolein equivalents" were; triolein: 4.34; cholesterol: 3.88; mixed: 3.36 (Figure 2.2C). The lower slope for cholesterol compared to triolein suggests a lower reactivity of cholesterol molecules, as has been previously suggested (Knight et al., 1972). The lowest slope of the mixed TAG standard supports my expectation that a decrease in reactivity will result from increasing degrees of saturation and polyunsaturation.

The vanillin assay with a custom mixed TAG standard (Table 2.1) resulted in slightly less tight correlations between concentration and absorbance (Figure 2.2D), with R^2 of 0.90-0.94 (for three separate standard curves, data not shown). I hypothesise that this is due to the variable reactivity of the polyunsaturated TAG molecules (trilinolenate) which make up 23% of this standard. This variability is likely to be present when measuring biological samples and will increase at higher levels of polyunsaturation, thus technical replicates are highly recommended. When concentrations measured by the vanillin assay were calculated using the custom mixed TAG standard I detected 97.3 % of the spiked TAG (Table 2.3), although the variability of return of the spike was twice as large (11.2 vs. 4.6 %, Table 2.3) as that of TLC-FID. When the lipid concentrations were calculated against the triolein or cholesterol standard curves, the return of the spike dropped to 70.8 % and 84.2 % respectively (Table 2.3). This indicates that the slopes of the cholesterol and triolein standard curves were not appropriate for calculating lipid concentrations of the

lipid composition of my custom mixed TAG standard (which is similar in composition to *E. propertius*).

There was a significant positive relationship between lipid concentrations estimated using the vanillin assay with any of the three standards (custom mixed TAG, triolein, or cholesterol) and lipid concentration measured by TLC-FID (Table 2.5; Figure 2.4B). There was no significant difference between lipid concentrations estimated by the vanillin assay using any of the three standards and TLC-FID (Table 2.4). As the slopes and intercepts of the standard curves are significantly different for each standard, this result (and the ranking of the samples) could change depending on the position of the sample concentrations on the standard curve. A non-parametric test showed a marginally non-significant ranking of lipid content compared with TLC-FID lipid concentrations (Table 2.5). The Spearman's coefficients are identical for the three different standards because each data point came from the same absorbance data with one of three equations applied to it (the equations describing the relationship between absorbance and concentration for the custom mixed TAG standard, the triolein standard, or the cholesterol standard). Although in this case the concentrations were not significantly different (and thus error due to standard choice would be low in this case), this error could increase with increasing ratios of polyunsaturated and saturated TAGs, and increasing sample concentrations.

When using a TAG standard, the vanillin method assumes that all fatty acids originate from TAGs. In this study I did not find any measureable FFA, DAG, MAG, glycerol or cholesterol; but in some insect species these molecules can make up significant proportions of the total lipid mass (Fast, 1970). Acyl chains from phospholipids would also produce a sulfo-phospho-vanillin reaction – if I assume that phospholipids make up 20 % of total lipid mass (Fast, 1970) then my estimate for TAG mass using the custom TAG standard drops by 20 % to 0.084 $mg.mg^{-1}$ DM, bringing it exactly into line with mean lipid content from TLC-FID (0.085 mg.mg DM^{-1}). Thus I believe that the custom mixed TAG standard did give accurate lipid quantitation, and advise the use of mixed standards for quantitative analyses. A natural oil such as cod liver oil which contains a

mixture of fatty acids (Carvalho et al., 2008) would be preferable to a standard containing only one type of acyl group. If the extraction of phospholipids is to be avoided, I recommend using a less polar solvent (e.g. petroleum ether or diethyl ether) for the lipid extraction, or partitioning the lipid fraction into neutral and polar lipids prior to analysis (Hahn, 2005).

Even where the influence of standard composition is acknowledged (Knight et al., 1972; Cheng et al., 2011), researchers assume that the vanillin method will rank samples effectively even if it does not provide accurate quantitative values. I found support for this assumption, and thus if the assay is to be used only as an index the choice of standard is not critical. Systematic biases may however be introduced if a treatment changes the fatty acid composition of the study organism. Changes in fatty acid profiles of insects through either plasticity or evolution have been documented in response to changes in diet (e.g. Taipale et al., 2011), thermal regime (e.g. Overgaard et al., 2008), stress (e.g. Goto et al., 2010), age (e.g. Schneider and Dorn; 1994), life-stage (e.g. Khani et al., 2007) and season (e.g. Bennett et al., 1997). Thus if treatment groups differ in any of these factors, an alternative method (e.g. TLC) would be preferable.

These considerations underline the importance of understanding the reaction mechanism when calculating sample lipid concentrations from standard curves in this assay – if cholesterol is assumed to have the same reactivity as a molecule of (monounsaturated) TAG, then lipid concentrations will be overestimated three-fold if calculations are performed on a molar basis. If calculations are performed on a per mass basis, the fact that the cholesterol molecule (M_r 0.387 x10^3) is close to one third the mass of a TAG molecule (e.g triolein: M_r 0.885 x10^3) will partially obscure the error, although incorrect lipid estimates will result.

2.3.5 **Evaluation of Enzymatic assay**

Using the enzymatic assay, standard curves for glycerol showed a very tight relationship between concentration and absorbance (R^2 >0.99; Figure 2.2E), however TAG standards showed no relationship between concentration and absorbance (Figure 2.2F). This

suggests that the weakness of this assay lies in the hydrolysis step. Lipid concentrations measured by the enzymatic assay were significantly lower (less than 20 %) than those measured by TLC-FID (Table 2.4), and less than 50 % of spiked TAG was returned (Table 2.3). Neither regression nor Spearman's rank correlation indicated any relationship between lipid concentrations from this method and lipid concentrations measured in the same samples by TLC-FID (Figure 2.4C and Table 2.5). Two-fold dilution of standards and samples, or doubling the volume of triglyceride reagent did not result in improved recovery of TAG standards (data not shown); so the amount of lipase present does not appear to be limiting the reaction.

The lack of relationship between lipid concentration of TAG standards and absorbance, despite trying several permutations of detergents, enzyme concentrations, and substrate concentrations, parallels the findings of Al-Anzi and Zinn (2010), who also demonstrated the lack of a linear response to lipid concentration in a GPO assay using lard, butter, extracted *Drosophila* fat, and triolein. The Sigma triglyceride assay was validated for use in insect tissue when it was first used on *Drosophila* sp. in the mid-1980s (Clark and Gelman, 1985), when a linear relationship between concentration of TAG extracted from flies (validated by gravimetric methods) and absorbance was demonstrated. There are a number of possible explanations for the discrepancy between the original validation and more recent studies. Different lipases vary in their ability to fully hydrolyse TAGs, and the original studies on which the commercially available assays are based found that complete hydrolysis of TAGs was only seen in the presence of a protease such as α-chymotrypsin (Bucolo and David, 1973) or an esterase (Megraw et al., 1979). The lipase in the Sigma Triglyceride assay is a microbial lipoprotein lipase (*Pseudomonas* sp., Sigma Technical support personal communication), which is not ideal for fully hydrolysing TAGs (Bucolo and David, 1973), and no proteases or esterases are present in the Sigma Triglyceride reagent, nor in the other commercial kits, where information on composition was available. It is possible (although difficult to verify), that the composition of the triglyceride reagent has changed in the intervening years between the initial validation (Clark and Gelman, 1985) and the present. However, this would not explain the ability of some researchers to generate biologically reasonable lipid values

from this assay under identical conditions (e.g. Marshall and Sinclair, 2011), including some who routinely run TAG standards (e.g. Clark and Wang, 1994; Montooth et al., 2003). Poor recovery of TAGs may be caused by lack of ATP availability due to ATPase contamination (Megraw et al., 1979), but it seems unlikely that ATPases would have been present in the distilled water or the Free Glycerol or Triglyceride reagent themselves, which were the only common reagents in all assays. I am not able to come to a satisfactory conclusion on the basis of these differences but suggest that careful use of TAG (not only glycerol) standards and spikes is essential to verify the validity of any enzymatic TAG assay prior to data collection.

2.4 Conclusions and Recommendations

TLC-FID is a simple, accurate and quantitative method to measure storage lipids, and is not sensitive to saturation, chain length, or the presence of non-TAG lipids. The choice of standard is nonetheless important, as the slightly different elution times of different TAG molecules results in a spreading of the peak and underestimation of samples containing mixtures of TAGs. Using a TAG standard with at least 3 TAG components with divergent elution times resolves this problem and is simple to implement. This method requires very little tissue (~ 1 mg DM) and can quantify not only TAGs but also cholesterol esters, mono- and di-acylglycerides, free fatty acids, and cholesterol using the same solvent system. Although the throughput for TLC-FID is limited, it is possible to process > 6 samples per hour in triplicate with two sets of chromarods, thus for medium-throughput (e.g. <100-500 samples) the method is not prohibitive.

Although vanillin is known to react with all lipid classes with different sensitivity, similar values were obtain for TAG concentration in small tissue homogenate and standard mixes using both vanillin assay and TLC-FID analysis. The vanillin assay is therefore an accurate method to quantify TAGs in small samples of whole insect homogenate regardless of the presence of other lipid classes. If extraction prior to the assay is performed with chloroform: methanol 2:1 v/v, the assay will measure polyhydric alcohols and phospholipids in addition to neutral lipids. The former can be avoided by following a procedure for separating organic substances prior to analysis (Lorenz, 2003); the

phospholipids may be eliminated by utilising solid phase extraction and eluting just the neutral lipids (Hahn, 2005). If fatty acid composition differs markedly among treatment groups, this assay will introduce systematic errors (although the magnitude may be small), and thus is not recommended.

The format of the vanillin assay in this study (reactions performed in glass test tubes then transferred to a 96-well plate for rapid spectrophotometric analysis) is compatible with high-throughput analysis. Although a recent report has also modified the vanillin assay for high-throughput in microplates (Cheng et al., 2011), the current method has the advantage of not requiring the transfer of organic solvents to the plastic plate (which can cause corrosion of the plastic), and not requiring special apparatus for heating the plate (as the heating step here is done in test tubes). Performing the reactions in test tubes then pipetting the solution into 96-well plates prior to measuring absorbance on a plate reader is a relatively high-throughput modification which would allow hundreds of samples to be processed efficiently.

A gravimetric method may be useful as an index (with chloroform: methanol 2:1 v/v as a solvent) or a semi-quantitative method (diethyl ether or petroleum ether) where it is not necessary to have high confidence in the total lipid values, but the limitations of these methods must be kept in mind when interpreting results. For analyses demanding the resolution of small effect sizes, or where quantitative accuracy is important, gravimetric methods are not appropriate.

I have not found the GPO enzyme assay to be a suitable method for measuring storage lipids in insects, although I acknowledge that some researchers measure biologically-reasonable lipid concentrations using this assay. Since the weakness appears to lie in the hydrolysis step, if researchers have confidence that the hydrolysis is proceeding to completion (evidenced by linear TAG standard curves and complete recovery of spiked TAG) then this assay offers excellent throughput and is very simple to perform. However, I am not able to attest to its accuracy and emphasise the need for appropriate TAG standards.

As the lipid composition of overwintering insects is likely to change during dormancy (e.g. Bennett et al., 1997), and accurate lipid measurements are of paramount importance due to the small magnitude of changes I anticipate, none of the high-throughput methods are suitable for my application. I will use TLC-FID to quantify storage lipids throughout this dissertation.

2.5 Literature cited

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Chapter 3: The effect of winter warming on three butterfly species.

I measured physiological and life-history parameters of three butterfly species in response to warm and cool winters, to assess the vulnerability of each species to negative impacts of winter warming. This manuscript is in preparation for submission to Ecological Entomology.

3.1 Introduction

Anthropogenic climate change is altering mean temperatures and the relationships among environmental variables (IPCC 2007a, Jackson et al. 2009); leading to shifts in species' phenology (Thackeray et al. 2010) and geographic range (Chen et al. 2011). Inter-specific differences in sensitivity to climate are leading to asynchronies within ecosystems (Singer and Parmesan 2010, Walther 2010), the disassembly of communities due to differing mobilities of species moving to track their preferred climates, and novel biotic interactions, as previously allopatric species move into contact (Thuiller 2004). There is a need for a comprehensive framework that can predict the physiological consequences of warming on focal species based on life-history and demographic traits, to guide conservation efforts and highlight potentially vulnerable species. The construction of such a framework will require information on vulnerability to various drivers of climate change (e.g. increases in mean, maximum or minimum temperatures) for large numbers of species, combined with information on species traits such as resource specialisation, geographic distribution or range size. These types of meta-analyses have been successfully applied to understanding the major drivers of diversity loss (Chown and Gaston 2008).

In North America, regional climate change projections include a relatively greater warming in winter than during the growing season (Bonsal and Kochtubjada 2009, IPCC 2007b). For temperate ectotherms, winter strongly influences fitness (Bergland et al. 2005), and changes in temperatures over the winter can strongly impact fitness. The intuitive outcome of winter warming is a release from cold-induced mortality (indeed,

this is important in some species: Crozier 2004, Jepsen et al. 2008). However, increased winter temperatures can also decrease fitness of dormant ectotherms by increasing rates of resource utilisation (Irwin and Lee 2003, Williams et al. 2003, Sorvari et al. 2011). Most temperate insects spend the winter in a state of dormancy (diapause or quiescence; Koštál 2006). In dormant insects, metabolic rate is suppressed (but still temperature dependent; Chapters 4-5; Irwin and Lee 2002), and activity and feeding cease. Many insects overwinter in late-larval or pupal stages, and post-winter feeding opportunities may be limited or non-existent (Boggs 2009); meaning that stored energy reserves not only have to sustain metabolic demands during the winter, but also have to supply energy for metamorphosis and reproduction in the spring. A depletion of stored reserves, mediated by increases in metabolic rate resulting from warmer overwintering temperatures (Hahn and Denlinger 2011), can thus negatively impact both survival and fecundity (Irwin and Lee 2003, Williams et al. 2003, Sorvari et al. 2011, Mercader and Scriber 2008, Koštál et al. 2011).

Not all species are equally susceptible to the effects of winter warming. Pupae of the cold-adapted *Papilio canadensis* (Lepidoptera: Papilionidae) suffered increased mortality and increased mass loss in response to warming of six degrees over winter (constant 10 °C compared to constant 4 °C for controls); while the closely-related, cosmopolitan species *P. glaucus* did not show any negative effects of warming on survival or mass loss (Mercader and Scriber 2008). A warm-adapted species of wasp (*Diplolepis variabilis*; Hymenoptera: Cynipidae) also failed to experience any negative effects of warming, while a congener with a distribution restricted to a cooler climate exhibited decreases in survival and fecundity in response to winter warming (Williams et al. 2003). Thus, factors such as geographic distribution may be useful in predicting responses to winter warming. Other traits, such as resource specialisation, dispersal ability, and overwintering stage predict the magnitude of phenological responses to climate change, with species with narrower larval diet breadth, more advanced overwintering stages, and smaller range size showing greater advances in phenology (Diamond et al. 2011). These traits and others may also predict tolerance of winter warming, although insufficient data exist to test preliminary hypotheses. To date, there are few studies comparing multiple species'

responses to ecologically-relevant levels of winter warming (but see Williams et al. 2003, Mercader and Scriber 2008). Comparative studies can help to identify species at risk of winter warming, and begin to compile a database for assessing vulnerability in terms of species traits such as resource specialisation, dispersal and gene flow, and cold-adaptation, in a phylogenetic context. Such databases have been successfully used to predict phenological responses to climate change (Diamond et al. 2011), and the vulnerability of terrestrial organisms to warming (Deutsch et al. 2008).

Here we use three species of Lepidoptera to compare vulnerability to winter warming. *Papilio troilus* (Spicebush swallowtail) and *P. glaucus* (Eastern Tiger swallowtail; Lepidoptera: Papilionidae) are sympatric butterfly species which share a northern range limit coinciding with the transition from temperate Carolinian forest to boreal forest and whose distributions extend south to Florida and the Gulf Coast. The distribution of *P. glaucus* is wider than that of *P. troilus*, extending further north and west into the Great Lakes region in northern Minnesota, North and South Dakota, and Colorado (Brock and Kaufman 2003). *P. glaucus* is polyphagous, feeding on 30-40 species in 14 plant families, including the Magnoliaceae and Rosacaea families (Wagner 2005), while *P. troilus* is oligophagous, feeding on a few species of plants from the family Lauraceae (Scriber et al. 2008). Both *Papilio* species overwinter as pupae. *Erynnis propertius* (Lepidoptera: Hesperiidae) is a skipper butterfly inhabiting Garry Oak ecosystems on the West Coast of North America from Vancouver Island in the north to Baja California in the south (Chapter 4). This species range is thus more restricted than that of *P. troilus* or *P. glaucus*, as it is confined to coastal oak ecosystems (Brock 2003). Populations are monophagous, although the host plant transitions from *Quercus garryana* in the northern part of the range, to *Q. kelloggii* in the range centre, to *Q. agrifolis* at the southern range boundary (Pelini et al. 2010). *E. propertius* overwinters as a late-instar larva.

Papilio glaucus is relatively insensitive to increases in overwintering temperature, showing no decreases in mass or survival in response to winter warming (Mercader and Scriber 2008). This was hypothesised to result from high energy reserves in this species, although energy reserves were not assessed directly (Mercader and Scriber 2008). *E.*

propertius is sensitive to changes in overwintering temperature, and shows local adaptation to overwintering conditions (Pelini et al. 2009). Nothing is known about the overwintering physiology of *P. troilus*. However, based on the lower climatic diversity of its geographical distribution compared to *P. glaucus*, I hypothesise that it will be more susceptible to warming than will *P. glaucus*. Thus, I predict that sensitivity to winter warming will rank as follows (from highest to lowest): *E. propertius*, *P. troilus*, *P. glaucus*.

3.2 Materials and Methods

3.2.1 Collection and rearing

Eggs of all three species were obtained from females wild-caught in Norfolk County, Ontario, Canada (*P. glaucus* and *P. troilus*) or lower Vancouver Island, British Columbia, Canada (*E. propertius*, as described in Pelini et al. (2009)). Larvae were reared on potted host plants (*P. glaucus*: *Liriodendron tulipifera*, tulip tree; *P. troilus*: *Lindera benzoin*, spicebush; *E. propertius*: *Quercus garryana*, Garry oak) in EGC-TC2 incubators (Environmental Growth Chambers, Chagrin Falls, Ohio, USA) in the Biotron Centre for Experimental Climate Change, at the University of Western Ontario, on a 12L:12D photoperiod, at diurnally cycling temperatures (19 °C day: 10 °C night). When either larvae had pupated (*P. glaucus* and *P. troilus*) or three days had passed without evidence of feeding (*E. propertius*), larvae (*E. propertius*), or pupae (*P. glaucus* and *P. troilus*) were considered dormant and transferred to MIR-153 incubators (Sanyo Scientific, Bensenville, IL, USA) in 40 mL plastic containers (Solo Cup Company, Highland Park, Illinois, USA) on a photoperiod of 11L:13D, with temperatures fluctuating between 14°C day: 10 °C night for two weeks; then 10L:14D, 10°C day: 6 °C night for two weeks; then kept at a constant 6 °C (8L:16D) until the 20 Nov 2008 (when all individuals had entered dormancy, determined as described above). This stepwise decline of temperature and photoperiod was designed to mimic natural seasonal changes and enable larvae and pupae to acclimate to winter conditions. On 20 November 2008, larvae were divided between warm and cool winter treatments, and placed in incubators cycling between 8 °C day: and 2 °C night (warm treatment); or between 4 °C day: -2 °C night (cool treatment). The

difference between the treatments (4 °C) is concordant with predicted global mean temperature increases over the next century under a range of emissions scenarios (IPCC 2007a). The cool treatment reflects mean daily maximum and minimum microclimate temperatures in London Ontario during the month of January (data not shown).

3.2.2 **Life-history measurements**

I measured survival (in *E. propertius* and *P. troilus* only, due to insufficient sample sizes of *P. glaucus)* and overwinter mass loss in overwintering pupae or larvae exposed to these warm or cold thermal regimes. Larvae and pupae were weighed (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; repeatability=0.1 μg) at the beginning, middle and end of winter (20 November 2008, 20 January and 20 March 2009). The length of *Papilio* pupae from cremaster to tip was measured to the nearest mm using digital callipers (Mastercraft, Toronto, Ontario, Canada). Mass at the end of winter was subtracted from mass at the beginning of winter to obtain overwinter mass loss. On the 17 Mar 2009, all remaining *E. propertius* larvae and *P. troilus* pupae were placed into an incubator cycling between 25 °C (day) and 15 °C (night), on a 16L:8D photoperiod, and checked daily for emergence. When adults emerged, the time to emergence was recorded, adults were killed at -20 °C, weighed, and the right wing was measured from proximal attachment point to apex (a size measurement that is not influenced by water content, and has fitness implications due to the requirements of flight for mate finding, and oviposition in females).

3.2.3 **Energy reserve assays**

To determine the effect of overwintering temperature on the consumption of energy reserves, I measured total protein, storage lipids, and carbohydrates of individuals from the warm and cool treatment at the beginning and end of winter (*P. troilus* and *E. propertius*); or, due to insufficient sample sizes, at the end of winter only (*P. glaucus*). *P. glaucus* pupae were divided randomly between treatments at the beginning of winter, so I assumed that energy stores were similar at that point and that any differences would be due to overwinter differences in metabolic rates over winter. *Papilio glaucus* and *P. troilus* pupae were sexed by the presence of a line intersecting the first abdominal sternite

(see Chapter 4 for validation of this method in another species). I weighed the pupae and larvae to determine their fresh mass (FM), then punctured them five times with a dissecting pin and transferred them frozen to be lyophilised (Lyocentre lyophiliser, Virtis, Warminster, PA, USA) for 48 h at ~13Pa, or until they were sufficiently dry to grind in a mortar and pestle with no sign of liquid. I then reweighed them to determine dry mass (DM) and calculated water content by subtracting DM from FM.

Lyophilised pupae and larvae were homogenised using a mortar and pestle, and aliquots of tissue were weighed for lipid (~1 mg), protein (~1 mg), and carbohydrate (~22 mg, *P. glaucus* and *P. troilus*; ~ 16 mg, *E. propertius*). The aliquots of tissue were placed directly into 2.5 mL chloroform: methanol (2:1 v/v; lipid assay), 1 mL Tween 20 0.05 % (protein assay), or 1.2 mL 30 % KOH (carbohydrate assay).

I quantified protein using the bicinchoninic acid (BCA) assay, as described by (Gefen et al. 2006). Triplicates of 10 µL of each sample were loaded on microplates and 200 µL BCA assay reagent (50 parts bicinchoninic acid: 1 part 4 % $CuSO_4$) were added to each well. Plates were incubated overnight, and absorbance at 562 nm measured the following day. Concentrations were determined by comparison to standards (0.2-1 $mg \cdot mL^{-1}$ bovine albumin serum, Sigma-Aldrich, St Louis, MO, USA). I validated the assay using three bovine serum albumin standards, of which 103 ± 8 % (mean ± SD) were recovered.

I quantified triacylglycerides (TAGs) by TLC-FID (Iatroscan MK-6, Shell-USA, Fredericksburg, VA, USA), as described in Chapter 2. Briefly, I used a modification of the Folch method (Folch et al. 1957) to extract the neutral lipids from the insect tissue, then spotted 1.5 µL of either a standard mix (containing a cholesterol ester, a mono-, di-, and tri-acylglyceride, a free fatty acid, and cholesterol) or sample in triplicate onto silica rods (Shell-USA), then developed the rods in benzene: chloroform: formic acid (70: 30: 0.5 v/v/v) for 35 min and quantified the lipids using TLC-FID. Because initial analyses did not detect monoacylglyceride in samples, so I used a monoacylglyceride (1-stearoyl-*rac*-glycerol; Sigma Aldrich, St Louis, Missouri) as internal standard, to correct for losses during extraction. I identified compounds by comparing positions on the rods to

standards, and quantified TAGs using previously determined reference curves (Chapter 2). This assay returns 99% of TAG (Chapter 2).

I quantified total carbohydrates using the anthrone method (Caroll et al. 1955). Briefly, the homogenised samples were vortexed then incubated in a heating block (100 °C, 20 min) to extract the glycogen from the tissue, then centrifuged (2000 × *g*, 10 min). A 1 mL aliquot was taken and ethanol (2 mL, 95 %) was added, then the glycogen was precipitated out of solution by the addition of 50 µL saturated $NaSO_4$ aqueous solution. Samples were centrifuged (2000 × *g*, 10 min) and the supernatant discarded, then the pellet was dried at 100 °C and reconstituted in 500 µL distilled water. An aliquot of 2.5 mL of cold anthrone reagent (stock solution of reagent: 0.05 % anthrone, 1 % thiourea, 1 L 72 % H_2SO_4) was added to samples, standards (0.1-0.5 $mg \cdot mL^{-1}$ glycogen), and a water blank and the tubes were immediately cooled in an ice bath. Once ice-cold, samples and standards were transferred to a water bath and incubated (100 °C, 15 min), before being returned to the ice bath then triplicate 200 µL aliquots were pipetted into an acid-proof polypropylene 96-well plate (Brandtech Scientific, Essex, Connecticut) and absorbance measured at 620 nm. The recovery of three glycogen standards carried through the entire extraction and assay procedure was 99 ± 2 % (mean ± SD). The anthrone method measures total carbohydrates including free glucose and bound glycogen, but does not measure polyols such as glycerol and sorbitol (Graham 1963).

I expressed TAG, carbohydrate and protein concentrations in $\mu g \cdot mg\ DM^{-1}$, then estimated whole-animal values by multiplying by total DM. I subtracted whole-animal TAG and carbohydrate from DM to give lipid- (and carbohydrate-) free DM. I calculated total energy content in J/pupa assuming 39.3 $J \cdot mg\ TAG^{-1}$ and 17.6 $J \cdot mg\ carbohydrate^{-1}$ (Djawdan et al. 1997).

3.2.4 **Data Analysis**

All statistical analyses were performed in R 2.13.0 (R Core Development Team 2009), and preliminary data exploration was performed according to (Zuur et al. 2010). My general modelling approach was to start with maximal models and drop terms

sequentially, using Akaike's Information Criterion to judge the improved fit of the simplified model (a difference of two points was the threshold for retaining the simplified model; Crawley 2007). I compared pupal length, mass (October, December, and January), overwinter mass loss, and adult mass, wing length and development time (where applicable) separately within each species using analysis of variance (ANOVA) with treatment (and sex, in the case of *P. troilus*) as a factor. Development time in *P. troilus* was $\log_{10}$-transformed to meet assumptions of normality. I compared rates of mass loss over time among the three species using a mixed general linear model with treatment, species and time (beginning, middle and end of winter) as fixed factors, mass at the beginning of winter as a covariate, and individual as an error term. I compared survival rates of *P. troilus* between treatments using a generalised linear model with a binomial error structure with treatment as a factor, and mass in November and overwinter mass loss as covariates.

For *P. troilus*, the only species for which I had robust adult size measurements, I assessed the multivariate structure of interactions among life-history variables (date of pupation, mass at the beginning, middle and end of winter, overwinter mass loss, development time, adult mass, and wing length) using a principal components analysis (PCA), using the "prcomp()" function. I centred and scaled the data to standardise variances then rotated the loading matrix to obtain orthogonal factors. Principal components (PCs) that were above the inflection point of a scree plot and had eigenvalues of greater than 1 were retained (Tabachnik and Fidell 2007), and the PCs were defined based on variables that loaded strongly (factor loadings > 0.3). Each individual's scores on each PC were saved as factor scores and factors scores on each PC were analysed for effects of treatment using ANOVA with treatment as a factor.

I used total protein as a proxy for metabolising tissue. Total carbohydrate (*P. glaucus*) was $\log_{10}$-transformed to meet assumptions of normality. The effects of treatment on total protein, water content, and TAG and carbohydrate concentration were analysed using ANOVAs with treatment (warm or cool) and sex (for *P. troilus and P. glaucus* only) as factors. The models contained the following covariates: date of dormancy (total protein,

E. propertius only; see results for details); total protein (TAG and carbohydrate content, all species); and dry mass (water, all species).

3.3 Results

3.3.1 Life-history traits

I excluded individuals that did not eclose as adults from the analysis of life-history variables (11 *P. troilus* pupae). Mass in November and March was bimodal, with small modes at very low values. For *Papilio troilus*, a generalised linear model (described below) showed that mass loss over winter was the only significant predictor of survival, with high mass loss being associated with failure to emerge as an adult (survived: 6.2 ± 2.7 % initial mass, range 3.4 - 15.5 %; died: 29.5 ± 25.8 % initial mass, range 4 - 79 %). I thus excluded *Papilio* pupae with water loss of greater than 16 % of their initial mass, as they were likely to represent individuals that would not have overwintered successfully, and I wanted to quantify sub-lethal effects of warmer overwintering temperatures. This resulted in the exclusion of three *P. glaucus* pupae from the warm treatment, and one *P. troilus* pupa from the warm treatment. Mortality is easier to detect in larval *E. propertius*, thus I did not exclude any individuals based solely on water loss in this species. All remaining mass loss measurements and life-history variables were normally distributed.

The temperature treatments did not affect pupal length (*P. glaucus* and *P. troilus*), mass at any given time-point (all three species), adult wing length, adult mass or development time (*P. troilus*) in any of the three species (Tables 3.1 & 3.2). Overwinter mass loss was significantly greater in warm than in cool conditions in all three species (Table 3.2). Mass declined significantly during the winter ($F_{2,138}$=597.1, p<0.001), and it declined significantly faster in the warm treatment than in the cool treatment (treatment × time interaction: $F_{2,138}$=25.0, p<0.001). Mass declined significantly faster for *E. propertius* than for either of the *Papilio* species (species × time interaction: $F_{4,138}$=32.8, p<0.001), but there were no inter-specific differences in the effects of overwintering temperature on the rate of mass loss (species × treatment interaction: $F_{2,68}$=1.8, p=0.167; Figure 3.1).

Table 3.1 - Life-history and experimental design parameters for three butterfly species overwintered in warm or cool thermal conditions. Asterisks indicate a parameter that differs significantly between warm and cool treatments for that species, based on ANOVA ($\alpha<0.05$; Table 3.2). Mass loss = Mass in November - Mass in March. Development = days to eclose as adult post-transfer to 25 °C. Values are mean ± SD, or values for each individual (*E. propertius*, adult mass, wing length, and development).

Species	***P. glaucus***		***P. troilus***		***E. propertius***	
Date of diapause	28 September 2008 ± 29 days		17 Sep 2008 ± 20 days		2 October 2008 ± 12 days	
Treatment	Cool	Warm	Cool	Warm	Cool	Warm
$N_{overwintering}$	19	18	43	42	28	31
Pupal length (mm)	27.0 ± 1.8	28.0 ± 2.7	29.0 ± 1.6	28.7 ± 2.0		
$Mass_{Nov}$ (mg)	678.2 ± 138.7	736.2 ± 210.4	800.7 ± 141.4	792.4 ± 142.1	273.3 ± 55.3	296.1 ± 48.3
Mass loss (mg)	31.6 ± 30.4	49.5 ± 14.8*	36.4 ± 9.1	54.9 ± 17.7*	72.7 ± 12.4	99.4 ± 30.6*
N_{adults}			13	12	2	1
Adult mass (mg)			212.4 ± 82.1	252.5 ± 74.9	18.1, 75.6	80.1
Wing length (mm)			41.6 ± 3.3	43.1 ± 3.4	8.7, 16.4	15.7
Development			21.4 ± 11.1	16.5 ± 4.4	28,18	16

Table 3.2 - ANOVA comparisons between thermal treatments for life-history variables of *P. glaucus, P. troilus,* and *E. propertius* kept at warm or cool overwintering conditions. Significant effects are highlighted in bold face. $Mass_{Nov}$, $Mass_{Jan}$ and $Mass_{Mar}$ refer to pupal mass in November, January and March. Mass loss is calculated as $Mass_{Nov} - Mass_{Mar}$. Development = days to eclose as adult post-transfer to 25 °C.

	Papilio glaucus			*Papilio troilus*			*Erynnis propertius*		
Variable	*F*	df	*p*	*F*	df	*p*	*F*	df	*p*
Pupal length	1.73	1,32	0.197	0.25	1,51	0.616			
$Mass_{Nov}$	0.94	1,32	0.341	0.05	1,51	0.832	2.74	1,55	0.103
$Mass_{Jan}$	0.72	1,32	0.402	0.23	1,51	0.635	0.09	1,35	0.766
$Mass_{Mar}$	0.62	1,32	0.436	0.06	1,21	0.814	0.48	1,15	0.497
Mass loss	**5.44**	**1,33**	**0.026**	**9.67**	**1,21**	**0.005**	**4.70**	**1,15**	**0.047**
Adult mass				0.91	1,22	0.349			
Wing length				1.24	1,22	0.276			
Development				1.05	1,22	0.317			

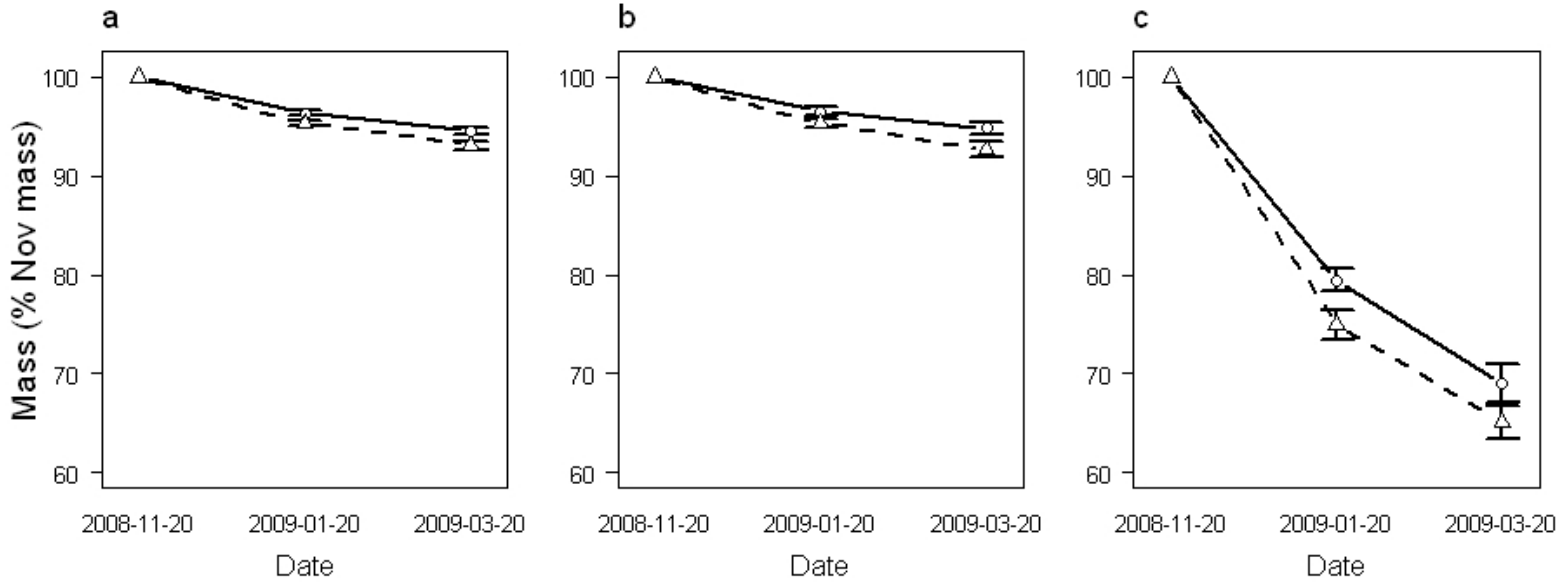

Figure 3.1 - Overwinter mass change of three butterflies exposed to cool or warm overwintering conditions. Mass of (A) *P. glaucus* (PG, N=34); (B) *P. troilus* (PT, N=24), and (C) *E. propertius* (EP, N=17) in November, January, and March under warm (triangles, dotted lines) and cool (circles, solid lines) conditions is expressed as percent of each individual's mass in November, to standardise for inter- and intra-specific size differences (although analyses were performed on raw mass data with mass in November as a covariate, see text for details). Data are mean ± SEM; if not visible error bars are obscured by symbol. Mass declines over time for all species, rates of decline are significantly faster in warm than in cool conditions, and *E. propertius* exhibits significantly greater rates of mass loss than do *P. glaucus* and *P. troilus*; see text for details.

I did not have sufficient sample size to perform survival assays in *P. glaucus,* and in *E. propertius,* only three individuals emerged as adults - two from the cool treatment and one from the warm (Table 3.1). Thus, comparisons of survival and adult size were made only for *P. troilus*. Survival in *P. troilus* remained constant at c. 30 % in both treatments ($F_{1,34}$=0.3, p=0.598; Table 3.1). Mass in November did not influence survival ($F_{1,34}$=0.1, p=0.773), but overwinter mass loss was a significant predictor of survival ($F_{1,34}$=8.0, p=0.009). Neither adult mass, wing length, nor development time differed significantly between overwintering treatments for *P. troilus* (Table 3.2).

A principal components analysis of life-history variables in *P. troilus* provided three principal components (PCs) that cumulatively explained 88 % of variation in the data set (Table 3.3). The first PC accounted for the effects of size, with all pupal and adult size measurements loading positively (Table 3.3). There was no significant effect of treatment on this PC ($F_{1,21}$=0.61, p=0.443). The second PC represented mass loss and development time, with mass loss loading positively while development time loaded negatively (Table 3.3). Individuals in the warm treatment had significantly higher factor scores on this component ($F_{1,21}$=6.41, p=0.019), indicating that they had higher mass loss and shorter development times than individuals in the cool treatment (Figure 3.2). The third principal component was defined by pupal length and date of diapause, whereby later diapause was associated with increased pupal length (Table 3.3). There was no effect of treatment on this PC ($F_{1,21}$=0.23, p=0.635).

Table 3.3 - Principal components (PCs) for life-history variables of *P. troilus*, subjected to warm or cool winters. Values are factor loadings for the first 3 PCs (total variation explained in parentheses) of a PCA. NS indicates variables that did not load significantly (factors loadings <0.3). Individuals in the warm treatment had significantly higher scores on PC2 (see text for details).

	PC1 loading (61.2%)	PC2 loading (16.0 %)	PC3 loading (16.0 %)
Name of PC	Size	Large mass loss, short development time	Late diapause, long pupae
Pupal $mass_{Nov}$	0.43	NS	NS
Pupal $mass_{Jan}$	0.42	NS	NS
Pupal $mass_{Mar}$	0.42	NS	NS
Pupal length	0.33	NS	0.46
Overwinter mass loss	NS	0.71	NS
Adult mass	0.37	NS	NS
Wing length	0.39	NS	NS
Development time	NS	-0.56	NS
Diapause date	NS	NS	0.81

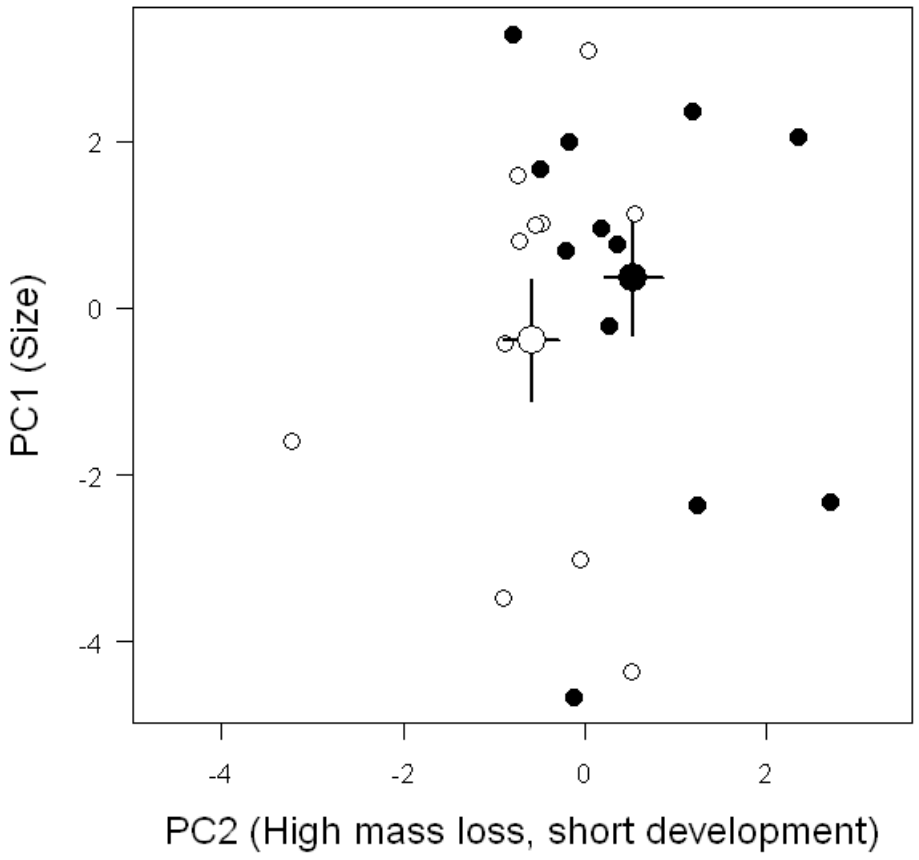

Figure 3.2 - The first two principal components of an analysis of life-history characteristics of *P. troilus* exposed to warm (filled) or cool (open) overwintering conditions. See Table 3.3 for details of components. Individuals overwintering at warm temperatures have significantly higher mass loss and shorter development times than those overwintering at cool temperatures.

3.3.2 **Energy reserves**

There was a strong correlation between total protein and lipid- and carbohydrate-free dry mass (*P. troilus*: r_{32}=0.63, p<0.001; *P. glaucus:* r_{16}=0.89, p<0.001; *E. propertius*: r_{35}=0.57, p<0.001). Date of dormancy did not affect total protein, TAG concentration, or carbohydrate concentration for *P. troilus* or *P. glaucus* (p>0.05, data not shown), while in *E. propertius* date of dormancy was negatively correlated with total protein (r_{35}=-0.35, p=0.033) and lipid- and carbohydrate-free dry mass (r_{35}=-0.64, p<0.001); but not with TAG or carbohydrate concentration (p>0.05, data not shown). Therefore I used date of dormancy as an additional covariate in the analysis of treatment effects on total protein for *E. propertius* only.

Total protein did not differ between overwintering treatments for *P. glaucus, P. troilus* or *E. propertius* (Tables 3.4 and 3.5). As suggested by the correlation between date of dormancy and total protein in *E. propertius*, date of entering dormancy significantly affected total protein (Table 3.5), with late entry into dormancy associated with lower total protein content. Water content was positively related to total protein in all three species, and declined significantly over time in *P. troilus* and *E. propertius* (Tables 3.4 and 3.5). TAG content was positively related to total protein in *P. glaucus* and *P. troilus,* with no effect of overwintering temperature on TAG content (Tables 3.4 and 3.5). In *E. propertius*, there was a three-way overwintering temperature × total protein × sampling time interaction, such that TAG concentration declined between the beginning and end of winter in the warm treatment but not in the cool treatment (Tables 3.4 and 3.5). Total energy content was strongly driven by TAG (r=0.99, df=16-35, p<0.001 for all three species), and in all cases the conclusions are identical for energy content and TAG content. Total energy content was not affected by overwintering temperature in *P. glaucus* or *P. troilus*, nor in *E. propertius* in the cool overwintering treatment (Figure 3.3). However, total energy content of *E. propertius* declined over the course of the winter in the warm overwintering treatment (Figure 3.3).

Table 3.4 - Physiological variables of *P. glaucus, P. troilus*, and *E. propertius* overwintered in warm or cool thermal conditions. Significant factors or covariates are derived from models in Table 3.5. TAG = triacylglyceride; Protein = total protein; Carb = total carbohydrate; Time = sampling time (beginning or end of winter).

	Beginning of winter		End of winter		Significant factors or covariates
	Cool	**Warm**	**Cool**	**Warm**	
P. glaucus					
N			9	9	
Protein (mg)			41.2 ± 8.4	41.0 ± 13.1	NS
Water (mL)			488.4 ± 82.5	517.2 ± 142.7	Dry mass
Water (% fresh mass)			78.3 ± 1.8	76.8 ± 4.0	
TAG (mg·mg protein^{-1})			0.380 ± 0.166	0.406 ± 0.241	Protein
Carb (mg·mg protein^{-1})			0.038 ± 0.031	0.043 ± 0.035	NS
P. troilus					
N	7	9	9	9	
Protein (mg)	41.2 ± 8.2	36.7 ± 10.5	61.6 ± 19.3	47.9 ± 14.3	NS
Water (mL)	665.9 ± 51.0	655.9 ± 107.3	612.2 ± 64.5	597.6 ± 114.5	Dry mass, Time
Water (% fresh mass)	83.0 ± 3.0	84.2 ± 2.2	79.5 ± 1.9	80.7 ± 2.1	
TAG (mg·mg protein^{-1})	0.248 ± 0.070	0.248 ± 0.074	0.236 ± 0.146	0.214 ± 0.112	Protein
Carb (mg·mg protein^{-1})	0.032 ± 0.041	0.071 ± 0.036	0.049 ± 0.025	0.050 ± 0.030	NS
E. propertius					
N	9	10	9	10	
Protein (mg)	19.9 ± 5.3	22.0 ± 7.5	23.8 ± 7.5	28.0 ± 10.2	Date of dormancy
Water (mL)	216.8 ± 46.9	236.0 ± 42.5	145.8 ± 45.0	147.9 ± 36.8	Dry mass, Time
Water (% fresh mass)	81.5 ± 2.1	80.3 ± 2.2	70.9 ± 4.8	70.7 ± 4.5	
TAG (mg·mg protein^{-1})	0.344 ± 0.120	0.356 ± 0.118	0.287 ± 0.096	0.274 ± 0.097	Treatment×Protein×Time
Carb (mg·mg protein^{-1})	0.059 ± 0.051	0.054 ± 0.045	0.047 ± 0.031	0.027 ± 0.027	Protein

Table 3.5 - ANOVA statistics describing the effect of overwintering treatment (warm or cool), sex, and sampling time (beginning or end of winter) on energy reserves of *P. glaucus* (PG) and *P. troilus* (PT) pupae, and *E. propertius* larvae (EP). Carb = total carbohydrate; Dormancy = date of dormancy; DM = dry mass; TAG = triacylglyceride.

Parameter (y)	Initial model	Minimal adequate model	Term	F	df	p
PG						
Protein	y=Treatment × Sex	Null model	NA			
Water	y=Treatment × DM × Sex	y=DM	Dry mass	43.24	1,16	<0.001
TAG	y=Treatment × Protein	y=Protein	Protein	8.33	1,16	0.011
Carb	y=Treatment × Protein × Time	Null model	NA			
PT						
Protein	y=Treatment × Sex ×Time	y = Time	Time	10.22	1,31	0.003
Water	y=Treatment×DM ×Time×Sex	y= DM+Time	Dry mass	24.15	1,31	<0.001
			Time	16.11	1,31	<0.001
TAG	y=Treatment × Protein × Time	y = Protein	Protein	4.50	1,32	0.042
Carb	log(y)=Treatment×Protein×Time	Null model	NA			
EP						
Protein	y=Treatment×Dormancy×Time	y = Dormancy	Dormancy	4.88	1,35	0.034
Water	y=Treatment×DM×Time	y = DM + Time	Dry mass	70.27	1,34	<0.001
			Time	126.12	1,34	<0.001
TAG	y=Treatment×Protein×Time	y=Treatment×Protein×Time	Treatment×Protein×Time	4.35	1,29	0.047
Carb	log(y)=Treatment×Protein×Time	log(y)=Protein	Protein	7.54	1,35	0.009

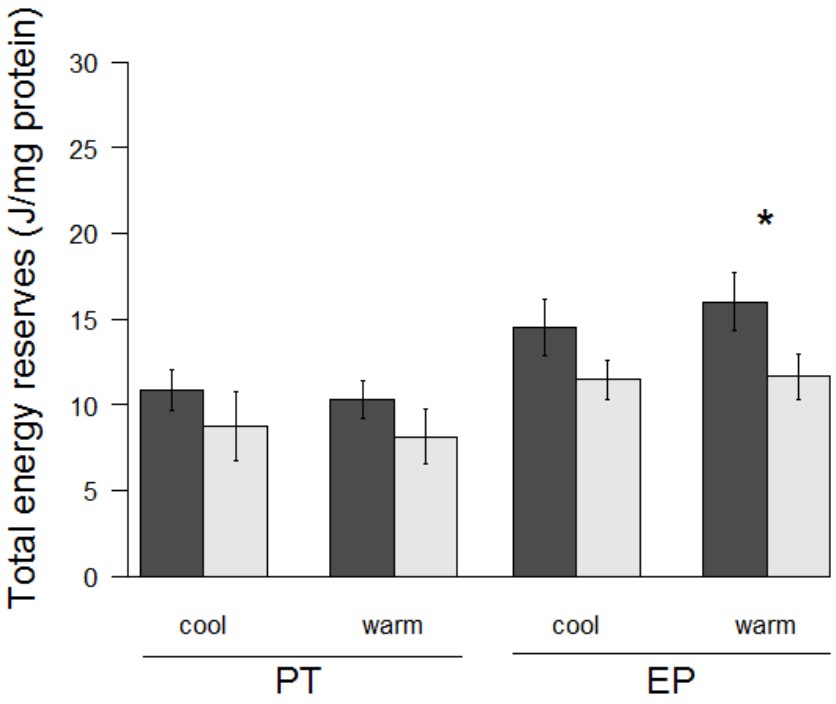

Figure 3.3 - Total energy reserves of *P. troilus* (PT) and *E. propertius* (EP) at the beginning (dark bars) or end (pale bars) of the overwintering period, for individuals exposed to warm or cool overwintering temperatures. Asterisk indicates significant energy depletion from the beginning to the end of winter.

3.4 **Discussion**

I examined the responses of the overwintering stages of three butterfly species to the degree of winter warming that is predicted over the next century (IPCC 2007a). I found that winter warming increased rates of overwinter mass loss in all three species, but that this mass loss was due to water loss rather than increased consumption of energy reserves. Although all species showed a trend towards lower energy content at the end of winter compared to the beginning (Figure 3.3), the only instance in which this trend was significant was for *Erynnis propertius* at warm temperatures. Both *Papilio* species were relatively insensitive to changes in their thermal environment over winter.

The decreased sensitivity to warming in the two *Papilio* species compared to *E. propertius* may be caused by caused by a deeper degree of overwinter metabolic suppression in *P. glaucus* and *P. troilus*, making them less sensitive to temperature. Mass-specific metabolic rates of *E. propertius* pupae are about twice those of *P. zelicaon* pupae under identical conditions (Pelini et al. 2009); a similar degree of metabolic suppression in *P. glaucus* and *P. troilus* would explain their decreased sensitivity to temperature. Measured metabolic rates of *P. canadensis* (a closely-related species to *P. glaucus*) in the field at 24 °C (Kukal et al. 1991) are an order of magnitude higher than estimates for *P. zelicaon* in the laboratory at 8 °C (Pelini et al. 2009; comparison of the two rates gives a Q_{10} of 55), which may be due to differences in measurement methods (open vs. closed system), physiological state of pupae (laboratory acclimated vs. field acclimatized), or inter-specific differences in diapause depth. I am thus not able to draw conclusions about the generality of extreme metabolic rate suppression in *Papilio* pupae, but consider differences in diapause depth between the *Papilio* pupae and *E. propertius* larvae to be a likely origin of the differences in sensitivity to overwintering temperatures.

Water content declined over time in *E. propertius* and *P. troilus*, and the decline in water content was sufficient to fully account for the observed mass loss in both species, as there was no significant sampling time × residual dry mass interaction. This raises the possibility that the relationship between high mass loss and mortality previously

documented in *P. canadensis* pupae in response to winter warming (Mercader and Scriber 2008) may be mediated by desiccation stress rather than energetic drain. However, in the present experiment, I did not find any increase in mortality in *P. troilus* pupae overwintered at warm temperatures. Moreover, a principal components analysis confirmed that the sublethal effects of warm winters were confined to an increase in mass loss (with no reduction in adult size) and a decrease in development time. A decrease in development time was also documented in *H. cunea* pupae in response to warm winters (Chapter 4), and may be a result of some development occurring during the winter under warmer temperatures (Denlinger et al. 2005). Although I was not able to assess water loss over winter in *P. glaucus* (there were insufficient sample sizes to perform destructive body composition analyses at the beginning of winter to provide a starting water content), whole pupa overwinter mass loss of *P. troilus* and *P. glaucus* were similar, suggesting that mass loss is likely attributable to water loss in *P. glaucus* as well. The higher water loss rates of *E. propertius*, compared to both *Papilio* species, likely results from the more permeable larval integument compared to the heavily sclerotised pupal case. The loss of almost 40 % of their initial water content may have been a cause of the high mortality in *E. propertius* larvae.

The negative relationship between date of dormancy and total protein content of *E. propertius* pupae may represent a general relationship between rates of growth and development and overall quality of individuals, such that higher quality individuals grow and develop faster (and thus reach the overwintering stage earlier), and also attain greater mass. Alternatively, later-diapausing individuals may be smaller due to a decline in host plant quality caused by seasonal fluctuations in phytochemistry (Bracho-Nunez et al. 2011) or inducible plant defences (Rieske and Dillaway 2008).

I classify the vulnerability of *P. glaucus* and *P. troilus* to energetic drain resulting from winter warming as low, since there were no discernable lethal or sub-lethal effects of the levels of warming that are predicted over the coming century. This is in concordance with a previous study that also found a low vulnerability of *P. glaucus* to winter warming (Mercader and Scriber 2008). *Erynnis propertius* has at least moderate vulnerability to

energetic drain due to winter warming - sub-lethal effects of warming were detected, and it was not possible to assess mortality due to very high levels of mortality across all treatments, possibly due to desiccation. These classifications are broadly in line with those predicted based on species traits - the species with the highest degree of resource specialisation and most restricted range showed the highest sensitivity to warming. However, data on many more species are required, before it will be possible to assess species traits that are predictors of vulnerability to winter warming in a phylogenetically-informed context. Classifications from the present study can be added to a modest but growing data set addressing the vulnerability of insects to energetic drain from winter warming (e.g. Chapter 4, Irwin and Lee 2003, Williams et al. 2003, Bosch and Kemp 2004, Mercader and Scriber 2008, Sorvari et al. 2011, Koštál et al. 2011), and once sufficient data are amassed it will be possible to conduct meta-analyses to identify species traits that predict vulnerability. This will help guide conservation attempts and identify species most at risk from winter warming.

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Chapter 4: Metabolic compensation and differential effects of temperature across life-stages result in low sensitivity to winter warming in the Fall webworm.

I examined the role of local adaptation and plasticity in modifying the life-history and physiological characteristics of a widespread, generalist pest species in response to overwintering microclimate conditions from the centre (Columbus, OH) or northern periphery (Ottawa, ON) of their range. This manuscript is in preparation for Functional Ecology.

4.1 Introduction

The abiotic environment varies over space and time, resulting in time- and location-dependent selective pressures. The main mechanisms by which organisms can respond to heterogeneous environments are through local adaptation and adaptive phenotypic plasticity. Local adaptation refers to genetic differentiation among populations leading to increased fitness in their natal environment, relative to non-adapted genotypes (Kawecki and Ebert 2004). Phenotypic plasticity is the environment-specific expression of multiple phenotypes by a single genotype, in response to changes in environmental conditions (Miner et al. 2005), which may or may not increase fitness (DeWitt and Scheiner 2004; Whitman and Agrawal 2009). The two strategies are not mutually exclusive, and whole-organism fitness may be a composite of local adaptation in some traits, and phenotypic plasticity in others. Local adaptation increases the fitness of individuals in their natal environment; phenotypic plasticity can (theoretically) lead to individuals having high fitness in many environments - yet local adaptation along environmental gradients has produced clines in physiological characteristics such as the critical photoperiod for

diapause induction (Bradshaw and Holzapfel 2001), thermal tolerance (David et al. 2003), and desiccation tolerance (Hoffmann et al. 2003). Reciprocal transplant experiments show that local adaptation is common and has substantial fitness benefits for adapted compared to non-adapted individuals (Hereford 2009, Auld et al. 2009). Phenotypic plasticity is also widespread, and sometimes has considerable adaptive value (DeWitt and Scheiner 2004). From an evolutionary standpoint, it is thus pertinent to investigate the costs and trade-offs that modulate the equilibrium between local adaptation and adaptive phenotypic plasticity, both intra- and inter-specifically.

Local adaptation may enhance performance in one environment, to the detriment of performance in other environments (possibly through trade-offs caused by antagonistic pleiotropy; Kawecki and Ebert 2004). Empirical data suggest that this cost to local adaptation is common but usually of small magnitude (Hereford 2009). Local adaptation will also be constrained by the presence of adaptive phenotypic plasticity, which will lessen the strength of selection on a trait (Forrest and Miller-Rushing 2010). Shorter generation times and greater genetic diversity will produce faster rates of evolution, and thus greater degrees of local adaptation (Chevin et al. 2010). Low levels of migration and a relatively homogeneous environment will also promote local adaptation (Sultan and Spencer 2002). In addition, if there are costs associated with phenotypic plasticity (Auld et al. 2009), evolution may favour individuals whose phenotypes are canalised to the mean environmental optimum, resulting in local adaptation. However, in metapopulations linked by migration, where the environment is spatially heterogeneous, plasticity is favoured even in the presence of moderate global or environment-specific costs, and even if there is considerable inaccuracy in the plastic response (i.e. the phenotype of plastic

individuals is frequently mismatched to the environment; Sultan and Spencer 2002). Ecological breadth is positively correlated with degree of phenotypic plasticity (Sultan 2001), and invasive species tend to be more plastic than non-invasive species, as plasticity enables them to persist in novel environmental conditions long enough for populations to become established (Davidson et al. 2011).

Despite local adaptation and phenotypic plasticity being frequently presented as mutually exclusive alternatives, whole organism life-history strategies are the composite outcome of trade-offs among multiple, interacting selection pressures on individual traits and life stages (Woods and Harrison 2002b), thus any individual may utilise a mixture of phenotypic plasticity in some traits, and environmental canalisation in others. Also, selection may act distinctly on different life stages, producing trade-offs. For example, high metabolic rates and thermal sensitivity promote fast rates of growth and development during the summer growing season (Ayres and Scriber 1994), but penalties may be incurred through higher energy use overwinter (Chapter 5, Pelini et al. 2009). This may prevent organisms from realising the fitness benefits of phenotypic changes, and higher-level measures such as fitness or life-history variation may obscure local adaptation and plasticity that occurred in physiological traits in order to maintain homeostasis (Schulte et al. 2011). To understand the mechanisms regulating plasticity and local adaptation it is thus necessary to track the effects of environmental conditions across multiple life stages, traits at various levels of organisation (i.e. whole-organism and physiological), and genetic backgrounds. The effects of selection may be most pronounced in life-stages that represent population bottlenecks, and such bottlenecks can occur over winter due to high rates of mortality (Roy and Sparks 2003). Thus, I

investigated local adaptation and phenotypic plasticity in the overwintering and post-overwintering stages of an insect.

Holometabolous univoltine insects can spend more than half their lives in an overwintering state. Winter conditions are a strong determinant of fitness (Bergland et al. 2005), and because winter conditions vary considerably with altitude and latitude, they can be instrumental in setting range limits (Crozier 2004; Jepsen et al. 2008). Thus, the overwintering period imposes strong selection pressure which produces both high levels of phenotypic plasticity in thermal physiology (numerous examples may be found in Hahn and Denlinger 2011) and divergence in thermal reaction norms of populations (e.g. Kukal et al. 1991; Gomi 2000; Bradshaw and Holzapfel 2001; Schaefer and Walters 2010). As insects are ectotherms, their body temperature equilibrates rapidly with that of the environment, and metabolic rate is dependent in turn on body temperature (Hahn and Denlinger 2011). Therefore, an increase in temperature will generally elicit an increase in metabolic rate. Temperate insects typically spend the winter in diapause, a state of dormancy in which metabolic rate is suppressed, and activity and feeding cease (Koštál 2006). Insects enter the winter with energy reserves that must not only sustain them through the overwintering period, but also fuel subsequent development and reproduction. Thus, overwintering energy reserves can determine fitness (Irwin and Lee 2000; Irwin and Lee 2003; Williams et al. 2003; Bosch and Kemp 2004; Koštál et al. 2011). During diapause, metabolism is decoupled from development, and any increase in metabolic rate will increase the rate of use of stored energy reserves, without a concomitant acceleration of development (Gray et al. 2001). Longer winters or warmer overwintering environments decrease survival and fecundity due to energy depletion, in a range of insect

taxa (Irwin and Lee 2003; Williams et al. 2003; Bosch and Kemp 2004; Mercader and Scriber 2008; Koštál et al. 2011). Anthropogenic climate change is leading to disproportionate autumn and winter warming (Bonsal and Kochtubjada 2009). Understanding the factors that determine an organism's capacity to adapt to its overwintering environment is therefore crucial to predicting species responses to climate change. The impact of warmer winters may be mitigated by local adaptation or plasticity of the temperature – metabolic rate relationship. A general suppression of metabolic rate (i.e. diapause; Hahn and Denlinger 2011) or a decrease in thermal sensitivity (Chapter 5) will conserve stored energy reserves, and is predicted in populations originating from, or exposed to, warm winter conditions.

The Fall webworm (*Hyphantria cunea* Drury, Lepidoptera: Arctiidae) is a widely distributed pest species whose native range extends from Florida to sub-boreal Canada (Wagner 2005), and which has invaded much of East Asia (Gomi et al. 2007). Larvae are polyphagous, feeding on over 400 species of woody plants, including cherry (*Prunus* spp.), black walnut (*Juglans niger*), elm (*Ulmus americana*), ash (*Fraxinus* spp.) and some species of maple (*Acer* spp.; Wagner 2005). *H. cunea* larvae are black-headed, although there is a sympatric sibling species of undetermined taxonomic status that has red-headed larvae with markedly different ecology (Takeda 2005). *Hyphantria cunea* overwinters in pupal diapause, and adults emerge in early summer (Takeda 2005). Reproductive capacity in *H. cunea* depends solely on reserves sequestered as larvae, as adults have degenerate mouthparts and do not feed, meaning that fitness can be strongly influenced by changes in temperature and duration of the overwintering period (Gomi 2000).

Populations of *H. cunea* inhabit a wide range of climatic conditions, from sub-tropical to high-latitude temperate, which suggests that this species must have a substantial capacity for local adaptation or plasticity. The theoretical framework outlined above enables us to make predictions on the development of local adaptation and phenotypic plasticity based on the life-history and demographic characteristics of *H. cunea*. Traits suggesting a propensity for local adaptation include moderate dispersal (Yamanaka et al. 2001), genetic differentiation of populations across their native range (Gomi et al. 2004), and high genetic diversity (Tao et al. 2009). Rapid local adaptation of development time, critical photoperiod for diapause induction, and number of larval instars has been detected in populations of *H. cunea* in Japan (Gomi and Takeda 1996; Gomi et al. 2003; Gomi 2007; Gomi et al. 2007). Generation time is positively correlated with latitude in North American populations of *H. cunea*, where populations are multivoltine in the southern portion of the range, transitioning to bi- and uni-voltine in the northern regions (Wagner 2005). As faster generation times increase the speed of local adaptation, southern populations may experience greater degrees of local adaptation than do northern populations. However, given the invasiveness and wide ecological niche of *H. cunea*, I expect substantial phenotypic plasticity in physiological and life-history traits.

In this study, I employ a reciprocal common garden design, using *H. cunea* from the northern edge or centre of their native range, collected at the end of the larval growing season, and overwintered in the laboratory at temperatures approximating the northern range edge or the centre of their range. As the majority of development occurred in the field prior to collection, source effects are due not only to the genetic background, but are also a result of developmental effects prior to collection, and maternal effects (Nijhout

and Davidowitz 2009). Thus, I am investigating the importance of carry-over effects due to both genetic and maternal factors, and developmental plasticity, in mediating an organism's response to overwintering conditions. As winter temperatures are predicted to change more than summer temperatures, at least in some regions (Bonsal and Kochtubjada 2009), understanding the relative impacts of changes in overwintering and growing season temperatures is highly salient. I will henceforth refer to the source populations as "ecotypes", to emphasize the joint impacts of genotype and environment. Local adaptation and adaptive phenotypic plasticity both have the potential to buffer organisms from deleterious consequences of changes in the thermal environment, thus I am looking for changes in inter-population differentiation in thermal reaction norms (functions describing the effects of temperature on a phenotype expressed by a population; Angilletta et al. 2003) of physiological and life-history traits, to provide evidence for adaptive changes in the thermal physiology of *H. cunea*. Acute thermodynamic effects on rates of biochemical reactions are expected to cause increased energy use in warm compared to cool overwintering conditions, with associated declines in energy reserves, mass, and consequently fitness (Hahn and Denlinger 2011). This is the null hypothesis against which I will evaluate my results.

I address the following questions: 1) How have local adaptation and phenotypic plasticity shaped overwintering physiological and life-history traits in two ecotypes of *H. cunea*, and what are the overall fitness consequences of these processes? 2) How well do demographic traits predict the evolution of local adaptation and plasticity? 3) Which traits are phenotypically-plastic, vs. locally-adapted, in this species? and 4) Do trade-offs

operate among life-stages, and how are these resolved at the physiological and life-history levels?

4.2 Materials and Methods

4.2.1 Microclimate data

I collected microclimate data using iButton thermochron data loggers (Model DS1922L, Maxim-Dallas Semiconductor; Sunnyvale, CA, USA), recording the temperature (± 0.5 °C) every hour from October 2008 - May 2009. I placed the data loggers in 10 mL plastic containers filled with silica gel to protect them from moisture damage, and deployed three loggers on the soil surface beneath the leaf litter layer in one woodlot near Ottawa, Ontario, and one near Athens, Ohio. The woodlots were dominated by black walnut (*Juglans nigra*), ash *(Fraxinus spp.)*, and cherry *(Prunus spp.*; Ottawa) or black walnut (Athens). *H. cunea* were present in these woodlots, and the placement of the loggers was designed to mimic the overwintering microhabitat of the pupae. I calculated bi-weekly mean daily maxima and minima for each location from the microclimate data, and used these to determine the temperature regimes used in the laboratory experiments.

4.2.2 Collection and rearing

Black-headed late-instar *Hyphantria cunea* larvae were collected by removing 20 entire nests from walnut trees in Columbus, Ohio, USA (CO: 40.06°N, 82.57°W) and Ottawa, Ontario, Canada (OT: 45.23°N, 75.43°W) in August 2009. One nest represents the entire reproductive output of one singly inseminated female (Jaenike and Selander 1980). The larvae were transported to the Biotron Experimental Climate Change Facility at the University of Western Ontario, where they were counted and reared to pupation on *ad*

libitum freshly cut local black walnut leaves in 3.7 L plastic containers (one nest per container) in temperature controlled chambers (EGC-TC2, Environmental Growth Chambers, Chagrin Falls, Ohio, USA) under a short day (12L:12D) photoperiod and a 20 °C:12 °C thermocycle, at 80% relative humidity. I checked the larvae every day for pupation, and upon pupation, the date was recorded and each pupa was randomly assigned to the warm (Columbus) or cool (Ottawa) overwintering treatment, giving rise to four treatment groups: CO/warm, OT/warm, CO/cool, and OT/cool. Throughout, I will use CO (Columbus, Ohio) and OT (Ottawa, Ontario) to refer to the source of the populations (i.e. the ecotype), and warm (Columbus, Ohio) and cool (Ottawa, Ontario) to refer to the overwinter thermal profiles (the environment). Host plant quality declined and the leaves started to senesce in late October, thus rearing was ended on 28 October 2009 and remaining larvae were discarded and included in larval mortality estimates. Pupae from each nest were allocated evenly between overwinter environments, but some nests were underrepresented in some treatments because of mortality.

The pupae were kept in the dark in 6-well cell culture plates with a moist paper towel on the lid to maintain high humidity, in MIR-153 incubators (Sanyo Scientific, Bensenville, IL, USA). Temperatures fluctuated on a 12:12 thermoperiod between the mean daily maximum and minimum microclimate temperatures for Ottawa and Ohio calculated from hourly microclimate data (Figure 4.1A-B). Incubators were adjusted every two weeks to track seasonal changes in microclimate temperatures. I weighed the pupae in November and April (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; d=0.1 μg) and measured their length (± 0.5 mm) using digital calipers (Mastercraft, Toronto, Ontario, Canada). At the same times (Figure 4.1B), 20 pupae from each treatment group were

flash-frozen in liquid nitrogen and stored at -80°C for metabolite analysis. At the beginning of April, all pupae were placed on moist vermiculite, and transferred to EGC-TC2 chambers on a long day photoperiod (16L:8D) under a 25 °C:15 °C thermocycle, at 80% relative humidity. Emergence was checked daily, and, when adult moths emerged, time taken to emerge following transfer to 25 °C was recorded, the moths were killed at -20 °C, and the length of the right forewing was measured from the proximal wing attachment point to the apex. Throughout the experiment, causes of any deaths were recorded as fungus (presence of fungus on pupa), parasitoid (presence of fly or wasp in plate with empty pupal case) or other (failure of pupa to develop, unsuccessful eclosion of adult from pupal case or adult with seriously deformed wings).

4.2.3 **Energy reserve assays**

To determine the effects of ecotype and overwintering environment on energy reserves, I measured triacylglycerides (TAGs), total carbohydrates, and protein in overwintering pupae at the beginning (October) and end (April) of winter. I weighed the pupae (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; d=0.1 μg) to determine fresh mass (FM) and then determined their sex by the presence (female) or absence (male) of a line intersecting the first abdominal sternite. I validated this method of sexing pupae on 77 pupae that were subsequently allowed to develop into adults, and sexed by the presence (males) or absence (females) of feathered antennae and claspers (Resh and Cardé 2009), with a success rate of 95%. Following initial sexing, I punctured each pupa five times with a dissecting pin, transferred them frozen to be lyophilized for 48 hours at ~13 Pa (Lyocentre lyophiliser, Virtis, Warminster, PA, USA), and reweighed them to determine dry mass (DM). I calculated water content as FM-DM.

Lyophilised pupae were homogenised using a mortar and pestle, and aliquots of tissue were weighed for the protein (~1 mg), lipid (~1 mg), or carbohydrate (~20 mg) assays. The tissue for each assay was placed into 1 mL 0.05% Tween 20 aqueous solution (for the protein assay), 2.5 mL chloroform: methanol (2:1 *v/v*; for the lipid assay), or 1.2 mL 30% KOH aqueous solution (for the carbohydrate assay).

I quantified protein using the BCA assay (described in Chapter 3), lipids by TLC-FID (as described in Chapters 2 and 3, with a monoacylglyceride standard), and carbohydrates by the anthrone method as described in Chapter 3. The anthrone method measures total carbohydrates including free (glucose) and bound (glycogen) carbohydrates, but does not measure polyols such as glycerol and sorbitol (Graham 1963).

I expressed TAG, carbohydrate and protein concentrations in $\mu g \cdot mg\ DM^{-1}$, then scaled them up to whole-animal values by multiplying by total DM. I subtracted whole-animal TAG and carbohydrate from DM to give lipid- (and carbohydrate-) free DM. I calculated total energy content in J/pupa assuming 39.3 $J \cdot mg\ TAG^{-1}$ and 17.6 $J \cdot mg\ carbohydrate^{-1}$ (Djawdan et al. 1997).

4.2.4 **Respirometry**

To assess plasticity and local adaptation in the temperature-metabolic rate relationship, I measured the CO_2 emission of six pupae from each treatment group over a range of temperatures in November (beginning of winter) and April (end of winter). I measured each pupa five times: at 5, 10, 15, 20 and 25 °C. The order of temperatures and times of day of measurement (8am-8pm) were randomized, and there was no less than 48 hours between measurements on any individual. Pupae were weighed before each measurement.

I measured CO_2 emission as a proxy for metabolic rate using flow-through respirometry as previously described (Williams et al. 2010). Briefly, air was scrubbed of CO_2 and water vapour using a Drierite-Ascarite-Drierite column and passed through a 4 cm^3 chamber at 50 $mL \cdot min^{-1}$, controlled by mass-flow valves (Sierra Instruments, Monterey, California, USA) and a mass-flow controller (Sable Systems International [SSI], Las Vegas, Nevada), and measured by a SS3 subsampler (SSI). I used an RM-8 multiplexer (SSI) to route the air flow through either an empty reference chamber (baseline) or one of six chambers containing a single pupa, then to an Li7000 infrared CO_2 analyser (LiCor; Lincoln, NE, USA) that measured water vapour and CO_2 emission. I controlled the temperatures (± 0.1 °C) using a PELT-5 temperature-controlled cabinet (SSI) in which all chambers were contained. Data were acquired with a UI2 interface (SSI). I drift-corrected water and CO_2 measurements to the baseline chamber and simultaneously inter-corrected for dilution effects (Lighton 2008), then converted into $mL \cdot min^{-1}$ by multiplying by instantaneous flow rates. As the dormant pupae all showed continuous and steady respiration over the time of measurement, I calculated mean CO_2 emission rate of each pupa over a 30 min period after a minimum of 1 h acclimation and a 10 min washout. I converted CO_2 emission to energy equivalents using the equation

$$E = V_{CO_2} \times RER \times \text{oxyjoule equivalent} \times 60 \quad (1)$$

where E is energy in Watts ($J \cdot s^{-1}$); V_{CO_2} is the whole-animal rate of CO_2 production; RER is the respiratory exchange ratio (CO_2 produced to O_2 consumed), here, assumed to be 0.8; and the oxyjoule equivalent is the number of joules of heat energy produced per unit of O_2 consumption (Lighton 2008), given here by the equation

$$\text{oxyjoule equivalent} = 16 + 5.164 \times \text{RER} \quad (2)$$

Both equations are from Lighton (2008). As RERs of non-assimilating organisms vary from 0.7 - 1 depending on the metabolic substrate, error ranging from -3 to +5% may be introduced in equation (1) by an incorrect assumption of RER (Lighton 2008). However, as the value of the oxyjoule equivalent in equation (2) also depends on RER, and the error introduced at this step is in the opposite direction, the assumption of an RER of 0.8 will cause less than 0.6 % error in metabolic rate estimates over the entire physiological range of RER (Lighton 2008).

4.2.5 **Data Analysis**

All statistical analyses were performed in R 2.13.0 (R Development Core Team 2010) unless otherwise noted. Preliminary data exploration was performed according to Zuur et al. (2010). My modeling approach involved starting with maximal models (all main effects and all possible interactions) and terms were dropped sequentially based on statistical non-significance. Akaike's information criterion (AIC) was used to confirm the improved fit of the reduced model, with a threshold difference in AIC of less than two points as the criteria for retaining the simplified model (Crawley 2007). Terms which are not reported were non-significant, and thus did not remain in the minimal adequate model. The "drop1()" function was used to compare Type III sums of squares. In all cases, I used Tukey's *post hoc* tests to evaluate differences amongst treatment groups. Significant relationships between response variables and covariates were explored using Pearson's product-moment correlations. Analyses were performed on transformed data

where necessary to meet assumptions of statistical tests, although raw data are presented for display purposes.

All life history variables were normally distributed except for date of pupation, mass loss over winter, and mass of adults upon eclosion. Date of pupation was bimodal but the modes did not correspond to any of my grouping variables (sex, survival, source, or overwintering environment). Normality of adult mass was restored after arcsine transformation, and that of overwinter mass loss after $\log_{10}$ transformation. I was not able to transform date of pupation to approximate a normal distribution, but as the sample size was large (n=542 individuals in total), ANOVA is adequately robust to cope with this deviation (Crawley 2007).

I first assessed whether pupal mass in October, date of diapause, and sex were significant predictors of overwinter survival using a generalised linear model (GLZ) with a binomial error structure. For variables that were significant predictors of survival, I used only survivors for subsequent analyses; however for variables that did not significantly predict survival I used the whole data set (all pupae). In all cases, results from the two data sets gave the same conclusions. I compared larval mortality between ecotypes, and overwinter mortality among ecotypes and overwintering environments, using GLZs, again with binomial error structures. I compared cause of mortality among ecotypes and environments using a Chi-Square test.

I compared date of pupation (as Julian day) among treatment groups (in all pupae) using ANOVA with overwintering environment, ecotype and sex as factors. I correlated pupal mass in October and date of pupation to check for covariance between these variables. I

compared pupal mass (in October and April), adult mass, and post-winter development time amongst treatment groups using ANOVA with overwintering environment, ecotype and sex as factors. Overwinter mass loss was compared among ecotype × overwintering environment combinations using ANCOVA with overwintering environment, ecotype and sex as factors, and mass at the beginning of winter as a covariate.

In addition to univariate comparisons, I assessed the interactions among life-history variables (pupal mass at start and end of winter, pupal length, adult mass, wing length, diapause date, and development time) using principal components analysis (PCA) using the "prcomp()" function. I centered and scaled the data to have equal means and variances, prior to rotating the loading matrix to obtain orthogonal factors. Principal components (PCs) were retained based on eigenvalues >1, and a position above the inflection point of a scree plot (Tabachnik and Fidell 2007); and defined based on factors with loading >0.2 on each component. Responses related to these factors were saved as factor scores defining the position of each individual on each PC, and multivariate analyses of variance were performed on these factor scores with the grouping variables sex, source population, and overwintering environment. Univariate statistics (ANOVA) with the grouping variables above were also performed on each PC to determine the dimensions that best separated each treatment group.

I used matrices of Pearson's product-moment correlations to identify correlated energy reserves. I compared total protein and water among treatment groups using ANOVA with ecotype, overwintering environment, sex and time (beginning or end of winter) as factors. To estimate overwinter water loss for each ecotype × sex combination, I subtracted mean

water content at the end of winter from mean water content at the beginning of winter. I compared TAG, and carbohydrate among groups using ANCOVA with ecotype, overwintering environment, sex and time as factors and date of diapause and total protein as covariates.

At the end of winter, the metabolic rate at 15 °C for one individual from OT/cool was lost due to equipment malfunction. I interpolated to this value using a linear regression of $\log_{10}$ metabolic rate against measurement temperature for that individual. Metabolic rate was $\log_{10}$-transformed to meet assumptions of normality. I compared $\log_{10}$-transformed metabolic rates among treatment groups using repeated measures ANCOVAs with measurement temperature and mass as covariates and ecotype and overwintering environment as factors, and individual as an error term. I first performed linear regression of order of measurement within a respirometry trial on metabolic rate to ensure there was no effect of measurement order within each trial.

To summarise the evidence for local adaptation and phenotypic plasticity, I plotted the thermal reaction norms for overwinter life-history and physiological fitness correlates (size measurements, development time, water content, lipid reserves, and overwinter mortality). I used predicted values from each model for females only, as the strongest selection for size is on females (Oberhauser 1997), and there were no significant interactions between sex and any other parameter. If sampling time was a significant factor, or there were significant interactions between time and water or TAG content, I separated the time points and repeated the models without sampling time or related

interaction terms to obtain predicted values, dropping non-significant terms within each time point as described above.

4.3 Results

4.3.1 Microclimate temperatures

Within each site, temperatures at the three data loggers varied by less than 0.4 °C, and are summarized in Table 4.1 and Figure 4.1. Mean microclimate temperatures at the Athens site (near Columbus) were warmer than those at the Ottawa site in every month from October to May (Figure 4.1, Table 4.1). At the Athens site, the soil was presumed to be covered by snow (because microclimate temperatures remained close to 0 °C with very low variability) for only a few weeks in January, while at the Ottawa site, there was some snow in late November, and continuous cover from mid December to late March (Figure 4.1, Table 4.1). This resulted in microclimate temperatures remaining at 0 °C with very little variability at the Ottawa site during December - March (Table 4.1). In months without snow cover, thermal variability of microclimates at the two sites was similar. The absolute minimum temperature was lower at the Athens site than the Ottawa site, and the absolute maximum was higher in Ottawa (Table 4.1).

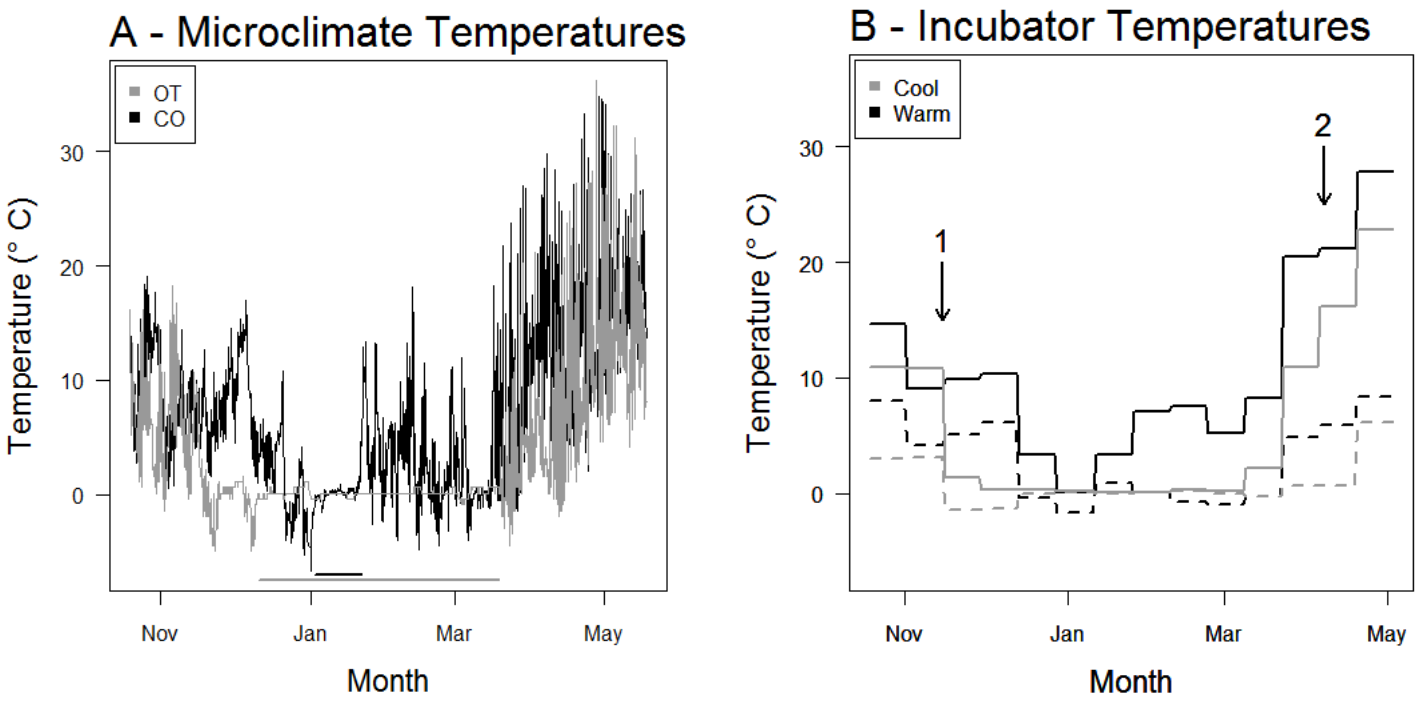

Figure 4.1- Microclimate temperatures for sites near Ottawa, Ontario (OT) and Athens, Ohio (CO), and incubator temperatures derived from those data. (A) Representative traces of microclimate temperatures from under the leaf litter in woodlots where *H. cunea* occur near the collection sites in OT or CO from October 2008 to May 2009; measured by iButton data loggers. Horizontal lines below indicate the period of presumed continuous snow cover at each site, determined by continuous zero temperatures and low thermal variability. (B) Day- (solid lines) and night-time (dashed lines) temperatures of incubators (on a 12:12 thermoperiod) used to house *H. cunea* under conditions approximating CO (warm) or OT (cool). Incubator temperatures were derived from fortnightly mean daily minima and maxima for Oct 2008 - May 2009, calculated from microclimate temperatures from three iButtons per site. Arrows indicate sampling time points for the beginning (1) and end (2) of winter, when I took life-history and respirometry measurements, froze samples for metabolite analysis, and moved pupae into emergence conditions (end of winter only).

Table 4.1- Summary of microclimate temperatures from *H. cunea* habitat in Ottawa, Ontario (origin of OT ecotype) or Athens, Ohio (near Columbus, origin of CO ecotype). Data are soil surface temperatures in winter 2008 - 2009, from iButton data loggers in the leaf litter (N=3 per site). N= number of loggers per site; Snow days = days of snow cover. Daily minima, mean and maxima in °C (mean ± SD); Location in latitude, longitude; Cumulative snow cover is in weeks.

	Ottawa				**Athens**			
	Daily minimum	Daily Mean	Daily maximum	Snow days	Daily minimum	Daily Mean	Daily maximum	Snow days
October	0.4 ± 2.2	6.0 ± 3.5	18.7 ± 4.2	0	2.3 ± 3.5	11.3 ± 4.2	20.0 ± 2.7	0
November	-5.2 ± 3.3	2.9 ± 4.3	18.5 ± 5.8	0	-0.5 ± 2.2	6.9 ± 3.9	16.3 ± 2.2	0
December	-4.4 ± 1.5	0.0 ± 1.0	2.1 ± 0.6	18	-7.2 ± 5.2	3.3 ± 6.3	18.8 ± 5.4	0
January	-0.4 ± 0.2	0.0 ± 0.2	0.4 ± 0.2	31	-5.0 ± 2.1	1.6 ± 3.3	14.8 ± 4.3	28
February	0.1 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	28	-6.1 ± 2.5	2.8 ± 4.3	19.8 ± 4.4	6
March	-4.7 ± 1.5	1.7 ± 3.9	23.7 ± 5.4	25	-5.8 ± 2.5	4.0 ± 5.5	25.8 ± 8.9	5
April	-2.4 ± 3.0	8.7 ± 7.5	39.2 ± 8.1	0	2.8 ± 2.8	12.7 ± 5.5	33.5 ± 7.7	0
May	3.9 ± 2.6	13.7 ± 5.7	33.7 ± 7.2	0	9.3 ± 2.2	17.2 ± 4.1	33.7 ± 5.5	0
Absolute min	-5.4				-9.1			
Absolute max	42.1				34.8			
Cumulative snow cover	14.5				5.5			
Location	45.2°N, 75.4°W				39.2°N, 82.0°W			

4.3.2 **Life history**

Over 1800 larvae, from at least 19 females, were collected from each location (Table 4.2). Larval survival was significantly lower in CO larvae (8.3 %) than in OT larvae (13.2 %; Tables 4.2 and 4.3). Overwintering survival was highest in the CO ecotype and the cool environment, and there was a significant ecotype × environment interaction such that the CO ecotype had higher survival in the cool environment, while the other three treatment groups had similar survival (Tables 4.2 and 4.3). Survival ranked as follows (from lowest to highest): OT/warm, CO/warm, OT/cool, CO/cool (Table 4.2). There were no differences in the cause of mortality (parasitoid, fungus, or failure to eclose successfully) among treatment groups (χ^2=7.0, df=6, p=0.321). High pupal mass was a significant predictor of overwintering survival ($F_{1,185}$=39.61, p<0.001), while date of pupation and sex were not significant factors (p>0.05). Models describing treatment effects on date of pupation were thus fitted for all pupae (to ensure that sample sizes were sufficiently large that ANOVA was adequately robust to the bimodal distribution), while size variables were analysed using only those individuals that survived to adulthood.

Table 4.2 - Experimental design and life-history variables of *Hyphantria cunea*, collected from woodlots near Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. Eco = Ecotype; Env = overwintering environment; N_{nests}, N_{larvae}, N_{pupae} and N_{adults} = numbers of nests, larvae collected, individuals successfully pupating, and individuals successfully eclosing as adults respectively. Significant effects refer to the outcome of models described in Table 4.5; those beside sample sizes refer to survival of that life stage to the subsequent stage (i.e. a significant effect on N_{larvae} refers to numbers of larvae surviving to pupation, in other words larval mortality). Values are mean ± SD.

Eco	**CO**		**OT**		**Significant effects**
N_{nests}	19		20		
N_{larvae}	2563		1896		Eco
Env	**warm**	**cool**	**warm**	**cool**	
N_{pupae}	141	71	119	132	Eco, Env
Diapause date	Sep 14 ± 11	Sep 16 ± 12	Sep 30 ± 16	Sep 27 ± 16	Eco
Survival (%)	9.2	28.6	7.6	10.6	Eco, Env
Sex (M/F)	6/7	8/11	3/6	9/5	

Table 4.3 - Effects of ecotype (CO or OT), overwintering environment (warm or cool), and sex on life-history variables of *H. cunea* from Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. Models are generalized linear models (GLZ) for larval and overwinter mortality; or analyses of variance (ANOVA) for all other variables. See text for details of modeling approach that gave the minimal adequate model according to AIC and ANOVA. Eco = Ecotype, Env = Environment. $Mass_{Oct}$, $Mass_{Apr}$ = pupal mass in October or April; Mass loss = pupal mass loss over winter; Development = days to emerge after transfer to 25 °C.

Variables (y)	Maximal model	Minimal adequate model	Term	F ratio	df	p
Larval mortality	y=Eco	y = Eco	Eco	43.18	1,4457	<0.001
Winter mortality	y=Eco×Env×Sex	y=Eco+Env+Eco×Env	Eco	12.04	1,462	<0.001
			Env	15.23	1,462	<0.001
			Eco×Env	4.35	1,462	0.042
Diapause date	y=Eco×Env×Sex	y=Eco	Eco	157.45	1,461	<0.001
$Mass_{Oct}$	y=Eco×Env×Sex	y=Eco+Sex	Eco	5.87	1,52	0.019
			Sex	12.29	1,52	<0.001
$Mass_{Apr}$	y=Eco×Env×Sex	y=Eco+Sex	Eco	4.07	1,52	0.049
			Sex	4.35	1,52	0.042
Mass loss	$\log_{10}(y)$=Eco×Env×Sex×$Mass_{Oct}$	$\log_{10}(y)$=$Mass_{Oct}$	$Mass_{Oct}$	6.27	1,53	0.015
Pupal length	y=Eco×Env×Sex	y=Eco	Eco	13.77	1,53	<0.001
Adult mass	arcsin(y/100)=Eco×Env×Sex	arcsin(y/100)=Sex	Sex	41.20	1,53	<0.001
Wing length	y=Eco×Env×Sex	y=Sex	Sex	4.41	1,53	0.041
Development	y=Eco×Env×Sex	y=Eco+Env	Eco	20.28	1,52	<0.001

Larvae from CO pupated earlier than larvae from OT (Tables 4.2 and 4.3). There was no relationship between mass and date of pupation ($p>0.05$). All pupal and adult size measurements were highly correlated (Table 4.4). In October, when pupae were split between overwintering treatments, females were heavier than males, pupae from Columbus were heavier than those from Ottawa, and the treatments did not differ (Tables 4.3 and 4.5). In April, females and pupae from CO were still heavier, although the magnitude of the difference between the sexes was reduced (Tables 4.3 and 4.5). Mass loss did not differ between sexes, source populations or overwintering environments (Tables 4.3 and 4.5). Pupae from CO were longer than those from OT, with no effect of sex or overwintering environment on length (Tables 4.3 and 4.5). Adult females were heavier than adult males, and although adults from CO tended to be heavier than adults from OT, the main effect of ecotype on adult mass was marginally non-significant (Tables 4.3 and 4.6). The wing length of adult females was also significantly longer than that of males, with no effect of ecotype or overwintering environment on wing length (Tables 4.3 and 4.6). Post-winter development time was shorter for pupae that overwintered at southern temperatures than for those that overwintered at northern temperatures, and was also shorter for pupae from OT than for those from CO (Tables 4.3 and 4.6).

Table 4.4 - Pairwise Pearson's product-moment correlations among size measurements for pupal and adult *H. cunea*. $Mass_{Oct}$ and $Mass_{Apr}$ = pupal mass in October and April respectively. df = 53 for all correlations.

$Mass_{Oct}$	r=0.69 p<0.001	r=0.83 p<0.001	r=0.74 p<0.001	r=0.54 p<0.001
	Pupal length	r=0.75 p<0.001	r=0.50 p<0.001	r=0.35 p=0.009
		$Mass_{Apr}$	r=0.64 p<0.001	r=0.45 p<0.001
			$Mass_{Adult}$	r=0.51 p<0.001
				Wing length

Table 4.5 - Pupal size measurements of *Hyphantria cunea*, collected from woodlots near Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. Eco = Ecotype; Env = overwintering environment; $Mass_{Oct}$, $Mass_{Apr}$ = pupal mass in October or April; Mass loss = pupal mass loss over winter. Significant effects refer to the outcome of models described in Table 4.5, NS = no significant effects. Values are mean ± SD.

Eco	**CO**				**OT**				
Env	**warm**		**cool**		**warm**		**cool**		
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**Significant effects**
$Mass_{Oct}$ (mg)	102.80 ± 7.73	126.67 ± 11.14	95.30 ± 9.63	109.11 ± 21.81	102.22 ± 3.78	97.30 ± 20.69	87.20 ± 12.06	109.08 ± 13.21	Sex, Eco
$Mass_{Apr}$ (mg)	104.57 ±16.50	108.98 ± 17.72	90.52 ± 9.74	100.69 ± 25.41	87.90 ± 19.70	93.24 ± 19.58	82.39 ± 10.67	101.82 ± 12.45	Sex, Eco
Mass loss (mg)	4.16 ± 1.90	7.86 ± 4.08	4.78 ± 2.67	6.17 ± 1.85	4.69 ± 0.69	4.06 ±1.53	4.80 ± 1.83	7.26 ± 2.77	NS
Mass loss (%)	4.07 ± 2.01	6.25 ± 3.15	5.05 ± 2.78	6.02 ± 2.52	4.53 ± 0.82	4.13 ± 1.24	5.44 ± 1.36	6.64 ± 2.39	
Length (mm)	12.2 ± 0.5	12.0 ± 0.3	11.2 ± 0.8	11.7 ± 1.0	11.1 ± 0.7	11.0 ± 0.6	11.0 ± 0.3	11.0 ± 0.7	Eco

Table 4.6 - Adult size and development time of *H. cunea*, collected from woodlots near Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. Significant effects refer to the outcome of models described in Table 4.5. Values are mean ± SD. Eco = Ecotype; Env = overwintering environment; Development = development time post-transfer to 25 °C.

Eco	**CO**				**OT**				
Env	**warm**		**cool**		**warm**		**cool**		
Sex	**M**	**F**	**M**	**F**	**M**	**F**	**M**	**F**	**Significant effects**
Mass (mg)	45.85 ± 10.98	86.70 ± 11.50	42.50 ± 7.25	65.78 ± 25.78	34.91 ± 13.23	62.80 ± 18.90	35.35 ± 2.60	70.01 ± 10.72	Sex
Wing length (mm)	12.1 ± 1.3	13.7 ± 2.8	12.5 ± 1.0	12. 6 ± 2.8	12.2 ± 0.5	13.1 ± 1.0	11.3 ± 1.7	13.0 ± 1.9	Sex
Development (days)	43 ± 3	42 ± 3	46 ± 2	47 ± 3	36 ± 0	38 ± 2	43 ± 6	43 ± 6	Eco, Env

A principal components analysis of the life history variables provided three principal components (PCs) that cumulatively explained 79 % of the variation in the data set (Table 4.7). The first PC represents size, with all five size variables loading positively (Table 4.7). The second PC represents the effect of length of dormancy on adult size, with diapause date and wing length loading positively while emergence date loaded negatively (i.e. positive scores on this axis represent long periods of dormancy and small adults, Table 4.7). The third PC describes the influence of phenology on the relationship between pupal and adult size; diapause date, emergence date, adult mass and wing length load positively, while pupal mass and length load negatively (Table 4.7). Thus, delays in phenology (later diapause and emergence dates) are associated with larger adults compared to their pupal size. MANOVA indicated that the components differed significantly between ecotypes, overwintering environments, and sexes (Table 4.8). Univariate analysis of each component indicated that PC1 (Size) was significantly affected by source and sex (Table 4.8), and, in concordance with my univariate analyses described above, females and those from CO were generally larger (Figure 4.2). PC2 (Length of dormancy) was affected by both ecotype and overwintering environment (Table 4.8), such that individuals from OT and those overwintered at southern temperatures had shorter periods of dormancy and larger adult size (Figure 4.2). PC3 (Phenology) did not differ between ecotypes, overwintering environments, or sexes (Table 4.8). In summary, the CO ecotype was larger in all pupal size measurements, but not in adult size, and had longer winters due to earlier entry into diapause and longer development times (Figure 4.3). A warmer overwintering environment decreased development time and thus length of dormancy in both ecotypes (Figure 4.3).

Table 4.7- A summary of principal components describing the relationship among life-history characteristics of *H. cunea* collected from Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. Values are factor loadings (total variation explained by principal component in parentheses). NS indicates factors that did not load significantly (factor loadings <0.2). Env = overwintering environment (warm or cool), Eco = ecotype (CO or OT); Development = days to develop post-transfer to 25 °C.

	PC1 loading (49.3 %)	PC2 loading (19.7 %)	PC3 loading (10.3 %)
Name of PC	Size	Length of dormancy	Phenology
Pupal $mass_{Oct}$	0.501	NS	NS
Pupal $mass_{Apr}$	0.487	NS	-0.241
Pupal length	0.436	NS	-0.205
Adult mass	0.442	NS	0.237
Wing length	0.352	-0.252	0.312
Development	NS	0.661	0.712
Diapause date	NS	-0.681	0.489
Significant effects	Eco, Sex	Eco, Env	none

Table 4.8 - Effects of ecotype (CO or OT), environment (warm or cool), and sex on factor scores on principal components (PCs) from a principal components analysis of life-history characteristics of *H. cunea*. Models are MANOVA (where there are multiple variables) or ANOVA. Insects were collected from woodlots near Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) microclimate temperatures in a reciprocal common garden experiment. See text for details of modeling approach that gave the minimal adequate model according to AIC and ANOVA. PC = principal component (from Table 4.7), Eco = Ecotype, Env = Environment. NS = no significant terms.

Variable (y)	Maximal model	Minimal adequate model	Term	F	df	p
Factor scores on PC1, PC2, PC3	y = Eco × Env × Sex	y = Eco + Env + Sex	Eco	6.37	3,49	<0.001
			Env	13.62	3,49	<0.001
			Sex	5.49	3,49	0.002
Factor scores on PC1	y = Eco × Env × Sex	y = Eco + Sex	Eco	8.15	1,52	0.006
			Sex	11.85	1,52	0.001
Factor scores on PC2	y = Eco × Env × Sex	y = Eco + Env	Eco	36.59	1,52	<0.001
			Env	36.35	1,52	<0.001
Factor scores on PC3	y = Eco × Env × Sex	Null model	NS			

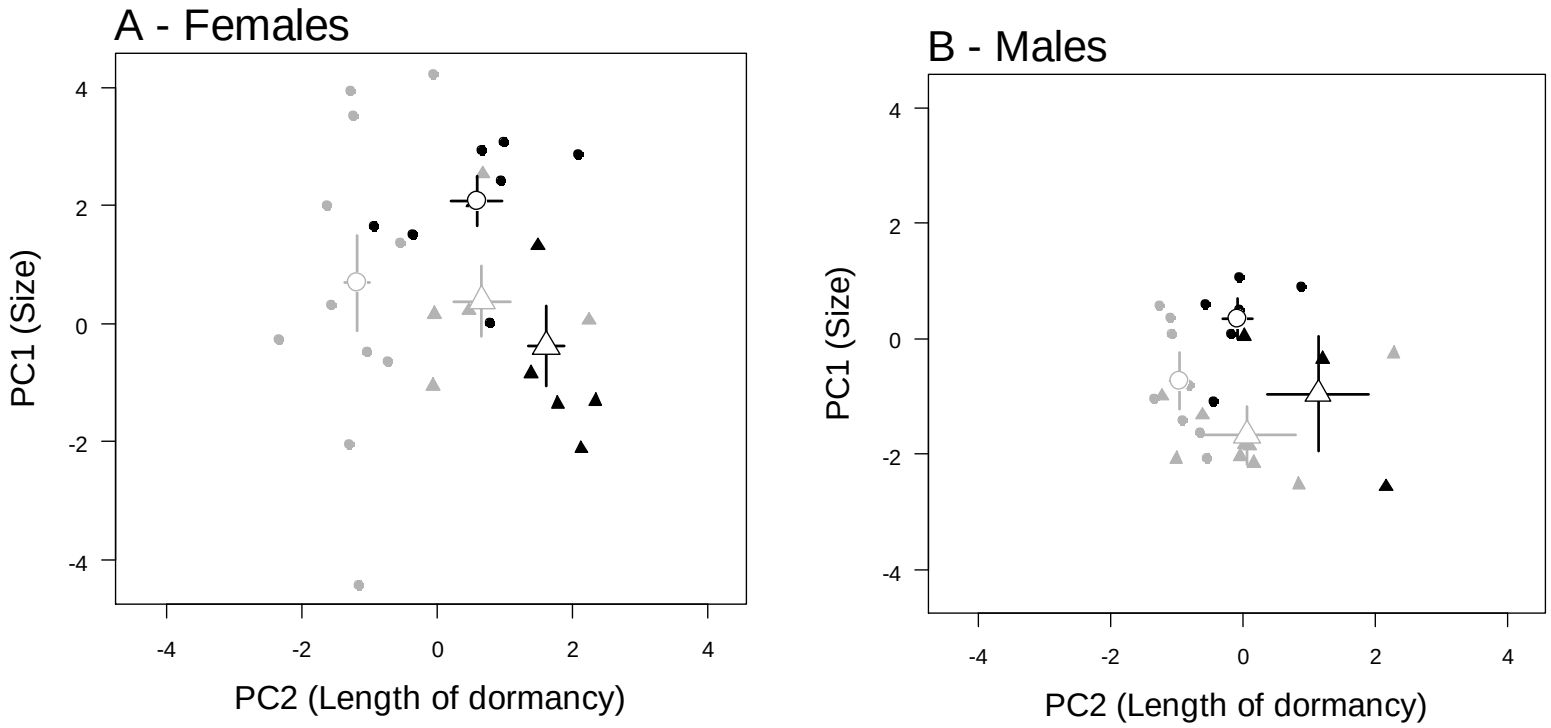

Figure 4.2 - Principal components analysis of life-history variables of *H. cunea*, describing effects of ecotype (Columbus, Ohio [CO] or Ottawa, Ontario [OT]) and environment (temperatures approximating CO [warm] or OT [cool]) on size and length of dormancy. (A) Females; (B) Males. Individuals from CO (circles) or OT (triangles) were overwintered in the laboratory at warm (black) or cool (grey) temperatures in a reciprocal common garden experiment. PC1 and PC2, represent size and length of winter (with an adult size decrease associated with longer winters), and account for 49 % and 20% of the variance, respectively (Table 4.7). Large open symbols show means for each group, error bars are SEM; if not visible they are obscured by the symbol.

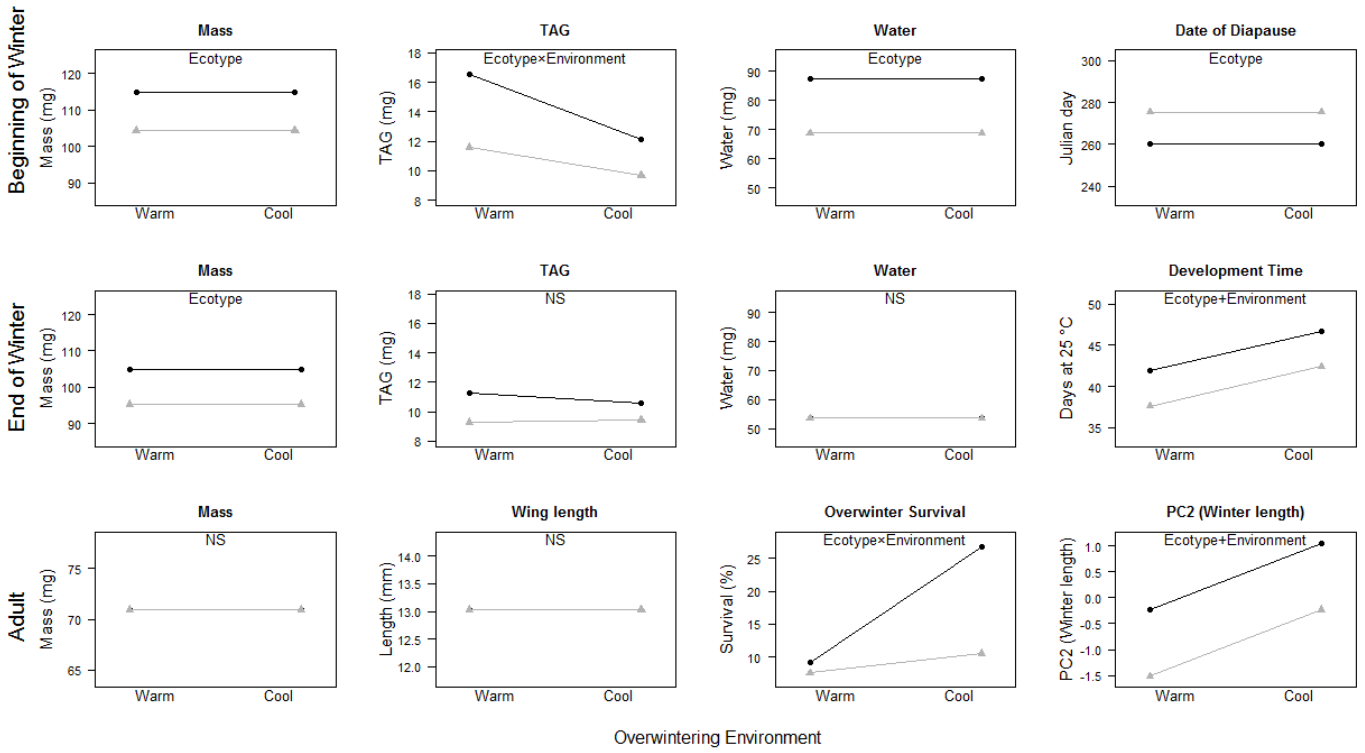

Figure 4.3 - Thermal reaction norms for life-history (Tables 4.2, 4.5 and 4.6) and physiological variables (Figures 4.4 and 4.5) of *H. cunea* from Columbus, Ohio (CO, black circles) or Ottawa, Ontario (OT, grey triangles); overwintered in the laboratory at temperatures approximating CO (warm) or OT (cool) microclimates in a reciprocal common garden experiment. Beginning of winter = pupal measurements from October; End of winter = pupal measurements from April; Adult = Adult measurements (Mass and Wing length), survival to adulthood (Overwinter Survival), or the second principal component from principal components analysis (PC2 [Winter length]; Figure 4.2, Table 4.7), which illustrates a trade-off between winter length and adult size, such that longer winters = smaller adults. Significant effects of ecotype, environment, or their interaction are indicated at the top of the figure; NS = no significant effect of ecotype or environment (from Tables 4.3, 4.8 and 4.9). If black lines are not visible, they are obscured by grey lines.

4.3.3 **Energy Reserves**

Fresh mass, lipid-free dry mass, triacylglyceride content, and protein content were all normally distributed. Carbohydrate content had a left-skewed distribution, and was ln-transformed to meet assumptions of normality. There were significant positive correlations ($r>0.4$, $p<0.001$) between each size variable (fresh mass, lipid-free dry mass, and total protein) and both triacylglyceride and carbohydrate. Therefore, I chose total protein as a size covariate since it does not include water or non-soluble (cuticular) protein, and is likely a good proxy for amount of metabolising tissue. There was a significant correlation between total protein and LFDM ($r_{77}=0.465$, $p<0.001$), and the general trends (sex and ecotype effects) were the same for both variables. Fresh mass was closely correlated with water content ($r_{77}=0.96$, $p<0.001$). There was a strong positive correlation between fresh mass and total TAG ($r_{77}=0.632$, $p<0.001$).

Total protein content was significantly higher in female than male pupae, and in pupae from CO than those from OT (Table 4.9, Figure 4.4A). Water content decreased over the course of the winter, was higher in females than males, and was higher in pupae from CO than those from OT (Table 4.9; Figure 4.4B). There was a significant ecotype × sampling time interaction on water content (Table 4.9), so I calculated average water loss for each ecotype across the entire winter, and found that those from CO lost far more water over winter (39 mg) than those from OT (14 mg); so that although pupae from CO tended to start the winter with greater water content than those from OT, their water content was lower at the end of the winter (Figure 4.4B).

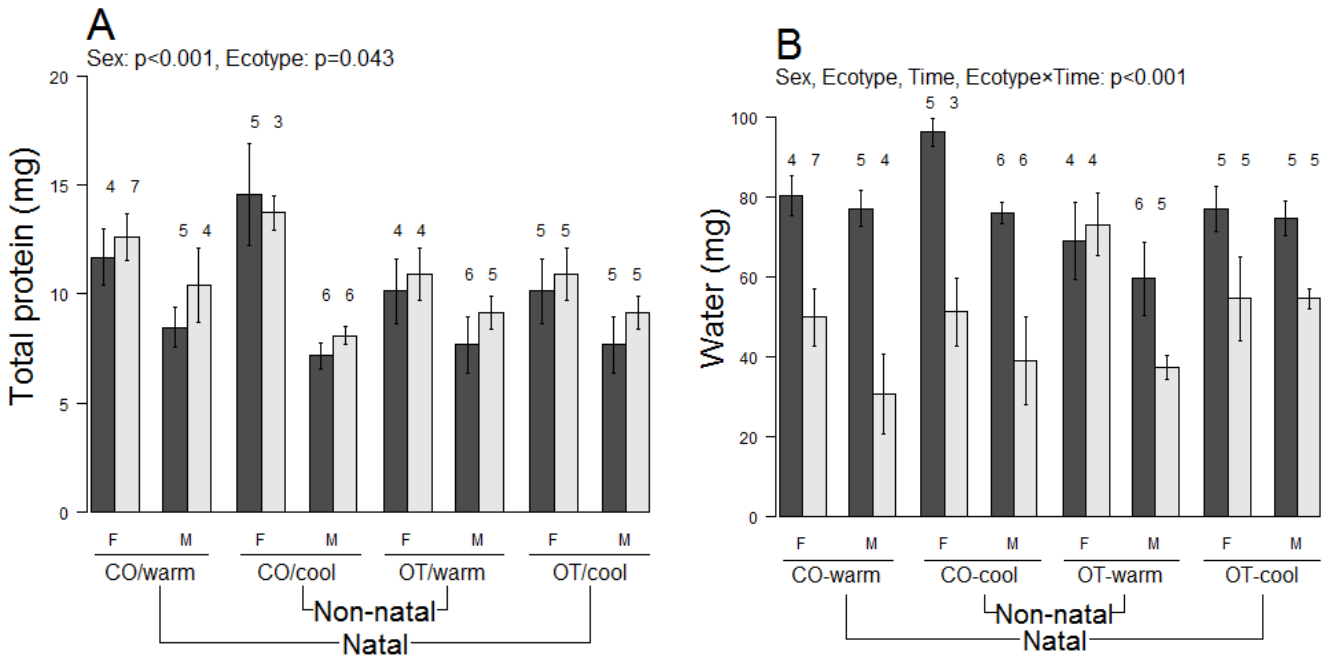

Figure 4.4 - (A) Total protein and (B) water content at the beginning (dark bars) and end (light bars) of winter for *Hyphantria cunea* pupae from Columbus, Ohio (CO) or Ottawa, Ontario (OT); overwintered in the laboratory at temperatures approximating CO (warm) or OT (cool) microclimates in a reciprocal common garden experiment. Significant effects indicated above are from ANCOVAs (Table 4.9): Ecotype = source population (CO or OT); Time = sampling time point (beginning of end of winter). M=males, F=females. Natal refers to populations experiencing an overwintering environment similar to the microclimate from their source region, Non-natal refers to those experiencing an overwintering environment similar to the other source region. Bars show mean ± SEM per pupa. Sample sizes above bars.

Table 4.9 - Analysis of variance (ANOVA) examining for effects of ecotype (CO or OT), environment (warm or cool), and sex on metabolite content of *H. cunea*. Insects were collected from woodlots near Columbus, Ohio (CO) or Ottawa, Ontario (OT) and overwintered in the laboratory at CO (warm) or OT (cool) temperatures in a reciprocal common garden experiment. Minimal adequate model determined by comparison of AIC and ANOVA (see text for details). Carb = carbohydrate; Eco = Ecotype; Env = Environment; TAG= triacylglyceride; Diapause = date of diapause; Water = water content. Metabolic rates (Met rate) were measured by respirometry in October and April. Temp = measurement temperature, Mass = pupal mass before respirometry, Ind = individual. Continued on next page.

Variable (y)	Initial model	Minimal adequate model	Term	F	df	p
Protein	y=Eco×Env×Sex×Time	y = Eco + Sex	Eco	4.23	1,76	0.043
			Sex	41.16	1,76	<0.001
Water	y=Eco×Env×Sex×Time	y=Eco+Sex+Time+Eco×Time	Eco	14.24	1,74	<0.001
			Sex	11.95	1,74	<0.001
			Time	64.38	1,74	<0.001
			Eco × Time	12.81	1,74	<0.001
TAG	y=Eco×Env×Sex×Time ×Protein×Diapause	y=Eco+Env+Protein+ Diapause+Env×Time+ Eco×Diapause+Env×Diapause	Eco	22.77	1,70	<0.001
			Env	5.26	1,70	0.025
			Protein	18.26	1,70	<0.001
			Diapause	8.09	1,70	0.006
			Env×Time	3.67	2,70	0.030
			Eco×Diapause	25.03	1,70	<0.001
			Env×Diapause	5.89	1,70	0.018

Table 4.9 continued.

Variable (y)	Initial model	Minimal adequate model	Term	F	df	p
Carb	y=Eco×Env×Sex×Time×Protein	y=Sex+Time+Protein	Sex	7.17	1,75	0.009
			Time	5.18	1,75	0.026
			Protein	10.06	1,75	0.002
Met $rate_{Oct}$	log10(y)=Eco×Env×Temp×Mass+Error(Ind)	log10(y)=Env+Temp+Mass+Error(Ind)	Env	5.28	1,20	0.032
			Temp	120.85	1,94	<0.001
			Mass	3.11	1,20	0.093
Met $rate_{Apr}$	log10(y)=Eco×Env×Temp×Mass+Error(Ind)	log10(y)=Temp+Temp×Eco+Error(Ind)	Temp	294.51	1,94	<0.001
			Temp×Eco	6.17	1,94	0.015

Triacylglyceride was the dominant neutral lipid component present in *H. cunea*, with trace amounts of cholesterol and cholesterol esters (likely membrane constituents; Fast 1970), alkanes (likely cuticular hydrocarbons, Fast 1970), and free fatty acids. Triacylglyceride content was positively correlated with total protein (Table 4.9, r_{77}=0.514, p<0.001), was lower in pupae from OT than in those from CO (OT: 0.42 ± 0.21 mg lipid·mg^{-1} LFDM; CO: 0.51 ± 0.23 mg lipid·mg^{-1} LFDM; Table 4.9), and also lower in pupae overwintered at warm temperatures compared to those overwintered at cool temperatures (warm: 0.44 ± 0.21 mg lipid·mg^{-1} LFDM; cool: 0.50 ± 0.23 mg lipid·mg^{-1} LFDM; Figure 4.5A; Table 4.9). There was a significant overall negative relationship between TAG content and date of diapause, and significant interactions between date of diapause and both ecotype and overwintering environment (Table 4.9) such that TAG and date of diapause exhibit a negative relationship in pupae from OT, particularly when kept in warm southern temperatures (OT/warm: r_{17}=-0.864, p<0.001; OT/cool: r_{18}=-0.619, p=0.005; while pupae from CO have no relationship between TAG and date of diapause under cool temperatures (p>0.1) and a positive relationship under warm temperatures (r_{18}=0.624, p=0.003; p-values adjusted for multiple comparisons using a false-discovery rate correction, Benjamini and Hochberg 1995). Thus the CO ecotype has more TAG if they enter diapause later (but only in a warm overwintering environment), while the OT ecotype has more TAG if they enter diapause earlier. There was a significant overwintering environment × sampling time interaction (Table 4.9), such that pupae in the cool overwintering environment lost TAG over the course of the winter, while pupae in the warm environment did not (Figure 4.5A).

Carbohydrate content was significantly affected by size and sex, controlling for size differences (Table 4.9), such that females had more carbohydrate than males (Figure 4.5B). Total carbohydrate also dropped significantly over the course of the winter (Figure 4.5B). There was a perfect correlation between TAG and total energy content (r=1), and the statistical analyses for the effects of ecotype, environment and sex on total energy content had identical conclusions to the corresponding analyses for TAG (Table 4.9). Total energy content, like TAG, declines over the course of the winter under cool temperatures, but remains constant in warm temperatures (Figure 4.6).

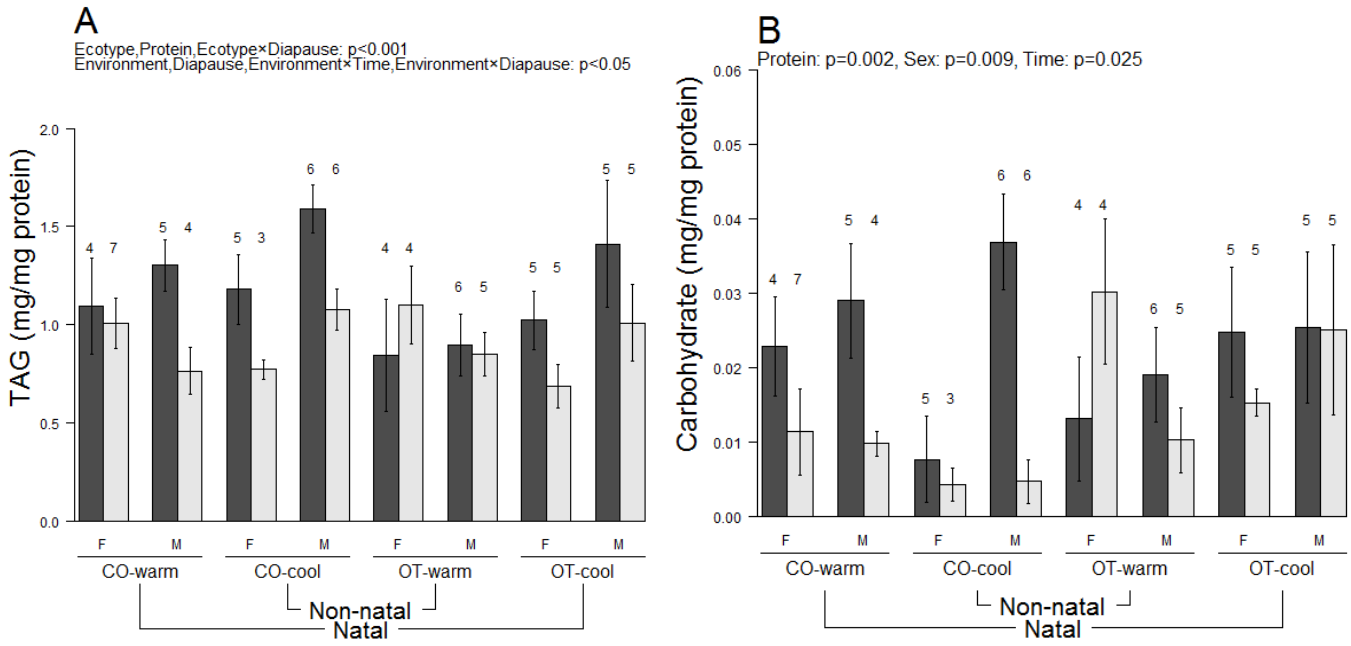

Figure 4.5 - Lipid and carbohydrate content of *H. cunea* pupae from Columbus, Ohio (CO) or Ottawa, Ontario (OT); overwintered in the laboratory at temperatures approximating CO (warm) or OT (cool) microclimates in a reciprocal common garden experiment. (A) Triacylglycerol and (B) carbohydrate content at the beginning (dark bars) and end (light bars) of winter. Significant effects indicated above are from ANCOVA (Table 4.9); Ecotype = source population, Environment = overwintering environment, Protein = total protein (size covariate), Diapause = date of diapause, Time = sampling period (beginning or end of winter). M=males, F=females. Natal refers to populations experiencing an overwintering environment similar to the microclimate from their source region, Non-natal refers to those experiencing an overwintering environment similar to the other source region. Bars show mean ± SEM per pupa, sample sizes above bars.

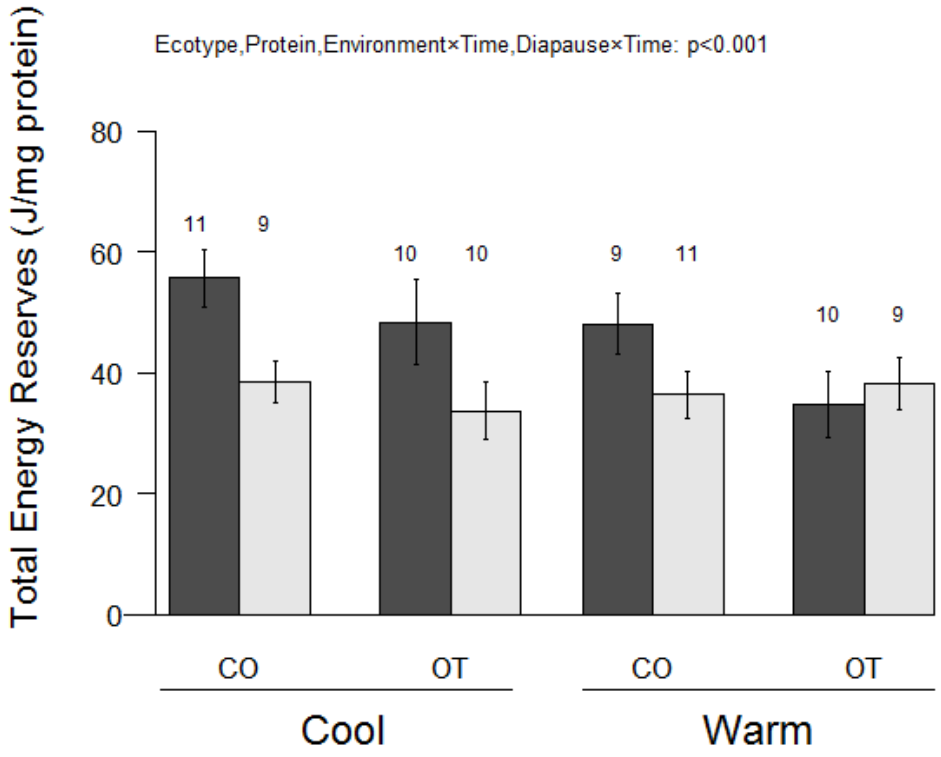

Figure 4.6 - Total energy reserves (J) of *H. cunea* pupae from Columbus, Ohio (CO) or Ottawa, Ontario (OT); overwintered in the laboratory at temperatures approximating CO (warm) or OT (cool) microclimates in a reciprocal common garden experiment. Energy content was calculated assuming 39.3 $J.mg^{-1}$ TAG and 17.6 $J.mg^{-1}$ carbohydrate (Djawdan et al., 1998). Significant effects indicated above are from ANCOVA (see text). Sexes are pooled as there was no significant effect of sex on energy content. Bars show mean ± SEM per pupa, sample sizes above bars.

4.3.4 **Metabolic rate**

All pupae showed continuous gas exchange at all measurement temperatures. There were no effects of measurement order on metabolic rate at either time point (beginning of winter: $F_{1,118}$=0.261, p=0.610; end of winter: $F_{1,117}$=0.1147, p=0.735). At the beginning of winter, metabolic rate increased with increasing measurement temperature, and mass was negatively correlated with metabolic rate (Table 4.9; r_{118}=-0.216, p=0.018, Figure 4.6A). At the beginning of winter, metabolic rate was significantly lower in pupae that were overwintering at warmer southern temperatures (Table 4.9; Figure 4.7A). At the end of winter, there was a positive relationship between measurement temperature and metabolic rate, and a significant measurement temperature × ecotype interaction (Table 4.9), such that the thermal sensitivity of metabolic rate was lower in individuals from Columbus compared to those from Ottawa (Figure 4.7B).

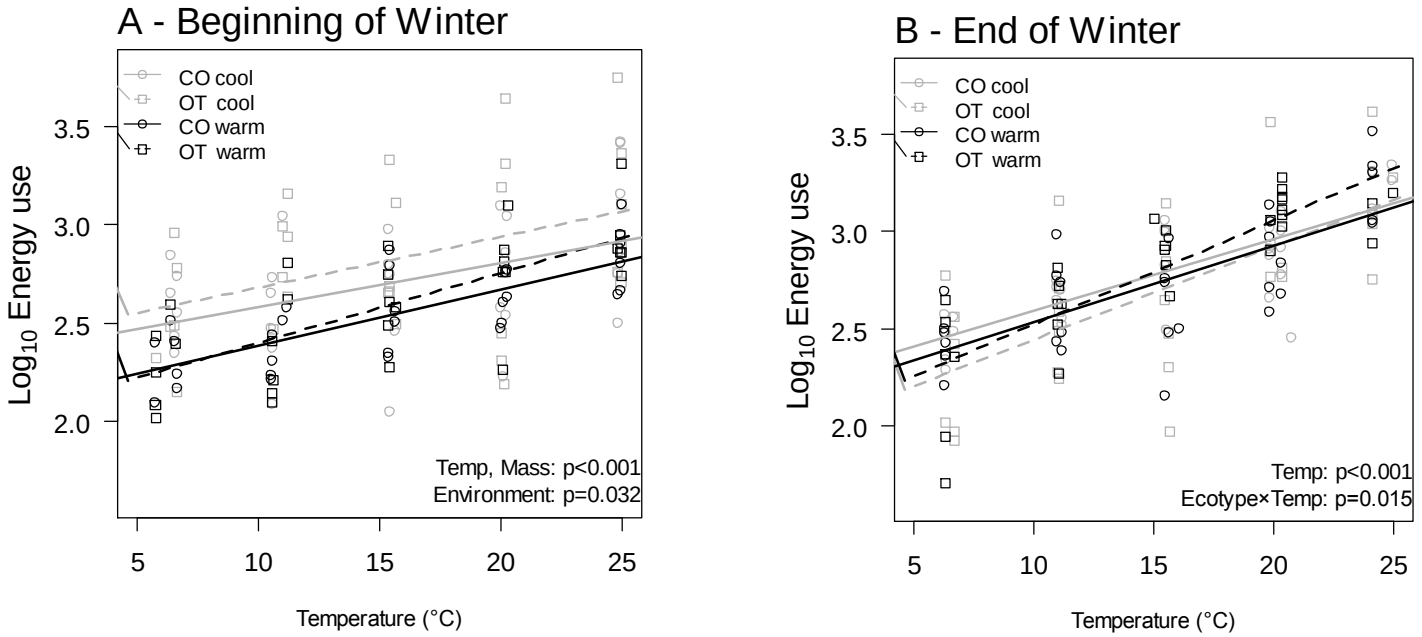

Figure 4.7 - Metabolic rate of diapausing *H. cunea* pupae from Columbus, Ohio (CO) or Ottawa, Ontario (OT), overwintered in the laboratory at temperatures approximating CO (warm) or OT (cool) microclimates in a reciprocal common garden experiment. Metabolic rate was measured in (A) October (beginning of winter) or (B) April (end of winter). Environment = overwintering environment, Ecotype = source population. The trend lines indicate the predictions of linear models of $\log_{10}$ energy use on temperature (Table 4.9).

4.4 Discussion

4.4.1 Overview

I examined response of life-history and physiological traits to warm or cool overwintering conditions in *Hyphantria cunea* pupae from the northern range edge or centre of their range, to evaluate the degree of local adaptation and phenotypic plasticity present in their overwintering energetics. I detected phenotypic divergence between ecotypes of *H. cunea*, from the northern range edge (Ottawa, Ontario; OT) and centre (Columbus, Ohio; CO) of their range, in most (11/17) life-history and physiological traits (Figure 4.3). These changes were particularly pronounced at the beginning of winter, when individuals from CO were larger, had greater lipid stores, advanced autumn phenology, and delayed development (and thus longer winters) compared to individuals from OT. The size differences between ecotypes at the beginning of the winter could be interpreted that the CO ecotype had higher potential fitness, as size and energy stores correlate well with fitness in Lepidoptera (Leather 1995; Oberhauser 1997; Berger et al. 2008), but the longer period of dormancy experienced by the CO ecotype as a result of early diapause entry resulted in the loss of their initial size advantage by the adult stage. As mortality of the ecotypes was also similar in their natal environment, there was no suggestion of a fitness advantage for either ecotype at home, although the CO ecotype had markedly higher survival in a cool environment. Thus, the acute effects of changes in the overwinter thermal environment were not realised in *H. cunea* due to phenotypic plasticity in their metabolism.

4.4.2 **Autumn phenology and origin of size differences**

The earlier entry into diapause in the CO ecotype likely resulted from a combination of earlier emergence of the parental generation, and faster growth and development, both resulting from warmer growing season temperatures in CO. The threshold temperature for initiation of development in *H. cunea* is 11 °C (Gomi et al. 2003) - my microclimate data show that mean temperatures would cross this threshold in March in the range centre, but not until April at the northern range edge. Spring emergence date is generally correlated with latitude, with earlier spring phenology in more southerly populations (e.g. Hodgson et al. 2011). Warmer temperatures during the growing season are likely to have promoted faster growth and development in the CO ecotype (Ayres and Scriber 1994; Davidowitz and Nijhout 2004), leading to earlier pupation, although based on my study design I am unable to differentiate between developmental, genetic and maternal effects on ecotype divergence.

Contrary to my findings, growth at higher temperatures generally leads to smaller size in ectotherms (the temperature-size rule; Kingsolver 2009), as higher temperatures reduce the time to cessation of growth after a critical weight is reached, thus reducing the accumulation of mass (Davidowitz and Nijhout 2004). However, in species whose generation time forms a substantial proportion of the growing season (e.g. Lepidoptera), body size frequently declines with increasing latitude (Ayres and Scriber 1994; Kingsolver et al. 2007; Pelini et al. 2009), and results from decreased energy availability due to the shorter growing seasons at higher latitudes (Chown and Gaston 1999). Despite the initial size advantage in the CO ecotype, the realisation of a fitness benefit due to increased fecundity (estimated by adult size) may have been prevented by potential trade-

offs imposed on overwintering life-stages, as longer winters (resulting from early diapause and late emergence) caused decreased adult compared to pupal size. Pupae from CO clearly consumed more TAG over the course of the winter than did pupae from OT (Figure 4.5A), although this was mediated by the earlier diapause dates in the CO ecotype (significant diapause date × sampling time interaction), rather than any other attributes of the CO ecotype (non-significant ecotype × sampling time interaction). A similar effect is seen in partially trivoltine populations of *H. cunea* in Japan, where second generation pupae, which enter diapause earlier and emerge slightly later than third generation pupae, suffer increased mass loss, and decreased survival and fecundity over winter compared to third generation pupae (Gomi 2000). However, in a univariate framework, I did not detect significant differences in overwinter mass loss; pupal mass at the end of winter was still higher for CO pupae, and I did not observe increased overwinter mortality in the CO compared to the OT population. Thus increased energy demands resulting from a longer period of dormancy ameliorated the CO ecotype's initial size advantage.

I found that females were larger than males in most measures of size, had greater concentrations of carbohydrate, but equal concentrations of lipid. Among insects, the fecundity of females (but not males) increases with increasing size (e.g. Gomi 2007). This leads to stronger selection on female size, and female insects are generally larger than males (Lease and Wolf 2011). Contrary to my findings, female lipid concentrations are usually higher than those of males (Lease and Wolf 2011), but there is a great deal of interspecific variation in relative lipid concentrations (Fast 1970). The extra carbohydrate reserves of females could act a fuel source for vitellogenesis, as carbohydrates are readily

converted to lipids in Lepidoptera and can be rapidly incorporated into developing eggs (Kozhantshikov 1938).

4.4.3 **Adaptive plasticity of metabolic rate**

Contrary to my null hypothesis of increased energy use in warmer overwintering environments, I did not document any reduction of size or energy reserves in pupae overwintering under warm conditions, presumably due to the phenotypically-plastic metabolic suppression that I documented at the beginning of winter in pupae from both populations. Metabolic suppression is a common part of the diapause programme (Koštál 2006), but this parallel and facultative metabolic suppression in diapausing pupae from two source populations illustrates that the depth of suppression can be modulated by conditions experienced after the onset of diapause. Since the rate of utilisation of stored energy reserves (measured by depletion of storage lipids) was actually lower in pupae overwintering at warm temperatures, this metabolic suppression seems to have more than compensated for the increased energy demands of a warm environment. *H cunea* pupae in Japan also show pronounced metabolic suppression, and no detectable decline in energy reserves over the course of a winter in the field (Li et al. 2001). By the end of the winter, the metabolic suppression had abated, and there was no difference in metabolic rate among overwintering environments, which is concordant with my predictions as spring is less energetically demanding than autumn (Chapter 5). The plastic suppression of metabolic rate was in the predicted direction, at the predicted time point, and was expressed similarly by two separate populations. This response resulted in the complete amelioration of the predicted negative effects of winter warming on energy stores in both populations; thus I believe this is an example of adaptive phenotypic plasticity.

There are two possible adaptive hypotheses to explain the decreased thermal sensitivity of metabolism in CO pupae at the end of winter: 1) Suppression of thermal sensitivity has become fixed in the CO ecotype as a consequence of relatively warm and variable overwintering conditions (Lynch and Gabriel 1987; Chapter 5); or 2) Increased thermal sensitivity in the OT ecotype facilitates faster development in spring, due to the selection pressures of a shorter growing season near the northern range edge (Jeong et al. 2010). An increase in thermal sensitivity in northern populations is predicted by the metabolic cold adaptation hypothesis (Clarke 1993), to promote faster rates of growth and development in energy-limiting environments. Since I saw the difference only in spring, and only between ecotypes but not environments (despite the presence of plasticity in the metabolic rate-temperature relationship in *H. cunea*), I favour the second hypothesis. The increased thermal sensitivity of OT pupae was associated with shorter development times upon transfer to warm, long-day conditions, supporting this hypothesis.

The proximate mechanisms for the observed metabolic suppression in *H. cunea* pupae exposed to a warm environment over winter may include a change in active enzyme quantities; either resulting from transcriptional, post-translational or allosteric modifications (Storey 2004), or microRNA degradation of mRNA transcripts (Biggar and Storey 2011). By contrast, the increased thermal sensitivity in the OT population in the spring may result from amino acid sequence changes or alterations to the three-dimensional structure of metabolically active enzymes (e.g. Fields and Somero 1997).

4.4.4 **Winter Mortality**

Fitness may be calculated as the product of fecundity and survival (Orr 2009). If I assume that fecundity is equal among ecotypes and environments (a reasonable assumption as there were no differences in adult size or mass, which are good correlates of fecundity in Lepidoptera; Leather 1995; Oberhauser 1997; Berger et al. 2008), then mortality has strong implications for fitness. Larval survival, prior to the separation of ecotypes into overwintering treatments, was higher for the OT ecotype. This may have been due to increased mortality resulting from higher initial density of CO larvae (there were more larvae in each nest when the nests were collected), or some pre-adaptation to laboratory conditions in OT larvae. The response of larval mortality to warming during the growing season seems to be highly variable among species: decreased larval survival has also been noted in *Papilio zelicaon* (Lepidoptera: Papilionidae) in response to warming, in contrast to increased larval survival for *Erynnis propertius* (Lepidoptera: Hesperiidae) under warmer conditions (Pelini et al. 2009). Overwinter mortality depended on both ecotype and overwintering environment; the CO ecotype was more fit, particularly in a cool overwintering environment, and cool environments increased fitness relative to warm ones. The increased fitness of the CO ecotype provides support for the "warmer is better" hypothesis (Frazier et al. 2006), which posits that warmer temperatures during development provide fitness advantages that cannot be overcome by adaptation to cooler temperatures.

The cause of the increased mortality associated with the OT ecotype and warmer winters does not seem to be associated with energy drain (pupae in a warm environment lost less TAG over the course of the winter than did those in a cool environment, and energy

reserves of the CO/cool group with high survival was no greater than any other ecotype × environment combination) or water loss (there was no evidence for increased water loss of pupae in warm compared to cool environments, and water loss of CO pupae, which had higher survival, was far greater that the water loss of OT pupae). The amount of water lost by pupae from CO (48 % of initial water content, cf. 22% in the OT ecotype) approaches limits of desiccation tolerance in most insects from mesic habitats (Chown and Nicholson, 2004), but the CO population had higher overwintering survival thus water loss did not affect fitness. In some species, water loss can be associated with increased cold-hardiness (Zachariassen 1991; Block 2002; Sinclair et al. 2007), but as there was no cold challenge in this experiment (diapausing *H. cunea* pupae have very high survival to -5 °C; Li et al. 2001), that cannot explain the increased survival of the CO/cool pupae. There was no difference in the causes of mortality among ecotypes or environments, thus increased proliferation of pathogens cannot fully explain the increased mortality in warm temperatures. It is possible that an energetic cost of reversing the metabolic suppression expressed in warm conditions manifested only after development resumed at warm temperatures (after the end of winter energy reserves measurements were taken), resulting in increased mortality in individuals expressing that suppression.

H. cunea pupae also exhibited a plastic response of development time to winter warming, with both source populations having shorter winters under warm southern microclimate conditions (Figure 4.2), mediated by decreases in development time upon transfer to 25 °C in pupae that had experienced a warm winter environment (Figure 4.3). A shortening of development time will help mitigate negative energetic consequences of warm winters. This is contrary to predictions of models that assume diapause development proceeds

faster at cooler temperatures (Gray et al. 2001), and may indicate that physiological development occurred faster during the winter at warm temperatures, enabling a faster response to the return of permissive temperatures in the spring.

4.4.5 **Local adaptation**

Despite marked phenotypic divergence between the populations, I detected few signatures of local adaptation (G × E interactions) to overwintering conditions in the life-history and physiological traits that I measured, and no local adaptation in fitness correlates such as adult size and survival. Warm winters increased mortality, and the CO ecotype had higher survival in either environment; thus the comparison between natal and non-natal ecotypes within each environment did not indicate that local ecotypes had higher fitness than foreign ecotypes; a key diagnostic for local adaptation (Kawecki and Ebert 2004). I note that local adaptation of physiological and life-history traits can only exhibit a G × E interaction if traits are phenotypically plastic; i.e. exposure to non-natal temperatures results in maladaptive phenotypic plasticity. There was some suggestion of local adaptation in the increased thermal sensitivity of metabolism in the OT ecotype at the end of winter, but to determine possible fitness benefits of increased thermal sensitivity it would be necessary to monitor performance of the subsequent generation throughout the growing season.

Greater local adaptation results from greater environmental divergence (Hereford 2009). It is thus possible that if I sampled more distant populations, I would detect a greater degree of local adaptation; however, my microclimate measurements showed considerable differences in the thermal profiles of the northern range edge compared to

the range centre, which are separated by over 800 km. I thus propose that the lack of local adaptation can be attributed to differential thermodynamic constraints across life-stages. Experimental studies of local adaptation have found that phenotypic variation is not well-correlated with local adaptation, in that populations with high degrees of phenotypic divergence are not necessarily locally-adapted (Hereford 2009). Hereford (2009) hypothesised that this was due to individuals in novel environments attaining increased values of certain fitness correlates (e.g. size), but not being able to realise the fitness benefits in that same environment. My results support this hypothesis: individuals from CO were larger, but were not likely to realise the fecundity benefits of increased size due to increased overwinter energy drain caused by longer periods of dormancy. Together, this illustrates the differential actions of temperature across life-stages: although warmer may be better during development (Frazier et al. 2006), warmer is worse during the overwintering period. Moreover, these differential effects of temperature across the lifecycle, at least in *H. cunea*, balance out the effects of changes in the seasonal covariance of temperatures: positive consequences of warmer growing seasons on mass gain are cancelled out by energetic demands of longer periods of dormancy. A trait-specific view has been espoused by several authors (Woods and Harrison 2002a; DeWitt and Scheiner 2004; Schulte et al. 2011), and in this study taking such a view has illuminated complex trade-offs involved in the maintenance of homeostasis in the face of environmental change.

4.4.6 **Impact of phenology on energy use**

Principal components analysis showed that a significant proportion (10 %) of the variation in life-history variables in this data set was due to phenology. In general,

advances in phenology resulted in smaller adult size than would be expected based on pupal size (Table 4.7), presumably due to increased consumption of stored energy reserves during early autumn (Chapter 5). Depending on their date of diapause, non-feeding pupae were required to support basic metabolic functions for a variable amount of time before they were sampled for analysis of body composition. Thus, all else being equal, I predicted a positive relationship between energy reserves and date of diapause, and I predicted that relationship to be strongest under warm winter conditions. I found the predicted positive relationship between TAG concentration and date of diapause in the CO ecotype in warm, but not cool conditions; suggesting that, for the CO ecotype, advanced autumn phenology increases energy use under energetically demanding (i.e. warm) overwintering conditions.

Conversely, TAG concentration was negatively related to date of diapause in the later-diapausing OT ecotype; most strongly so when they were kept at warm southern temperatures. I hypothesise that this negative relationship was caused by declining host-plant quality in the autumn, which has been shown to induce early diapause in *H. cunea* and other insects (Morris 1967, Ishihara and Ohgushi 2006). Thus, individuals entering diapause later in the season may have received cues to enter diapause without the chance to accumulate sufficient energy reserves. Host plant quality also reverses the temperature-size rule in *Manduca sexta* (Lepidoptera: Sphingidae) in the laboratory (Diamond and Kingsolver 2010). The current experiment had to be terminated prematurely before all larvae had pupated, due to the senescence of host plant, indicating that there could be mortality associated with delayed phenology in the wild. In this experiment however, the lack of energy reserves of the OT ecotype were balanced out by the decreased length of

dormancy resulting from the later entry into diapause, thus there were no fitness consequences of their initial lipid deficit compared to the CO ecotype.

4.4.7 **Voltinism**

Early instar larvae are the sensitive stage for photoperiodic induction of diapause in this species (Takeda 2005), thus, as there was no direct development, each population apparently experienced photoperiods shorter than the critical threshold photoperiod for diapause induction in the field prior to collection. Although in the past the northern populations were univoltine (Morris 1967), and are largely still considered so (Wagner, 2005), the timing of both larval and pupal stages in the north is consistent with that of second generation black-headed larvae and pupae in Missouri (Takeda 2005). I thus believe that both populations have at least a partial second generation, but that second generation may only be partially successful in Ottawa, where the onset of low temperatures or host plant phenology may be limiting (larvae were still feeding right up until leaf drop).

In Japan, the emergence of an additional generation of *H. cunea* has been driven by energy drain, with associated decreases in survival and fecundity, as a result of early entry into diapause (Gomi 2000). I documented a similar effect in CO larvae, while in OT larvae late entry into diapause led to decreased energy stores, possibly due to decline of host plant quality in the late autumn. This illustrates the mechanisms that can maintain mixed voltinism in transition zones: in warm years it is advantageous to have an additional generation, so as to avoid the energetic penalties of diapause in the warm autumn, while in cool years fitness will be higher in individuals of the penultimate

generation, who will avoid mortality or energetic penalties of not reaching the pupal period before the decline of host plant quality (Gomi 2000). I thus hypothesise that the Ottawa population is in a transition area between bivoltinism and univoltinism. If there were univoltine individuals present in the Ottawa population, they would have already dispersed from their nests by the time I sampled. The increased thermal sensitivity that I documented in the OT population in the spring is likely to have facilitated the development of an additional generation in the northern population, allowing earlier emergence of the first generation of adults. Increases in voltinism have been widely documented in response to climate change (Altermatt 2010), but to my knowledge this is the first study to demonstrate a physiological change facilitating such a shift.

4.4.8 **Conclusions**

I found little evidence of local adaptation of *H. cunea* populations from the northern range edge or centre or their range to their natal thermal conditions. Instead it seems that a large repertoire of developmental/genetic and plastic changes in life-history and physiological phenotypes in response to changes in the thermal environment render this species relatively insensitive to sub-lethal effects of temperature. The lack of responsiveness of *H. cunea* to changes in thermal conditions during overwintering may be reflected in the success of this insect as an invasive species. While conditions from the range centre enhance fitness during the growing season, those same conditions impose longer winters on CO ecotypes which largely cancel out initial fitness gains. Warmer winters have deleterious effects on fitness that counteract the benefits of warm growing seasons. However, this species does not appear to be at risk of climate warming no matter where in the life-cycle it occurs, due to many compensatory mechanisms. If winter warms

more than summer, *H. cunea* has the capacity to suppress metabolic rate to mitigate negative effects of energy drain. If growing season temperatures rise, *H. cunea* may reach final instars earlier (compounding overwinter energy drain), but will do so with a size advantage that will offset the longer periods of dormancy that result. I saw some suggestion of the development of a previously undescribed generation in the north, the development of which may enhance the fitness of northern individuals as climate warms. This study clearly illustrates the impact of multiple potential trade-offs imposed directly (thermal effects on water balance, metabolic costs, growth and development) or indirectly (through host plant) by climate; and how these trade-offs result in modifications to life-history and metabolic traits.

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Chapter 5: Thermal variability drives metabolic depression and increases the impact of autumnal phenological shifts in an overwintering insect.

I test the hypothesis that increased daily thermal variability will induce a decrease in thermal sensitivity of metabolism in overwintering *Erynnis propertius* larvae, and model overwintering energetics using long-term meteorological data. A similar version of this manuscript is under review at PLoS ONE.

5.1 Introduction

Climate change is affecting insects (Chen et al. 2010), and winter warming has driven range expansions in several species of Lepidoptera (Crozier 2004, Jepsen et al. 2008). In holometabolous insects, energy reserves derived from larvae supply resources for vitellogenesis and somatic maintenance (Boggs 2009), and some essential amino acids are solely derived from the larval diet (O'Brien et al. 2002). Many temperate univoltine insects overwinter in late juvenile life stages, with limited or no opportunity to feed before metamorphosing into adults in the spring. Thus, although the intuitive outcome of warmer winter temperatures is a release from cold-induced stress, warmer conditions increase the rate of consumption of stored energy reserves (Irwin and Lee 2003), reducing reserves available for post-winter reproduction and somatic maintenance, thereby affecting performance and fitness (Hahn and Denlinger 2011).

Mean rates of metabolism in ectotherms are influenced by both mean temperature and thermal variability. Metabolic rate has a curvilinear relationship with temperature in ectotherms (Gillooly et al. 2001), typically accelerating to an inflection point then decelerating until the thermal optimum is reached, after which it declines sharply until critical thermal limits are exceeded and death occurs (Huey and Stevenson 1979). Jensen's inequality states that the mean value of metabolic rate (over the accelerating portion of the curve), will increase with increasing variance in temperature (Ruel and Ayres 1999). Thus, the warm part of daily thermal cycles will disproportionately influence metabolic rate, and therefore ectotherm performance and fitness; beyond what

would be predicted by mean temperatures. The magnitude of this effect will depend on the degree of curvature in the rate-temperature function over the range of temperature fluctuations (Pazstor et al. 2000). Jensen's inequality underlies predictions of increased sensitivity of tropical organisms to temperature increases (Dillon et al. 2010), and can explain discrepancies between experiments conducted at fluctuating and constant temperatures (e.g. Paaijmans et al. 2010, Kingsolver et al. 2009; Folguera et al. 2011, Estay et al. 2011).

Temperate insects modify the shape of the temperature-metabolic rate curve while in diapause by suppressing metabolic rate and reducing its thermal sensitivity (Hahn and Denlinger 2011). Global suppression of metabolic rate may be accomplished by a coordinated down-regulation of energetically demanding processes such as protein synthesis, gene transcription, and ATP-dependant ion pumps, regulated by reversible protein phosphorylation of metabolic enzymes and/or ion pumps (Storey and Storey 2004) or potentially microRNAs (Biggar and Storey 2011). Decreases in thermal sensitivity may result from the expression of allozymes (over evolutionary time) or isozymes (as plastic response) of key metabolic enzymes with different thermal properties, or by the modification of the three-dimensional structure of key metabolic enzymes (Fields and Somero 1997). As decreased thermal sensitivity lessens the metabolic impact of variability by reducing the relative impact of the warm portions of thermal cycles (Ruel and Ayres 1999), temporally heterogenous environments are theoretically predicted to decrease the thermal sensitivity of performance functions (Lynch and Gabriel 1987). However, this has not been empirically supported with regards to variability on a daily time-scale (Niehaus et al. 2011), nor has it been explored in dormant organisms, for whom energy conservation is of the highest priority. I thus hypothesised that high daily thermal amplitude would elicit a decrease in the thermal sensitivity of overwintering insects, in order to compensate for the increased energetic demands of thermally variable environments.

To test this hypothesis, I used larvae of *Erynnis propertius* (Lepidoptera: Hesperiidae). This insect overwinters in both thermally-stable and -variable environments, and I reared

the larvae in the laboratory in a reciprocal common garden experiment with stable and variable thermal regimes. I measured thermal sensitivity of metabolic rate, and used this sensitivity to parameterise a model of overwintering energetics. This model allowed me to explore the consequences of metabolic suppression and thermal variability over 27 winters. I show that winter metabolic rate is suppressed by variable environments, and that most of the energy use during dormancy occurs during the autumn. Thus, shifts in autumn conditions and phenology may be critical in the impact of climate change on overwintering ectotherms.

5.2 Materials and Methods

5.2.1 Study animals and rearing

Erynnis propertius larvae feed on oak (*Quercus* sp.) throughout the summer, then overwinter as dormant sixth instar larvae in the leaf litter (although southern Californian populations have additional generations), and do not feed again before metamorphosis and adult flight in the spring (Prior et al. 2009). Eggs were collected from fertilised females caught in Garry Oak savannahs in Medford, OR and VI, British Columbia (Figure 5.1) in April-May 2009, shipped to the University of Notre Dame, and raised in growth chambers on fresh-cut Garry Oak leaves as described previously (Pelini et al. 2009) on a 12h:12h thermo- and photoperiod under diurnally fluctuating temperature regimes based on long-term climate normals from nearby climate monitoring stations for each locale (Figure 5.1, Table 5.6). Throughout, I refer to the source of the populations by name (OR and VI), and the rearing conditions by their thermal profile (variable or stable). Broods were split such that offspring from each mother were represented in each rearing condition, resulting in four treatment groups (origin/rearing conditions): 1) OR/variable; 2) OR/stable; 3) VI/variable; and 4) VI/stable. In late August 2009, larvae were transferred to the University of Western Ontario under ambient conditions and kept in Sanyo MIR-153 incubators (Sanyo Scientific, Bensenville, IL, USA) with *ad libitum* fresh-cut Garry Oak leaves until the larvae entered dormancy. A sixth instar larva was considered dormant when it did not feed for three days, was brown in colour, and was rolled in a leaf. Dormant larvae were removed from leaf rolls to facilitate weighing, and

transferred to 6-well plates with moist paper towel on the lid (to maintain high humidity) in constant darkness to approximate conditions on the ground in a leaf roll below leaf litter.

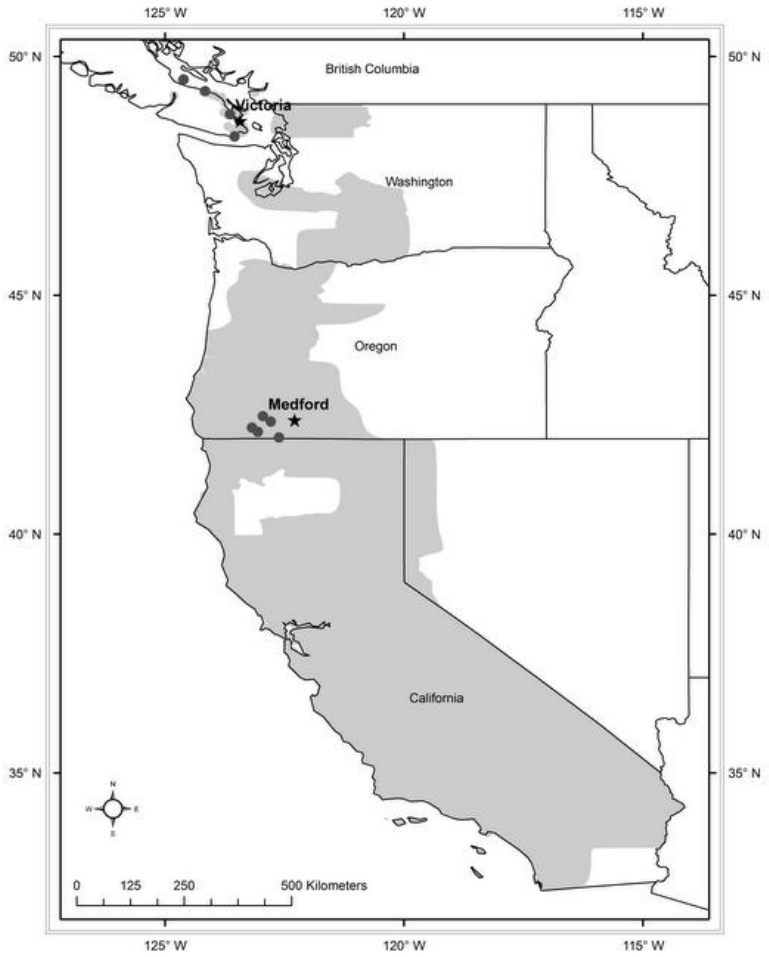

Figure 5.1 - The range of *Erynnis propertius* (grey shading). Butterflies were collected from the vicinity of Medford, OR, and Vancouver Island, British Columbia (VI). Circles indicate the sampling locales and stars the location of the climate stations from which meteorological data were obtained (Table 5.1, Figure 5.2a-b). *E. propertius* has also been reported from Baja California Norte, Mexico (not shown). Data from Opler et al. 2011; and Royal British Columbia Museum Entomology Collection, Canadian National Collection (CNC) of Insects, Arachnids and Nematodes, Lyman Entomological Museum, Nova Scotia Museum of Natural History, Halifax, NS, Canada, Lepidopterists Society Season Summaries 1973-1997, Crispin S. Guppy Collection, Royal Ontario Museum: Entomology, and the Spencer Entomological Museum (accessed through GBIF Data Portal, data.gbif.org).

5.2.2 **Physiological and biochemical measurements**

In November, after all individuals had entered dormancy, larvae were weighed (MX5 microbalance, Mettler-Toledo, Columbus, OH, USA; d=0.1 μg) and 5 larvae per treatment group frozen in liquid nitrogen and stored at -80 °C until lipid analyses were performed. Lipids were extracted using the Folch method (Folch et al. 1957) and quantified by TLC-FID using the solvent system benzene: chloroform: formic acid (70:30:0.5 v/v/v), as described in Chapter 1, using a monoacylglyceride (1-stearoyl-rac-glycerol; Sigma-Aldrich, St Louis, MO, USA) as internal standard.

5.2.3 **Respirometry**

Carbon dioxide production was measured using flow-through respirometry (Sable Systems International, Las Vegas, NV, USA), as previously described (Williams et al. 2010) in six larvae from each treatment group, each at 10, 15, 20, 25, and 30 °C (November) or 1, 8, 18, 25, and 30 °C (February) in randomised order at a randomised time of day between 8 am and 6 pm with at least three days between subsequent measurements for any one individual. Dry, CO_2-free air was passed through an 11 cm^3 chamber containing a caterpillar, or a blank reference chamber, at 50 $ml \cdot min^{-1}$, controlled by mass-flow valves (Sierra Instruments, Monterey, California, USA) and a MFC-2 controller (Sable Systems International, S.S.I.). CO_2 and water vapour were quantified in excurrent air with a LiCor 7000 infra-red gas analyser (LiCor, Lincoln, NE, USA). Activity of the animal was monitored by an AD-1 infrared activity detector (S.S.I.). Three caterpillars were measured in each run (RM-8 multiplexor, S.S.I.), which included a 1 h acclimation period, 10 min baseline measurement of the reference chamber at the beginning and end of the run, and 40 min of measurement of each caterpillar after a 10 min wash-out period. Data were acquired by a UI2 interface (S.S.I.), drift corrected to baseline values, and CO_2 data corrected for the dilution of water vapour. To generate resting CO_2 production for each larva at each temperature, I analysed a 10 min section with no detectable movement, where CO_2 production was low and stable.

5.2.4 **Meteorological data**

Hourly thermal data were obtained from the meteorological stations closest to the locations where the butterflies were collected: Victoria International airport, VI (Environment Canada, Toronto, ON, Canada) and Rogue Valley International-Medford airport, Medford, OR (National Climatic Data Centre, Asheville, North Carolina, USA) (Figure 5.2a-b, Table 5.1). Unless stated otherwise, a period of dormancy in my analyses ran from the autumnal equinox of one year to the vernal equinox of the following year. Thus the 2008 period of dormancy ran from September 2008 to March 2009. I excluded years where any month was missing more than 10 % of the hourly observations (Table 5.6), and replaced other missing values with the hourly means for the month in question (when available). When the hour in question was missing for an entire month (3pm and 11pm in April 2000 and July 1999 for Vancouver Island), those values were replaced with the mean temperature for that day and time from the complete dataset for that location.

Table 5.1 - Summary of climate data from OR and VI. Meteorological data are from the climate monitoring stations closest to the study locations (Figure 5.1). Procedures for excluding meteorological data are described in supporting information. Temperature during dormancy is the mean of temperatures occurring between 12 August and 23 March each year; the approximate dates over which larvae are in dormancy at both locations. Values are latitude, longitude; year; or mean ± SD.

	OR	VI
Location of weather station (°)	42.23°N, 122.52°W	48.38°N, 123.25°W
Elevation	405 m	19 m
Coverage	1973-2010	1973-2010
Missing winters	1996-1997	1984, 1992-1994, 2000-2002, 2005-2007
Annual mean temperature (°C)	12.3 ± 9.1	8.6 ± 3.9
Temperature during dormancy (°C)	10.0 ± 8.4	7.9 ± 3.4
Annual mean daily range (°C)	14.1 ± 4.42	3.7 ± 0.9
Absolute maximum (°C)	44.4	26.8
Absolute minimum (°C)	-19.4	-12.2

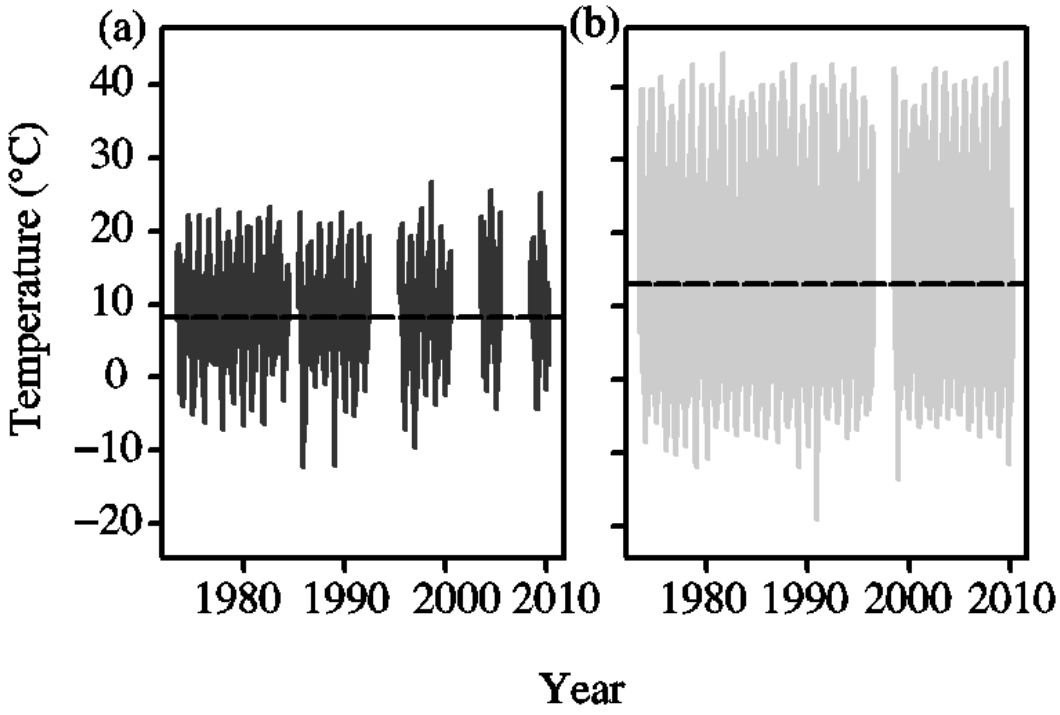

Figure 5.2 - Hourly meteorological data from (a) Victoria International airport, VI and (b) Rogue Valley International-Medford, OR. Horizontal dotted lines indicate mean winter temperatures.

5.2.5 **Statistics and modelling**

Statistical analyses were performed in R 2.13.0 (R Core Development Team 2009) unless otherwise noted. Terms were dropped sequentially from maximal models based on statistical significance and Akaike's information criterion (AIC) was used to confirm the improved fit of the reduced model, with a less than two point difference in AIC being the threshold for retaining the simplified model (Crawley 2007). Mass and date of dormancy were compared among treatment groups using ANCOVA with rearing conditions and source population as factors and date of dormancy as covariate (mass) or by ANOVA with rearing conditions and source population as factors (date of dormancy). Lipid concentration was compared among treatment groups using ANCOVA with rearing conditions and source population as factors, and date of dormancy as a covariate. Tukey's *post hoc* tests for the final model from preceding analyses were performed using PROC GLM in SAS 9.1 (SAS Institute Inc., Cary, NC). Extra energy use above that predicted by mean temperature was analysed by ANCOVA with location as a factor and daily thermal amplitude as covariate, and then separately by location by regressing extra energy use above that predicted by mean temperature against daily thermal amplitude.

Modelling of the relationship between carbon dioxide production and temperature was conducted using a maximum likelihood approach to choose appropriate nonlinear models, implemented with the nlme package (Pinheiro et al. 2009). This modelling was conducted in two discrete steps. First, exponential and power law nonlinear models (both with and without body mass as a covariate, see Table 5.2) were fitted to all metabolic rate data using the "gnls()" function without rearing treatment or source population membership defined, and with a variance structure that assumed variance increased as a power of the temperature (Pinheiro and Bates 2000). All models were specified with an overall scaling factor (S), a temperature-scaling factor (T_s), and a final coefficient representing the minimal life-supporting metabolic rate ("L_{met}") for *E. propertius* as an additional term (Makarieva et al. 2006). Some models additionally contained a mass (M), with or without a mass-scaling factor (M_s). Starting values for coefficients were selected graphically, and the same values were used for every model. The best-fitting model was selected based on

Akaike's Information Criterion (AIC) and likelihood ratio tests (Angilletta 2006). The first exponential model investigated (Model 1, Table 5.2) was based on the metabolic theory of ecology, a commonly postulated model relating metabolic rate to temperature and body mass (Gillooly et al. 2001), but this model was not a good fit for my data compared to other models, based on AIC and log-likelihood ratios. The model with the lowest AIC included body mass (Model 4, Table 5.2), however a likelihood ratio test showed no significant difference in model fit between Model 4 and Model 5 (likelihood ratio test, $F_{1,5} = 3.29$, $p = 0.07$) and the coefficient value for body mass was not significantly different from zero, so Model 5 (the simpler model that did not include body mass) was selected for further investigation. The values for minimum life supporting metabolic rate ("L_{met}") and scaling coefficient ("S") determined from this model were $1.66 \times 10^{-5} \pm 2.85 \times 10^{-6}$ mL·min^{-1}, and $1.78 \times 10^{-7} \pm 7.00 \times 10^{-8}$ mL·min.°C^{-1}, respectively. These values were then used for all following models.

Table 5.2 - Generalized nonlinear models relating measured *E. propertius* CO_2 production rate ("CO_2", mL/min) to measured body temperature ("T", °C). Caterpillar body mass ("M", mg) was used as a covariate in some models. Coefficients estimated by the maximum likelihood function include: "S", an overall scaling factor, "M_s", a mass scaling factor, "T_s", a temperature scaling factor, and "L_{met}", a coefficient representing the minimum life supporting metabolic rate. All models are better than a trivial linear model ($CO_2 = S \times T$) using a log-likelihood test ($p < 0.05$). The model with the lowest AIC is bolded, however Model 5 was finally selected since there is no significant difference in explanatory power between Model 4 and Model 5 (likelihood ratio test, L = 3.06, p = 0.08) and Model 5 is simpler. A power of the mean model for variance was used in all models. AIC = Akaike's Information Criterion, df = degrees of freedom.

Model number	Model	AIC	Log likelihood	df	Parameter p-values
1*	$CO_2 = S \times M^{-0.25} \times e^{Ts \times T} + L_{met}$	-3291.195	1650.598	5	S: < 0.001 T_s: < 0.001 L_{met}: < 0.001
2*	$CO_2 = S \times M^{Ms} \times e^{Ts \times T} + L_{met}$	-3296.919	1654.459	6	S: < 0.001 M_s: 0.781 T_s: < 0.001 L_{met}: < 0.001
3*	$CO_2 = S \times e^{Ts \times T} + L_{met}$	-3300.129	1655.064	5	S: < 0.001 T_s: < 0.001 L_{met}: < 0.001
4*	$CO_2 = S \times M^{Ms} \times T^{Ts} + L_{met}$	**-3306.704**	**1659.352**	**6**	**S: < 0.001** **M_s: 0.7979** **T_s: < 0.001** **L_{met}: < 0.001**
5*	$CO_2 = S \times T^{Ts} + L_{met}$	-3305.645	1657.823	5	S: < 0.001 T_s: < 0.001 L_{met}: < 0.001

Second, Model 5 (Table 5.2) was used to investigate model structure and the effect of incubator temperature regime and geographical origin on the metabolic rate reliance on temperature. I used the parameter estimates for L_{met} and S from the previous step to estimate group-specific temperature sensitivity (T_s). Generalized nonlinear and nonlinear mixed-effects models (Pinheiro and Bates 2000) using the "gnls()" and "nlme()" functions respectively in R were fitted to carbon dioxide production rate data from the autumn (Table 5.3) and the spring (Table 5.4). Because it was expected that there might be individual effects on this relationship (so the value of T_s might vary significantly between individuals), generalized nonlinear mixed effects models were initially investigated. However, 95% confidence interval plots of individual values of these coefficients indicated few differences among individuals, so models without mixed effects were also investigated. Mixed effects models were specified with either T_s as a random effect or not. All generalized nonlinear models were given a variance structure whereby variance increased as a power of mean temperature (Pinheiro and Bates 2000). The estimated power functions were 0.956 and 0.701 for autumn and spring respectively, which indicated that the power of the mean variance function was appropriate (Crawley 2007). Residual plots indicated homoscedasticity. The overall best-fit models for both time points based on AIC and likelihood ratio tests were generalized nonlinear models with larvae from VI raised at stable temperatures as one group, and all other larvae pooled in a second group (Tables 5.3 and 5.4). Extracted experimental group coefficients (Table 5.5) were used for all subsequent modelling of metabolic rate as a function of temperature.

Table 5.3 - A comparison of models relating *E. propertius* CO_2 production rate to body temperature during the autumn. All models are based on the general form of Model 5 (Table 5.2), and used the estimated values of S and L_{met} from that model, while T_s was estimated for the experimental groups noted. All generalized nonlinear mixed effects models were fitted with T_s and S as fixed effects. AIC = Akaike's Information criterion. df = degrees of freedom. The model with the lowest AIC is in bold type, and coefficients from this model were used in all subsequent analyses. Different letters beside log likelihood ratios indicate models that significantly differ in explanatory power within the generalized nonlinear models ($p < 0.05$). Coefficient p-value refers to the probability that T_s does not differ significantly from 0.

Model type	Random effects	Experimental groups	AIC	Log likelihood	df	Coefficient p-value
Generalized nonlinear mixed effects	T_s	All rearing conditions × source populations	-1741.769	874.885	4	< 0.001
Generalized nonlinear mixed effects	T_s	None (all data pooled)	-1725.219	866.609	4	< 0.001
Generalized nonlinear mixed effects	T_s	VI stable separate, other 3 groups together	-1744.758	876.379	4	< 0.001
Generalized nonlinear	NA	All rearing conditions × source populations	-1750.003[a]	881.002	6	< 0.001
Generalized nonlinear	NA	None (all data pooled)	-1727.219[b]	866.609	3	< 0.001
Generalized nonlinear	**NA**	**VI stable separate, other 3 groups together**	**-1752.134[a]**	**880.067**	**4**	< 0.001

Table 5.4 - A comparison of models relating *E. propertius* CO_2 production rate to body temperature during the spring. All models are based on the general form of Model 5 (Table 5.2), and used the estimated values of S and L_{met} from that model, while T_s was estimated for the experimental groups noted. All generalized nonlinear mixed effects models were fitted with T_s and S as fixed effects. AIC = Akaike's Information criterion. df = degrees of freedom. The model with the lowest AIC is in bold type, and coefficients from this model were used in all subsequent analyses. Different letters beside log likelihood ratios indicate models that significantly differ in explanatory power within the generalized nonlinear models ($p < 0.05$). Coefficient p-value refers to the probability that T_s does not differ significantly from zero.

Model Type	Random effects	Experimental groups	AIC	Log likelihood	df	Coefficient p-value
Generalized nonlinear mixed effects	Ts	All rearing conditions × source populations	-1604.600	806.300	4	< 0.001
Generalized nonlinear mixed effects	Ts	None (all data pooled)	-1601.278	804.639	4	< 0.001
Generalized nonlinear mixed effects	Ts,	VI stable separate, other 3 groups together	-1605.459	806.730	4	< 0.001
Generalized nonlinear	NA	All rearing conditions × source populations	-1610.525[a]	811.263	6	< 0.001
Generalized nonlinear	NA	None (all data pooled)	-1603.278[b]	804.639	4	< 0.001
Generalized nonlinear	**NA**	**VI stable separate, other 3 groups together**	**-1611.517[a]**	**809.759**	**4**	< 0.001

Table 5.5 - Group-specific coefficients for temperature sensitivity (Ts) from the best-fit generalized nonlinear model ($CO_2 = S \times T^{Ts} + L_{met}$ **, Tables 5.3 and 5.4) relating *E. propertius* CO_2 production rate to body temperature.** VI = Vancouver Island; stable = rearing conditions approximating Vancouver Island climate. Value indicated is mean ± SEM.

Group membership	Time of year	Coefficient
VI stable	Autumn	2.54 ± 0.029
All other rearing × source	Autumn	2.41 ± 0.018
VI stable	Spring	2.38 ± 0.025
All other rearing × source	Spring	2.46 ± 0.011

5.2.6 **Modelling CO_2 production from meteorological data**

Carbon dioxide production for every hour of each winter was estimated from hourly or monthly mean climate data from OR and VI using the general equation from Model 5 (Table 5.1) with group-specific coefficients as described in Table 5.5. Total CO_2 production/winter was estimated under variable (OR) and stable (VI) thermal regimes for larvae expressing high or low thermal sensitivity. To examine the effect of phenology changes, start and end dates of winter in the model were moved sequentially forward and backward from the fall and spring equinox respectively in daily intervals to the most extreme value observed for either population in the lab (Section 5.2.7) and CO_2 production was calculated for the resulting winter. CO_2 production was converted into lipid used assuming a respiratory exchange ratio (RER) of 0.7 (indicating complete reliance on lipid catabolism), and that 2 L of oxygen is converted to H_2O per mg of lipid oxidised (Schmidt Nielsen 1990).

5.2.7 **Estimating site-specific phenology**

Date of entry into dormancy was obtained from the laboratory portion of this study (Section 5.2.1), while the end of dormancy was estimated from a combination of field and lab data. Average first flight dates were April 3 and April 12 respectively for OR and VI, and average last sightings were on June 14 and June 19 respectively (dates obtained from transect-based population surveys, previously described by Prior et al. 2009, Hellmann et al. 2008). Adult *E. propertius* eclose on average 17 days after transfer to permissive conditions (16L:8D, 20 °C), and average adult lifespan is 28 days when provided with sucrose solution *ad libitum* in the laboratory. Thus energy use was calculated from 17 days before the first adult sighting to 45 days before the last adult sighting giving end of dormancy dates ranging from March 17 – May 10.

5.2.8 **Assumptions**

Our first assumption is that carbon dioxide production is a reasonable proxy for metabolic rate. Carbon dioxide production will not capture metabolic processes which do not result in the production of carbon dioxide such as anaerobic processes, and some insect species

do show a shift towards anaerobic metabolism during diapause (Hahn and Denlinger 2011). This would result in my estimates of energy use being too low, but provided the treatment groups did not differ in their proportion of anaerobic metabolism this will affect only absolute energy use values and not the relationships among groups and locations. I have no reason to believe that my treatment groups would differ in their propensity for anaerobic metabolism.

The second assumption concerns the relationship between carbon dioxide produced and lipid consumed. I have assumed a respiratory exchange ratio of 0.7 moles carbon dioxide for each mole of oxygen; the ratio observed in animals relying solely on lipid metabolism (Schmidt Nielsen 1990). Fuel use during diapause is dynamic and in addition to lipid insects may also rely on carbohydrate, protein, or a mixture of these components; the importance of which can change over the course of the winter (Hahn and Denlinger 2011). Respiratory exchange ratios for these substrates range from 0.8-1 moles carbon dioxide to each mole oxygen. I also ran all analyses using a respiratory exchange ratio of 1, and my conclusions about relationships among groups were unchanged. Total lipid use estimates are 30% lower if the respiratory exchange ratio was 1, thus my values represent maximum lipid use. The cost of variability decreases from 0.6 mg to 0.42 mg per degree of daily thermal amplitude assuming a respiratory exchange ratio of 1. I have no reason to believe that treatment groups would differ in their substrate utilisation, although different respiratory exchange ratios among groups would introduce systematic bias.

The third assumption is that climate measurements are a reasonable proxy for microclimate conditions. As *E. propertius* overwinters on the ground, the main modifiers of microclimate temperature will be leaf litter and snow (Zhang et al. 2008). Leaf litter may buffer variability to some extent, decreasing the highs and increasing the lows without substantially changing the mean (Parajulee et al. 1997), meaning my absolute values may be slightly overestimated. However, I assume that leaf litter will buffer microclimates to similar degrees in both locations. Snow cover generally increases temperature relative to air temperatures by preventing temperatures from dropping below

zero, and so would be expected to increase energy use. There is not extensive snow cover in these habitats in either Oregon or British Columbia.

5.3 **Results**

Larvae from VI or OR attained greater mass when reared under variable temperatures ($F_{1,109} = 11.9$, $p < 0.001$), and larvae from OR attained greater size than those from VI when reared under stable conditions (rearing conditions × source population interaction: $F_{1,109} = 7.08$, $p = 0.008$; Table 5.6). There was a significant positive relationship between mass and date of dormancy for larvae reared at variable temperatures ($F_{1,61} = 12.78$, $p < 0.001$), but not those reared at stable temperatures ($F_{1,50} = 1.08$, $p = 0.304$; date of dormancy × rearing conditions: $F_{1,109} = 18.93$, $p < 0.001$). Thus extended pre-winter dormancy resulted in smaller variable-reared larvae in November. Larvae kept at variable temperatures entered dormancy more than a month earlier than those kept at stable temperatures ($F_{1,112} = 267.78$, $p < 0.001$), but larvae from OR entered dormancy significantly later than those from VI under variable conditions ($F_{1,112} = 14.73$, $p < 0.001$; Table 5.6). Lipid concentrations in November were higher in larvae reared at stable temperatures ($F_{1,16} = 8.53$, $p = 0.010$; Table 5.6). At this time, immediately after the most variable temperatures were experienced, metabolic rate was depressed at high temperatures in individuals that came from OR or experienced variable temperatures, significantly decreasing thermal sensitivity relative to larvae from VI reared at stable temperatures (Figure 5.3a, Table 5.3). In February, the metabolic suppression of larvae which had experienced variable temperatures had been released, and in fact thermal sensitivity was now slightly but significantly higher in those groups compared to the VI/stable animals (Figure 5.3b, Table 5.4).

Table 5.6 - Life history, physiological and experimental design parameters of *Erynnis propertius* larvae used in my experiments. The number of mothers from which larvae for each group originated ($N_{mothers}$) is a minimum estimate as multiple females (1-4) were housed together. $N_{dormant}$ refers to the number of larvae surviving to enter dormancy. Values are mean ± SD, different letters indicate significant differences among treatment groups (see text for details).

Rearing	**Variable**		**Stable**	
Source	**OR**	**VI**	**OR**	**VI**
$N_{mothers}$	14	49	22	37
$N_{dormant}$	28	35	20	32
Mass (mg)	246.5 ± 43.4^{a}	244.1 ± 52.3^{a}	231.3 ± 31.7^{b}	210.5 ± 34.8^{c}
Date of dormancy	Aug 17 ± 8.7 days[a]	Aug 9 ± 7.9 days[b]	Sep 7 ± 7.6 days[c]	Sep 4 ± 6.3 days[c]
Lipid (mg·mg DM^{-1})	0.090 ± 0.028^{a}	0.099 ± 0.026^{a}	0.130 ± 0.026^{b}	0.129 ± 0.030^{b}

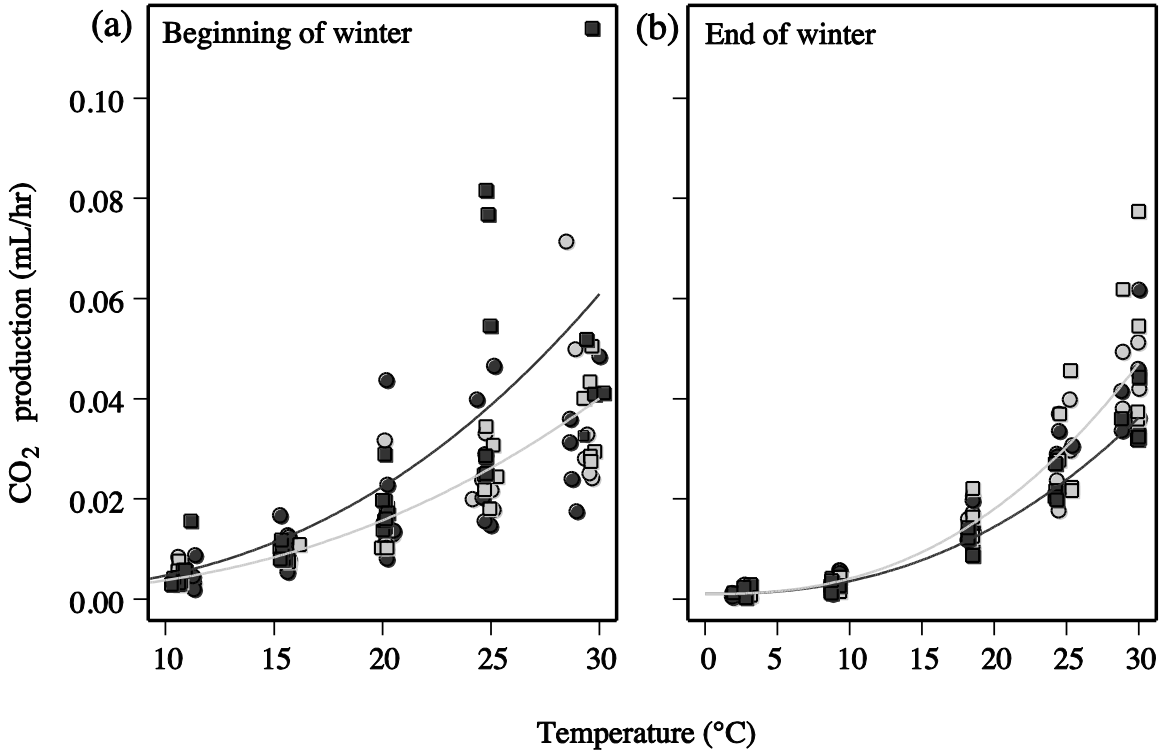

Figure 5.3 - Temperature-CO_2 production relationships of *E. propertius* larvae measured at (a) the beginning (n=24) or (b) the end (n=24) of winter. Light grey shading indicates larvae originating from OR, dark grey those from VI. Circles indicate variable, squares stable, rearing temperature conditions. The dark grey line shows the best model fit for larvae from VI reared at stable conditions; the light grey line the best model for the other three treatments.

5.3.1 **Predicted overwinter metabolism**

To predict the consequences of metabolic suppression on energy use by larvae from variable environments, I used the equations describing the metabolic rate - temperature relationship for each source population, and OR meteorological data. Predicted inter-equinox metabolic demand was approximately 20 % lower in larvae expressing the low thermal sensitivity phenotype compared to those with the high thermal sensitivity phenotype (Figure 5.4a). I then estimated metabolic demand of each source population, in their natal environment, and predicted metabolic demand in OR to be higher than on VI, based on both hourly temperatures (i.e. incorporating thermal variability; $t_{59.2}$= 8.70, $p < 0.001$) and monthly mean temperatures ($t_{56.3}$= 3.11, p = 0.003; Figure 5.4b). The magnitude of the difference between the locations was greatest and more consistent when variability was accounted for (Figure 5.4b). I subtracted the consumption predicted from mean temperatures (estimated from monthly mean climate data) from the consumption predicted from hourly temperatures for each year, and found that the additional energetic cost of thermal variability over a six month period of dormancy was expected to be greater in variable than stable conditions ($F_{1,58}$ = 15.14, $p < 0.001$; Figure 5.5). In the variable climate, the expected energetic cost of thermal variability was positively correlated with daily thermal amplitude ($F_{1,33}$=11.15, p=0.002), and the slope predicted a cost of 0.6 mg lipid/winter for every degree of additional temperature range (Figure 5.5). There was no relationship between the predicted energetic cost of thermal variability and daily thermal amplitude in the stable environment ($F_{1,25}$=1.20, p=0.284, Figure 5.5).

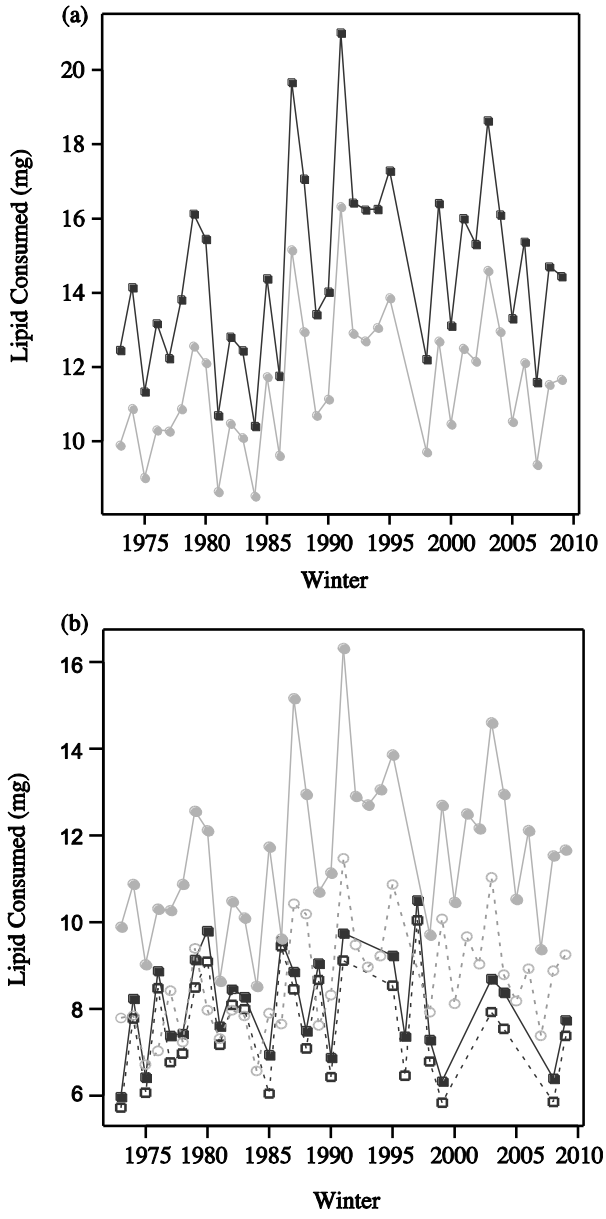

Figure 5.4 - Predicted overwinter energy use of *Erynnis propertius* from OR and VI. (a) Predicted overwinter energy use from 1973 – 2010 in OR for larvae expressing high (dark squares) or low (light circles) thermal sensitivity. (b) Predicted energy use in their natal environment for larvae expressing high (VI, dark grey squares) or low (OR, light grey circles) thermal sensitivity, based on either hourly (solid lines, filled symbols) or monthly mean (dotted lines, open symbols) temperatures.

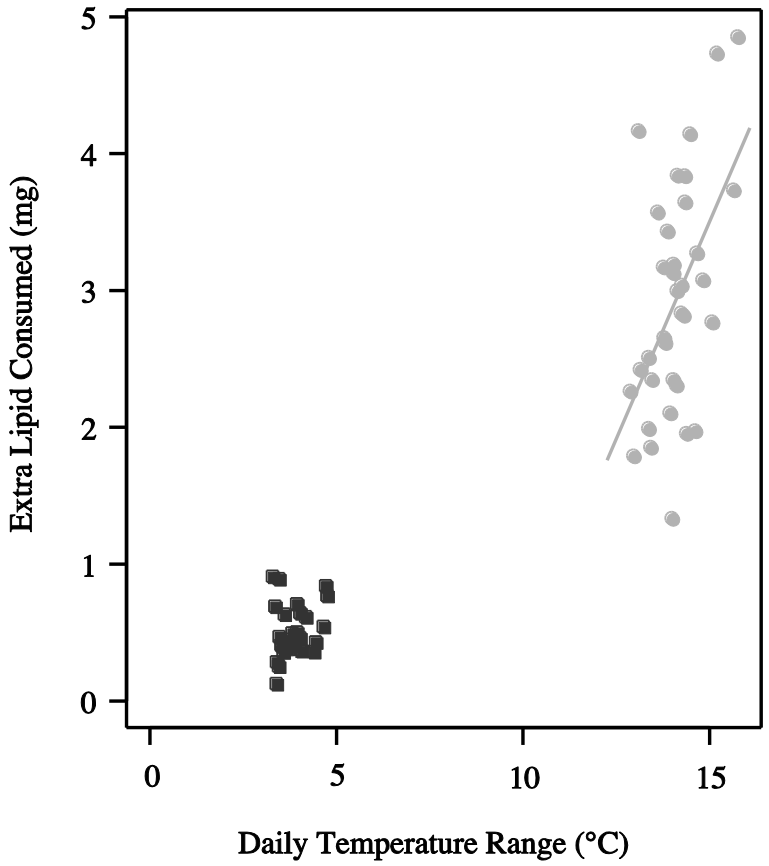

Figure 5.5 - Predicted extra lipid used overwinter by *E. propertius* larvae above that predicted by mean temperatures as a function of daily thermal amplitude in a given winter in OR or VI. Dark symbols: larvae with high thermal sensitivity in VI; light symbols: larvae with low thermal sensitivity in OR.

The rate of use of stored reserves is strongly influenced by phenology in the field (Han and Bauce 1998, Bosch et al. 2010). I therefore used my hourly estimates of lipid use in each location (1973-2010) to predict the energetic impact of phenological shifts under stable or variable climatic regimes by shifting the start and end dates of dormancy across the range of observed dates at which larvae cease and resume activity. At any given length of winter, variable OR winters are expected to be more energetically expensive than stable VI winters (Figure 5.6), despite the metabolic rate suppression expressed by OR-derived individuals. This discrepancy in energetic costs between locations increases with increasing length of winter, and phenological changes have more impact in variable conditions (Figure 5.6). At ecologically-relevant dates, lipid consumption in OR (27.3 mg) is predicted to be more than double that on VI (12.7 mg; Figure 5.6). Phenological shifts have more impact in autumn than in spring, as can be seen from the steeper decline in energy use with delaying start date compared to advancing end date (Figure 5.6).

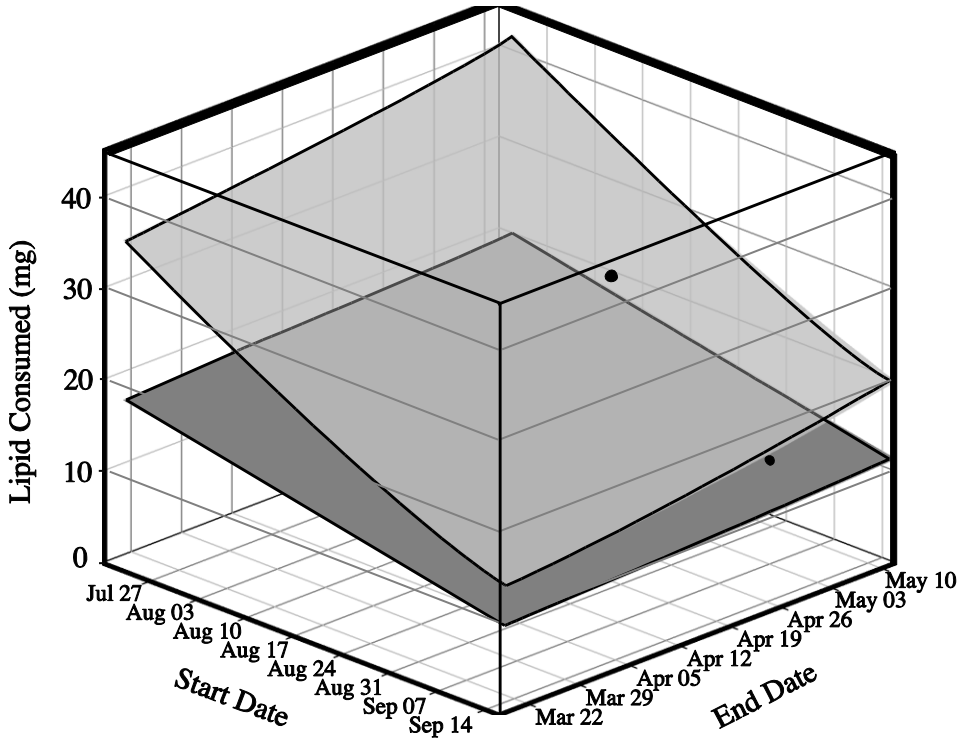

Figure 5.6 - The sensitivity of overwintering energy use of *E. propertius* larvae to phenological shifts in OR (low thermal sensitivity; light grey) or VI (high thermal sensitivity; dark grey). Dates encompass the full range of start and end times of dormancy in *E. propertius* (Table 2). Black dots indicate location-specific start and end dates of dormancy (Section 5.2.7).

5.4 **Discussion**

Lynch and Gabriel's (1987) model describing the evolution of thermal tolerance curves predicted a decrease in the thermal sensitivity of performance functions in response to increasing temporal environmental heterogeneity, particularly where the environment was spatially homogenous. This is likely to be the case in *E. propertius*, where populations experience a spatially restricted habitat due to low dispersal, indicated by considerable genetic structure across the range, particularly towards the northern range edge (Zakharov and Hellmann 2008). The observed decrease in thermal sensitivity demonstrated here is the first empirical support for this theory with regards to variability on a daily timescale. I show metabolic suppression in overwintering caterpillars in response to high daily thermal variability, which appears to be facultative in the VI population. By contrast, all larvae of OR origin expressed the suppressed metabolism phenotype, suggesting that metabolic suppression is genetically fixed in that population. I cannot rule out the possibility that larvae in VI express this decrease in thermal sensitivity in more variable years in VI. The facultative response in the VI population may be maintained due to the benefits of high thermal sensitivity in autumn in cool years, which could allow the VI population to develop faster and attain greater size than if they expressed the low thermal sensitivity phenotype. There may be a cost to the facultative decrease in thermal sensitivity that could lead to trade-offs in resource allocation (e.g. down-regulation of immune function) stemming from the costs of synthesising alternative isozymes with different thermal properties (Hochachka and Somero 2002).

Dormant insects are small-bodied, with limited thermoregulatory capabilities, and thus experience fluctuations in body temperature throughout the day that make average temperatures a poor estimate of energy expenditure. If mean temperatures are used, the difference between the stable and variable environments is reduced. For example, in some years the mean conditions predict lower energetic costs in the variable environment than in the stable environment, while accounting for variability reveals that the variable environment is always more energetically-demanding. The relative differences in energetics may be exacerbated in thermally variable years if VI larvae facultatively

suppress thermal sensitivity of metabolism in the field. I believe that my approach, which better incorporates the non-linear effects of temperature, will improve the accuracy of predicting whole-insect performance measures such as development time compared to degree-day models (Schlenker and Roberts 2009).

Despite the energy savings afforded by decreased thermal sensitivity, the variable environment was still more energy demanding - in a "typical" winter, larvae in the variable environment were predicted to have double the energy expenditure of those in more stable temperatures. Variable-reared larvae were larger than their stable-reared counterparts, likely due to enhanced growth during the warmer and more variable growing season. Larvae from OR were larger than those from VI under stable temperatures (in agreement with previous findings; Pelini et al. 2009), suggesting a genetic or maternally-mediated predisposition to increased size which could result from selection for greater energy stores (Chippendale et al. 1996), and would compensate for the increased energy drain of the more variable environment. Mass was measured after all larvae had entered dormancy, so there was a variable period of time over which larvae were dormant (non-feeding) before the mass measurements were taken. When larvae were reared at variable temperatures (but not when reared under stable conditions), there was a positive relationship between date of diapause and mass, such that larvae which entered diapause later were larger. This provides additional support for the increased energetic demand of variable thermal conditions. Lipid concentrations were lower in larvae reared at variable temperatures, making them more vulnerable to deleterious effects of energy depletion. Using the masses and lipid concentrations measured in this study and assuming a water content of 75 % for this species (Chapters 2 and 3), I predict that larvae reared at variable temperatures would have a total lipid content of approximately 7 mg remaining in November, larvae from OR (expressing low thermal sensitivity) reared at stable temperatures would have 9 mg, and larvae from VI reared at stable temperatures would have 8 mg. This supports my model predictions of increased energy drain as a result of variable temperatures in the autumn, as does the fact that larvae from OR, expressing decreased thermal sensitivity, have increased energy stores compared to larvae expressing high thermal sensitivity in the same (stable) environment.

Although the remaining lipid is low compared to my model predictions, I note that the time at which the measurements were taken (early November) was later than the latest start of dormancy date in my model, thus energy use from that point on is predicted to be inconsequential since the majority of lipid is consumed in the autumn. In addition to baseline metabolic costs, larvae in variable conditions may incur further energetic costs due to thermal stress, for example the cost of synthesising heat shock proteins or other molecular chaperones (Sørensen et al. 2003).

Here I show that phenological shifts will have more impact in variable thermal environments, particularly in the autumn. Variation in phenology is heritable and under strong selection (van Asch et al. 2007), and phenological shifts have been among the most well-documented consequences of recent climate change (Parmesan and Yohe 2003). I would therefore expect populations from variable environments to be selected for delayed entry into dormancy, but *E. propertius* from OR reared at variable temperatures actually enter dormancy almost three weeks earlier than do stable-reared larvae. Larvae reared under variable conditions are larger than those in stable environments, so the lack of delay could result from larvae entering dormancy at a pre-determined instar or size threshold (Nijhout 1975) that is reached earlier in variable-reared larvae due to higher metabolic and growth rates. Early dormancy could also be driven by declining host plant quality early in the autumn in variable environments (Ishihara and Ohgushi 2006), although no such cues were present in my laboratory rearing. When raised in a variable thermal environment, larvae from VI enter dormancy even earlier than larvae from OR (Table 5.5). This suggests that OR larvae may have been selected to delay the onset of dormancy, perhaps to mitigate the deleterious effects of high autumn temperatures.

My model explains why early entry into dormancy decreases fitness of overwintering insects, while extended cool springs have less impact (Han and Bauce 1998, Bosch et al. 2010). This suggests that although phenology research has focused on spring metrics such as first flight, flowering, or breeding dates (e.g. Thackeray et al. 2010), it is also important to thoroughly document autumn phenological events such as leaf drop, migration, and entry into dormancy because of the significant potential impact on

overwintering energetics. Although in my study system the centre of the range (OR) is more variable than the northern range edge due to oceanic influences on Vancouver Island, daily thermal range typically increases with increasing latitude and elevation (e.g. Sunday et al. 2011). Therefore, species following isotherms upwards or polewards in response to climate change may encounter changes in variability leading to increased energetic demands. In addition, predicted increases in mean temperatures (IPCC 2007) will shift daily thermal cycles to fluctuate about a steeper point on the metabolic rate - temperature relationship, compounding energetic drain (Dillon et al. 2010). If consumption of energy reserves over winter increases, this could precipitate life-history trade-offs such as decreases in adult size and fecundity that could decrease the fitness of individuals and increase likelihood of population declines in many temperate, univoltine, insects, and possibly in other temperate ectotherms as well.

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Chapter 6: General Discussion

As thermal regimes change rapidly, a theoretical framework predicting the outcomes of winter warming on insect populations is desirable, and such a framework must be based on empirical evidence. This can only be achieved through a robust understanding of the effects of changes in winter temperatures on insect physiology, the mechanisms by which insects mitigate the potentially negative consequences of winter warming, and species traits that may predict the degree of vulnerability (or, conversely, the degree of adaptation) to warming that is likely for any given species. In this dissertation I have presented data on vulnerability to warming for four species of Lepidoptera, and characterised, at the physiological level, novel mechanisms of adaptation to changes in the overwintering thermal environment for two species. I will now place these data into the context of a framework for assessing vulnerability to winter warming in insects.

6.1 Consequences of increased energetic demand overwinter

Energetic demand over winter may increase through either a rise in mean temperatures, or an amplification of thermal variability. An increase in mean or variability of temperatures will raise metabolic expenditure through Arrhenius effects on rates of biochemical reactions (Kingsolver 2009, Figure 1.1). With regards to energetics, there are two alternative outcomes of warmer or more variable temperatures over winter, which are not mutually exclusive: 1) energetic depletion, with associated lethal or sub-lethal effects, and 2) downregulation of metabolic rate to mitigate negative consequences of energetic depletion.

6.1.1 Energetic depletion as a consequence of winter warming

In the absence of complete metabolic compensation, increased energetic demands over winter will lead to increased rates of use of stored energy reserves, with associated sub-lethal and/or lethal effects. The only species that showed susceptibility to energetic depletion over winter was *Erynnis propertius*, where both empirical evidence (Chapter 3 and 5) and theoretical predictions suggested that warmer or more variable winters would result in lower energy reserves remaining in the spring. The ecological consequences of

less energy include increased overwinter mortality (e.g. Irwin and Lee 2000,Williams et al. 2003, Bosch et al. 2010, Musolin and Numata 2003, Koštál et al. 2011), decreased fecundity (Irwin and Lee 2000,Williams et al. 2003, Musolin and Numata 2003, Irwin and Lee 2003), and decreased longevity (Musolin and Numata 2003), although I was not able to assess fitness consequences in *E. propertius* due to insufficient sample sizes resulting from very high overwinter mortality in the laboratory. The insensitivity of *Hyphantria cunea*, and possibly also *Papilio glaucus* and *P. troilus*, resulted from adaptive modifications to the metabolic rate - temperature relationship.

6.1.2 **Adaptive modifications to metabolism**

The relationship between temperature and respiratory metabolism has been at the centre of attempts to explain global patterns in biodiversity, structure of species' geographical ranges, and growth rate and body size of animals (Chown and Gaston 1999, Gillooly et al. 2001, Allen et al. 2002, Clarke 2003). Organisms can modify their physiological responses to temperature in order to maintain optimal function despite a change in operative temperature (temperature compensation; Clarke 2003), and alterations to the metabolic rate - temperature relationship are important mechanisms allowing populations to persist in environments where this would not otherwise be possible (Chown and Gaston 1999). This metabolic compensation may occur as a result of acclimation (facultative responses) or as a result of evolutionary adaptation (obligate responses). Although many studies in the ecology and physiology literature invoke metabolic compensation to explain inter- and intra-specific differences in metabolism, particularly in the framework of metabolic cold adaptation (Clarke 1993), and seasonal dormancy (Hahn and Denlinger 2011), a closer inspection reveals that concrete demonstrations of metabolic compensation at the physiological level are scarce.

In this dissertation, I have documented two classes of modification to thermal physiology, which are potentially adaptive. The first involves a general metabolic suppression across all temperatures, which was demonstrated facultatively in *Hyphantria cunea* from two source populations in response to winter warming (Chapter 4). *Papilio glaucus* and *P. troilus* may also show a similar metabolic down-regulation, based on the lack of depletion

of storage lipids during warmer winters (Chapter 3), although I did not measure metabolic rates in these species. The second class of modification is a decrease in thermal sensitivity of metabolism. In *H. cunea*, a decrease in thermal sensitivity was an obligate response in the population from a warmer and more variable climate in the centre of the range, compared to the population from the cooler northern range edge (Chapter 4). In *Erynnis propertius*, a decrease in thermal sensitivity was a facultative response to increased daily thermal variability in a population from a stable environment, and an obligate response in a population from a variable environment (Chapter 5). I also saw a facultative decrease in thermal sensitivity of metabolism in *H. cunea* in response to increased daily thermal variability in the laboratory (unpublished data). I will now put these results into the context of other examples of facultative (phenotypically plastic) and obligate (evolutionary) metabolic compensation.

6.1.2.1 Phenotypic plasticity in metabolism

Perhaps the strongest evidence for the ability of insects to compensate their metabolic rates in response to changes in environmental temperatures comes from studies of thermal acclimation. Insects commonly raise or lower metabolic rates to compensate for acute changes in temperature (e.g. Terblanche et al. 2009, Le Lann et al. 2010, Terblanche et al. 2010). Diapause itself is commonly considered an example of metabolic compensation: insects when in diapause may have metabolic rates less than 10 % of their non-diapausing rates, at the same body temperature (Ragland et al. 2009). However, in this case, care must be taken not to confound seasonal changes in ATP demand with metabolic compensation (Clarke 1993). Respiratory metabolism consists of the utilisation of oxygen and the production of carbon dioxide resulting from the consumption of energy reserves in the production of ATP (Sibly and Calow 1986), and thus corresponds to the demand of an organism for ATP. The demands for ATP in a dormant organism are much reduced compared to those of an active organism, as movement, assimilation, growth, and development are halted. The remaining sources of ATP consumption are those related to basal metabolism; in insects mainly protein turnover and maintenance of proton gradients, both of which are reduced in winter as a consequence of lowered activity (Clarke 1993). Stress resistance, which, for overwintering insects, may require the production of

molecular chaperones, antifreeze proteins, or low-molecular weight cryoprotectants (Lee 2010), may impose additional costs on overwintering organisms in addition to basal metabolic costs. Notwithstanding additional demands of cold-stress, a large portion of reduced metabolism in overwintering insects is a consequence of decreases in ATP demands of biological processes such as maintenance, production, activity, and reproduction, and does not require metabolic compensation to be invoked (Clarke 1993). Thus, although intrinsic factors acting directly on enzymes or membranes may contribute to metabolic suppression during diapause, lowered metabolic rates in diapausing organisms are not in themselves sufficient evidence to infer metabolic compensation.

There is limited evidence for phenotypically plastic modifications of thermal sensitivity in insects. Active crickets (*Acheta domesticus*) acclimated to 25 °C had reduced thermal sensitivity compared to those acclimated to higher temperatures, although the effect was weak and requires confirmation (Lachenicht et al. 2010). Other studies have found no effect of acclimation to different mean temperatures in insects (Terblanche et al. 2009, Terblanche et al. 2010) and other ectotherms (Schaefer and Walters 2010). To my knowledge, only one other study has examined the effects of daily thermal variability on the acclimation of thermal sensitivity in an ectotherm (a larval anuran amphibian), finding no effect of acclimation on thermal sensitivity (Niehaus et al. 2011), but this was in an active, feeding animal. The reduction in thermal sensitivity expressed by *E. propertius* in response to increased daily thermal variability is, to my knowledge, the only example to date of a facultative modification of thermal sensitivity in insects (Chapter 5) or other dormant ecotherms. I hypothesise this decrease in thermal sensitivity to be adaptive in that it reduces the impact of daily thermal variability; particularly in autumn when temperatures are warm and variable but development does not occur (Chapter 5). Possible examples of acclimatization of thermal sensitivity in response to thermal variability may be found in diapausing insects: the thermal sensitivities of metabolism of dormant organisms are sometimes lower than those of active insects (Layne and TenEyck 1996, Bennett et al. 1999), but as multiple biotic and abiotic variables co-vary in the field it is impossible to make causal assumptions about the origin of this decrease.

6.1.2.2 **Metabolic cold adaptation**

The metabolic cold adaptation (MCA) hypothesis is the foremost example of an attempt to explain evolutionary variation in metabolic rate - temperature relationships. Metabolic cold adaptation posits that organisms living in relatively cold (high-latitude or -altitude) environments will have evolved higher metabolic rates at any given environmental temperature, and higher thermal sensitivity, compared to organisms from warmer environments (Clarke 1993). This allows organisms in energy-limited environments, with short growing seasons and long winters, to sustain high rates of growth and development despite lowered ambient temperatures. Metabolic cold adaptation, while well-established at the biochemical level (Hochachka and Somero 2002), has been controversial as the hypothesis has found mixed support at intra- and inter-specific levels, for several reasons: 1) respiratory metabolism reflects ATP demand at the time of measurement, and thus in active animals may incorporate the ATP demands of processes such as growth, development and performance in addition to basal metabolic costs (Clarke 1993); 2) study designs frequently confound developmental influences on metabolic rates with genetically-based variation by failing to account for acclimation state (Chown and Gaston 1999, Clarke 2003); and 3) inter-specific comparisons confound metabolic compensation with phylogenetic constraints due to differences in ecology and life-history (Chown and Gaston 1999). However, even accounting for these factors, MCA does appear to occur in ectotherms (e.g. Terblanche et al. 2009, Schaefer and Walters 2010, Ayres and Scriber 1994, Berrigan and Partridge 1997, Addo-Bediako et al. 2002), although it is not a universal pattern (e.g. Schaefer and Walters 2010, Nielsen et al. 1999). To my knowledge, Chapter 4 is the first study to address MCA in overwintering or dormant organisms, despite the fact that dormant animals represent an ideal test of metabolic compensation due to the uncomplicated interpretation of the significance of respiration rates, stemming from the cessation of almost all growth, development, and activity. My data from Chapter 4 provide some support for the MCA hypothesis: *H. cunea* from a cooler climate have higher thermal sensitivity than those from a warmer climate. I am not, however, able to discount the possibility that differences may be due to developmental effects (but not acclimation, as the OT ecotype expressed the increased

thermal sensitivity in two separate acclimation treatments). Further support for the MCA hypothesis comes from a general increase in the slope of the metabolic rate - temperature relationship with increasing latitude in insects (Irlich et al. 2009).

Similar to the MCA hypothesis, I also predicted higher metabolic rates in cooler climates in overwintering insects, but the proximate selection pressures leading to the pattern are different. Although there is no benefit to an increase in metabolic rate *per se* (Clarke 1991), particularly for dormant organisms that obtain no benefit from the ATP produced, lower metabolic rates in warmer populations likely arise from stronger selection on those populations to reduce rates of resource utilisation during energetically demanding winters. Thus, metabolic cold adaptation is perhaps a misnomer with regards to overwintering organisms - a more appropriate description would be metabolic warm adaptation. No evidence for decreased metabolic rates over winter in southern compared to northern populations or species yet exists, but I believe this will be an interesting extension to the field. There is evidence that minimum life-supporting metabolic rates across taxa are independent of temperature (Singer et al. 1993; Makarieva et al. 2006), providing support for the ability of animals from a wide range of taxonomic groups to compensate their metabolic rates to the lowest possible limit despite diverse temperatures experienced during dormancy.

6.1.2.3 Ability of insects to completely compensate for temperature changes

Metabolic rate is the summation of all energy-demanding processes (Clarke 2003), and thus represents the cost of living at a given environmental temperature. In general, Arrhenius plots of resting metabolic rates as a function of environmental temperature indicate that the cost of living is positively related to temperature, possibly due to higher rates of protein turnover at high temperatures and higher costs of maintaining mitochondrial proton gradients (Clarke 2003). High cost of living at high temperatures is matched by high rates of energy intake and more active lifestyles, which are accompanied by decreased longevity. Energy efficiency may decrease above an optimal temperature, if metabolic costs outpace increases in food intake (e.g. Vucic-Pestic et al. 2010); but in

conditions where energy-intake is not limiting, insects choose body temperatures that favour fast growth over efficient utilisation of resources (Miller et al. 2009). Thus, increased costs of living at higher temperatures may not negatively impact fitness. Indeed, inter-specifically, thermodynamic constraints result in insects that live at higher average temperatures having higher rates of population increase than those at low temperatures (Frazier et al. 2006). Thus it appears that temperature compensation is not able to entirely mitigate the thermodynamic disadvantage of life at lower temperatures, on a global scale (Clarke 2003). My work suggests that, intra-specifically, metabolic compensation may be either complete (in the case of *H. cunea*, where warmer winters resulted in overcompensation and a subsequent decrease in utilisation of energy reserves; Chapter 4), or incomplete (in the case of *E. propertius*, which still experienced greater energy drain in variable environment despite metabolic compensation; Chapter 5).

6.1.2.4 Proximate mechanisms of metabolic compensation

To maintain physiological function in diverse thermal environments, animals may attempt to maintain physiological rates by quantitative, qualitative, or modulatory mechanisms. Quantitative modifications involve alterations to quantities of active enzyme (Clarke 2003), which may be mediated by increased copy number (Hochachka and Somero 2002), decreased rates of degradation relative to synthesis (e.g. Sidell 1977), or post-translational or allosteric modifications to enzymes (Storey and Storey 2004). Qualitative enzyme modifications involve alterations to enzyme structure and thus function (e.g. lactate dehydrogenase and citrate synthase in cold-adapted notothenoid fish; Kawall et al. 2002). Small changes in the amino acid sequence of enzymes in key metabolic pathways (e.g. the citric acid cycle or the glycolytic pathway) can enhance performance at some temperatures, to the detriment of performance at other temperatures (Holland et al. 1997). The ability of enzymes to function at low temperatures depends on their retaining the flexibility to perform the conformational changes required to catalyse reactions (Hochachka and Somero 2002), and large scale comparisons of structure in psychrophilic enzymes has shown enhanced flexibility in these proteins in response to cold adaptation (Feller and Gerday 2003). This flexibility may be achieved by replacing the amino acids which provide the enzyme with structural stability, for example proline and arginine

residues (which restrict rotation and can form multiple hydrogen bridges); hydrophobic amino acids (which form relatively strong hydrophobic bonds which stabilize the core of the enzyme), or polar hydrogen-bond forming amino acids (Feller and Gerday 2003).

If gene duplication events result in extra copies of enzymes (isozymes), then the function of the isozymes may diverge (Hochachka and Somero 2002), thereby providing the building blocks for metabolic plasticity. However, the current view is that quantitative modifications are more common mechanisms of acclimation or acclimatization (e.g. (Schulte et al. 2000), while qualitative changes dominate over evolutionary time (Kawall et al. 2002). Thus the metabolic down-regulation seen in *H cunea* in response to warm winter temperatures (Chapter 4), may result from quantitative changes in enzyme levels, resulting from increased transcription and translation (e.g. Schulte et al. 2000), or decreased protein turnover rates (e.g. Sidell 1977). However, quantitative changes cannot account for changes in thermal sensitivity. Facultative changes in thermal sensitivity (e.g. *E. propertius* from Vancouver Island in response to variable rearing temperatures; Chapter 5) are likely to result from the expression of a different isozyme, as isozyme variants can have different degrees of thermal sensitivity (Fields and Somero 1997). *Erynnis propertius* from Oregon, by comparison, may not have the ability to synthesise the more thermally sensitive isozyme, due to the high energetic penalties of expressing high thermal sensitivity in a variable environment (Chapter 5). Higher thermal sensitivity of metabolism may facilitate faster rates of growth and development in an environment with a cooler autumn, maintaining the facultative response in the Vancouver Island population of *E. propertius* (Chapter 5).

An alternative origin of differences in enzyme function can arise from modulation of the membrane environment in which many enzymes reside. Decreases in temperature lower the fluidity of membranes and slow the function of enzymes embedded in the phospholipid bilayer (Hochachka and Somero 2002). The theory of homeoviscous adaptation posits that the temperature-induced restructuring of membranes conserves membrane fluidity at an optimal level (Sinensky 1974). Modifications of membrane composition in response to changes in the thermal environment may occur through

alterations to the saturation of acyl chains, the identity of phospholipid head groups, or the cholesterol content of membranes (Hochachka and Somero 2002). Unsaturated acyl chains and phosphatidylethanolamine head groups both enhance function at low temperatures relative to saturated fatty acids and phosphotidylcholine head groups respectively, by enabling the membrane to remain in a liquid-crystalline (fluid) state to a lower temperature, while cholesterol stiffens membranes and lowers fluidity; decreasing low temperature performance (Hochachka and Somero 2002). When the thermal environment changes, modifications of membrane lipids can be accomplished rapidly (on the scale of hours,e.g. Overgaard et al. 2005), and as a result of acclimation (over days or weeks, e.g. Overgaard et al. 2008, Goto and Katagiri 2011). These modifications are a component of seasonal acclimatization in some insects (e.g. Bennett et al. 1997, Tomcala et al. 2006, Pruitt and Lu 2008, Izumi et al. 2009, Cakmak 2010), and may contribute to the metabolic compensation seen in *E. propertius* and *H. cunea*.

Because oxidative phosphorylation takes place in the mitochondria, mitochondria are key organelles that are modified in response to thermal adaptation. Quantitative, qualitative and modulatory changes to mitochondrial membranes and enzymes (of the classes discussed above) have been documented in response to cold in fish (Clarke 2003), reptiles (e.g. Guderley and Seebacher 2011) and mammals (e.g. Muleme et al. 2006). Few studies on mitochondrial temperature adaptation have been conducted in insects (e.g. Thiessen and Mutchmor 1967), but this would be an interesting direction for future study. A decline in mitochondrial respiration during metamorphosis in the tobacco hornworm, *Manduca sexta*, is induced by targeted down-regulation of succinate dehydrogenase and cytochrome *c* oxidase (Chamberlin 2007), so these enzymes may also be involved in temperature compensation.

A comparative analysis of the lipid composition of the membranes of populations of *E. propertius* and *H. cunea* both pre- and post-exposure to warming or increases in thermal variability (e.g. De Schrijver and Vermeulen 1991) would determine whether modifications to the membrane environment were a potential mechanism underlying the observed changes in the metabolic rate - temperature relationship in these species. These

studies would ideally be performed in both whole-animal homogenate and isolated mitochondria, in combination with direct measurements of membrane fluidity (e.g. Najjar et al. 2007). I am using an oligonucleotide microarray (O'Neil et al. 2010) to identify differential gene expression in *E. propertius* expressing the decrease in thermal sensitivity. Examining the functional categorisation of gene families that are differentially regulated among phenotypes will illuminate pathways that may be involved in the biochemical changes (e.g. Zhang et al. 2011), which can then be further explored by metabolomic profiling and measurement of the function of key regulatory enzymes that change at the pathway level (Zera 2011). The end goal will be to elucidate the mechanisms by which individuals modify the thermal sensitivity of their metabolism, and examine inter- and intra-specific differences in these mechanisms.

6.1.2.5 Costs of biochemical adaptation to winter warming

Although decreased fitness at lower temperatures seems to imply a cost of, or a constraint to, adaptation to low temperatures over the whole life cycle (Section 6.1.1.3), the mechanistic origins of these costs are not well-defined, and in particular, few data exist on costs of facultative metabolic suppression. It is, however, possible to make some qualitative predictions on the costs of different types of thermal adaptation, based on the biochemical mechanisms involved. Energetic costs of physiological adaptation stem from demands related to synthesising new proteins and remodeling membranes; and these costs may manifest as direct energetic penalties (invoking allocation or acquisition trade-offs), or as specialist-generalist trade-offs, where function in one thermal environment is improved at the cost of function in other thermal environments (Angilletta 2009). If quantitative adjustments to enzymes are involved in metabolic compensation, the costs will relate to the relative amounts of protein that must be synthesised by each phenotype; thus if lowered metabolic rate results from less protein then energetic costs of protein synthesis will be lower for phenotypes expressing metabolic suppression (Hochachka and Somero 2002). In this case, the cost may arise from decreased rates of growth and development in the autumn, or latency to develop in the spring. If expression of a different isozyme is required, costs of protein synthesis may accompany a decrease in thermal sensitivity (e.g. facultative metabolic suppression in *E. propertius*, Chapter 5).

Turnover rates may differ among protein variants, in ways that are influenced by temperature, molecular stability, and physiological function, and are thus impossible to predict from first principles (Clarke 2003). These costs will influence the development of thermal adaptation strategies, and may induce life-history trade-offs that can change the fitness of individuals as climate changes. Before the costs of metabolic compensation can be addressed, the mechanisms must be elucidated. Then further steps (e.g. comparison of enzyme protein levels between phenotypes, time courses of flux through metabolic pathways; Zera 2011) can be taken to quantify the costs of compensation.

6.1.3 **Other consequences of winter warming for insect physiology**

6.1.3.1 **Increased water loss rates in warmer winters**

I found some evidence for increased water loss in some species overwintering at warm compared to cool winters. *Papilio troilus*, *P. glaucus*, and *E. propertius* had increased mass loss in warm winters (Chapter 3), and mean water content but not mean energy content of both species declines over time in the two species for which I collected both beginning and end of winter body composition measurements (*P. troilus* and *E. propertius*). This suggests that mass loss is predominantly due to water loss. Lower sensitivity of comparisons between groups (necessitated by destructive sampling techniques) rather than within individuals over time likely resulted in decreased power to detect treatment effects, as there were no differences in final mass or water contents between warm and cool winter treatments for *P. troilus*, *P. glaucus*, and *E. propertius*. This raises the possibility that the lack of treatment effects in energy reserves may also be obscured by intra-specific variability in initial body composition, but there were no clear trends in energy content between warm- and cool-overwintered insects, and if any effects existed the magnitude would be very small. Increased rates of water loss were not associated with overwintering temperature in *H. cunea*, but were instead related to source population, with the southern ecotype showing increased rates of overwinter water loss, likely related to the longer winters experienced by that ecotype due to early entry into diapause (Chapter 4). Increased rates of water loss were not accompanied by increased mortality in species for which I was able to measure overwinter mortality (*P. troilus*,

Chapter 3; *H. cunea*, Chapter 4), although inter-specific variability in desiccation tolerance means that increased desiccation-related mortality is a possible consequence of winter warming for desiccation-sensitive species (Chown and Nicolson 2004).

6.1.3.2 **Decreased development time following warm winters**

Decreased development time after overwintering at warmer temperatures, despite identical emergence conditions, was found in both species measured (*P. troilus*, Chapter 3; *H. cunea*, Chapter 4). A similar effect was seen in *Osmia cornuta* (Hymenoptera: Megachilidae), wherein higher overwintering temperatures also resulted in faster development (Bosch and Kemp 2004). This is contrary to the model of Gray et al. (2001), wherein colder winter temperatures result in increased rate of degradation of a development inhibitor and thus faster progression of diapause, leaving insects primed to develop quickly in the spring. There are two possible (non-mutually exclusive) proximate explanations for the observed decreases in development time after warmer winters, with different implications for predictions of vulnerability to warming.

The first possible explanation is that increased development rates, as seen in *H. cunea* and *P. troilus*, may result from differences in thermal sensitivity in the spring, with higher thermal sensitivity promoting faster development. This is supported with regards to *H. cunea* from the northern range edge, which showed increased thermal sensitivity and also faster development time (Chapter 4). The ultimate origin of the change in thermal sensitivity is unknown, although it may relate to sensing of physiological state, whereby decreased physiological condition results in changes in physiology that induce early exit from dormancy (Hahn and Denlinger 2011), or, alternatively, it may be an adaptation to promote early spring emergence (Section 6.1.1.2; Chapter 4). I do not have data on the thermal sensitivity of *P. troilus*, so I am not able to comment on the likely generality of this mechanism. However, an increase in thermal sensitivity of metabolic rate cannot explain the shorter development times of *H. cunea* after overwintering in warm conditions.

An alternative explanation invokes physiological development during winter in warmer conditions. Even under ecologically relevant levels of warming, chilling requirements of *P. troilus* and *H. cunea* were apparently still met, allowing diapause development to proceed as normal and not delaying emergence as predicted by Gray et al. (2001). Warmer temperatures promote physiological development after diapause is terminated and insects are in post-diapause quiescence (Koštál 2006), thus some development may have occurred during the winter, leaving less development to be completed upon the resumption of favourable conditions. Examining the stage of development of individuals upon transfer to emergence conditions would allow determination of what degree of development had been completed during the winter, allowing for control of the effects of physiological development during winter. I would expect a later developmental stage to have been attained following warm winters if the rapid emergence is due to development occurring during winter. An examination of thermal sensitivity of metabolism (e.g. Chapters 4 and 5) can also distinguish between the roles of overwinter development and changes in thermal sensitivity in facilitating faster development following warm winters.

Decreases in development time after warm winters will counter energetic drain by shortening periods of dormancy and thus reducing the impact of winter warming (Chapter 4), in species for which chilling requirements are still met during warmer winters. This introduces a mechanism by which winter warming may differentially affect cold-adapted compared to warm-adapted species. Chilling requirements are species-specific and correlate negatively with habitat temperatures (e.g. Neven et al. 2000, Irwin et al. 2001, Forrest and Thomson 2011, Papanastasiou et al. 2011); thus for species with high chilling requirements (i.e. cold-adapted species), increases in winter temperature may increase both overwinter energy use and development time. This means that a positive feedback loop could amplify the negative effects of warming on cold-adapted species, while a negative feedback loop could mitigate the effects of winter warming on warm-adapted species. This hypothesis could be addressed by a comparative approach, comparing the effects of winter warming on the post-winter development of warm-adapted compared to cold-adapted species.

6.2 Importance of phenology in mediating overwintering energetics

Autumn is the time of greatest energy drain for overwintering organisms, as temperatures are still warm and variable (e.g. Chapters 4 and 5), and do not contribute to development but only waste energy (Gray et al. 2001). Energy use in the pre-wintering period can be far greater than energy use over the rest of the winter combined (Chapter 5). Conversely, changes in spring phenology have little impact on overwintering energetics, likely because temperatures in spring are cooler and less variable than temperatures in the autumn (Chapter 5). I found inter- and intra-specific differences in the relationship between timing of entry into diapause and lipid stores, which are mediated by thermal conditions. In general, when entry into dormancy occurs during energetically demanding thermal conditions (warm, variable) there is a positive relationship between lipid stores and date of entry into dormancy. *Hyphantria cunea* from the centre of their geographic range, that enter diapause relatively early, have greater lipid reserves if they enter diapause later when overwintered under warm conditions; as does *E. propertius* from a variable, energetically demanding environment. Energy drain as a consequence of early entry into dormancy has been documented in several insects (Bosch et al. 2010, Musolin and Numata 2003, Han and Bauce 1998, Gomi 2000), but my model in Chapter 5 is the first prediction of the magnitude of the energetic drain, and description of the relationship between phenology and thermal variability.

However, later autumn phenology does not always decrease energy depletion. *Papilio troilus*, *P. glaucus* and *E. propertius* do not show any relationship between date of entry into dormancy and lipid content when kept under relatively constant and benign pre-wintering conditions (Chapter 3). Conversely, I found a negative relationship between date of diapause and lipid reserves in late-diapausing *H. cunea* from the northern edge of their geographic range, which I hypothesise to be mediated by declining host plant quality. Thus, stabilising selection on phenology in insects is likely to be strong for energetic reasons in addition to the requirements for synchrony with host plants (van Asch et al. 2007). Warmer temperatures in autumn, as are predicted in future climates (IPCC 2007), will make energetic penalties of early dormancy more pronounced, and

extensions of the vegetative growing season (Jeong et al. 2010) will lessen the penalties of decline of host plant quality in late autumn. This will select for increasing voltinism in species that do not have an obligate diapause (Altermatt 2010).

6.3 A framework for assessing vulnerability of insects to winter warming

There is a pressing need to identify species at high risk of negative effects of climate change (IPCC 2007). Winter warming is predicted to be particularly pronounced, and insects are already showing vulnerability to negative effects of winter warming (e.g. Irwin and Lee 2000,Williams et al. 2003, Bosch et al. 2010, Musolin and Numata 2003, Koštál et al. 2011). It is impossible to gather empirical evidence on responses of all species, but it may be possible to generalise vulnerability based on the life-history and demographic traits of species. An initial framework to predict vulnerability of temperate insects to winter warming must contain a classification of levels of vulnerability and how these will manifest experimentally, and some *a priori* predictions regarding traits with potential predictive value, to guide empiricists in their choice of model organisms. A species with vulnerability to winter warming must by definition experience a winter, thus I am excluding insects that are restricted to tropical climates from this framework. Although such insects may experience aestival diapause, during which increased temperatures may leave them vulnerable to energy drain, a different framework is required to assess vulnerability in these species. I will now outline the main components of a framework to assess vulnerability to winter warming in insects here, and provide an example of how this approach will be utilised, using currently available data.

6.3.1 Definition of vulnerability

My functional definitions of vulnerability to winter warming are as follows: 1) low vulnerability indicates a species that shows no lethal or sub-lethal effects of warming; 2) moderate vulnerability indicates a species that experiences sub-lethal effects due to energy drain, which may manifest as a reduction of energy reserves, smaller adult size, and ultimately decreased fecundity, but no evidence for an increase in overwinter mortality; and 3) high vulnerability refers to species that show both sub-lethal and lethal

effects (increased overwinter mortality in addition to previously mentioned sub-lethal effects). Thus, species with low vulnerability likely exhibit mechanisms for compensating for winter warming, e.g. metabolic compensation, and/or changes in phenology or life-history traits (Chapters 4-5).

6.3.2 **Species traits with predictive value**

Species traits must be assessed in light of where they will place the focal species on the continuum from complete compensation to increases in energetic demand over winter, either metabolically or through alterations to life-history traits such as energy storage or phenology; or death due to energetic depletion. Traits that may be useful for predicting the vulnerability of an insect species to warming include climatic diversity of current geographic range, extent of cold adaptation, degree of exposure to the environment of microhabitat, timing of resource acquisition, amount of post-winter development, and diet breadth. The system for classification of species traits I will suggest here is an example of the kinds of traits that may be useful and how they may be classified, and will no doubt be refined as more data are collected. I have suggested numerical scoring system for each trait on a scale from 1-3 (which can be applied using relatively readily-available information), and have arranged the scoring so that trait scores are positively correlated with (predicted) vulnerability to climate warming (Table 6.1). There will likely be a hierarchy among these traits, but here they are in no particular order, for example exposure of overwintering microhabitat may be nested within climatic variability of geographic range, but the relative importance of the traits will be determined as more data are amassed.

A geographic range that extends over a wide diversity of thermal environments indicates a high ability to adapt to diverse thermal conditions. The commonly-used Köppen climate classification scheme divides the world's climates into five classes: tropical; arid; temperate; cold (continental); and polar (Peel et al. 2007). For coherence with the cold-adaptation category (below), I will pool cold and polar climatic regions in this category. One of the criticisms of the Köppen classification is that the temperate category is too broad (Stern et al. 2000), containing, for example, both subtropical southern Florida and

highly seasonal Oregon. I will therefore add a sub-tropical region, as recommended by several authors (e.g. Stern et al. 2000), which will be superimposed on the existing classifications and will span from 23.5 - 40° latitude in each hemisphere. Thus, the five climatic regions will be: cold-polar, arid, temperate, subtropical, and tropical. I predict that the thermal diversity of a species' range, or number of these climatic regions occupied, will be negatively correlated with vulnerability (Table 6.1).

Table 6.1 - Species traits that are hypothesised to be correlated with vulnerability of insects to increased energetic demand over winter, and a suggested system for scoring these traits. The trait classifications are arranged so that the score correlates positively with hypothesised vulnerability. See text for further details of each trait category.

	Score and hypothesised vulnerability		
	1	2	3
Trait	Low	Moderate	High
Climatic diversity of species range	Range encompasses four or five climatic regions	Range encompasses two or three climatic regions	Range is confined to one climatic region
Extent of cold-adaptation	No cold-adaptation: distribution does not include cold/polar regions	Some cold-adaptation: distribution includes but is not restricted to cold/polar regions	Complete cold-adaptation: distribution is confined to cold/polar regions
Degree of exposure to environment of overwintering microhabitat	Buffered microhabitat (e.g. soil, persistent snow cover at high latitudes or altitudes)	Partially buffered microclimate (e.g. leaf litter, cambium)	Exposed microclimate (e.g. outer tree bark, exposed gall)
Origin of reserves that fuel reproduction	Adult diet is complete (adult income may fully determine reproductive output)	Adult diet deficient in essential nutrients (both juvenile capital and adult income fuel reproduction)	Non-feeding adults (complete reliance on capital (juvenile-derived) resources
Amount of (pre-feeding) development remaining post-winter	Overwintering stage is competent to feed	Some post-winter development is required before feeding may commence	Significant development (e.g. metamorphosis) required before feeding resumes
Diet breadth	Polyphagous	Oligophagous	Monophagous

Cold adaptation refers to the suite of physiological changes that enable species to inhabit cold and polar environments (Clarke 1991). As species that are adapted to cold temperatures are likely to be less suited to warm temperatures (Clarke 2003), it follows that species whose ranges are restricted to cold regions are likely to be less able to compensate metabolically for, and thus more likely to suffer from, winter warming. In addition, insects in cold regions are also more likely to be energy-limited, due to low energy availability resulting from short cool growing seasons and long winters (Chown and Gaston 1999, Clarke and Gaston 2006), exacerbating sensitivity to energetic drain. I suggest that cold-adapted species should thus be considered as species that occur in cold or polar regions (from the Köppen climate classification scheme; Peel et al. 2007). I predict cold-adaptation to be positively correlated with vulnerability (Williams et al. 2003, Mercader and Scriber 2008; Table 6.1). This is counter to predictions of increased sensitivity of tropical organisms to warming based on the likelihood of temperatures approaching upper critical limits (Deutsch et al. 2008), and increased metabolic expenditure (Dillon et al. 2010).

The degree of exposure to the environment of the overwintering microhabitat is another likely determinant of vulnerability to winter warming (Irwin and Lee 2003). Insects that burrow into the soil experience strong buffering of thermal fluctuations, and temperatures that rarely drop below freezing (Hoshikawa et al. 1988, Carcamo et al. 2009). Snow cover strongly insulates insects overwintering in the leaf litter or on the ground, beneath which temperatures remain constant at 0 °C (Zhang 2005; Chapter 4). However, snow cover is ephemeral and does not insulate insects during the autumn; the time of greatest energy demand for some species (e.g. Chapter 5). The remaining litter layer, which is a common overwintering location for insects (Leather et al. 1995), buffers thermal variability to some extent, dampening thermal fluctuations and raising minima (Carcamo et al. 2009, Parajulee et al. 1997). Thermal conditions beneath the bark of trees, in the cambium, may also be buffered from extremes of ambient temperature (Coeln et al. 1996), although the difference from ambient temperature may be slight (Crosthwaite et al. 2011). As insects that overwinter in protected microhabitats typically experience warmer conditions than do

insects overwintering in exposed microhabitats, and are thus likely to be adapted to warmer overwintering conditions, I predict that insects from more exposed microhabitats will be more vulnerable to warming (Table 6.1).

The origin of reserves that fuel reproduction is also likely to be an important factor. All insect species fall somewhere along a continuum from complete reliance on juvenile-derived reserves for reproduction and somatic maintenance as adults (capital breeders), to drawing considerably on adult-derived nutrients for reproduction (income breeders; Boggs 2009). All else being equal, capital breeders that accumulate their reserves prior to winter are likely to be more vulnerable to winter warming than are income breeders who can readily replenish depleted resources. The scoring system suggested in Table 6.1 is an oversimplification of the complex interactions between juvenile and adult nutrition and how they interact to determine fecundity, but in light of the complex range of resource allocation strategies present even within the Lepidoptera (Boggs 2009), and the absence of studies on the nutritional ecology of the majority of insect species, this a tractable compromise that I believe can still yield useful predictions.

Another potentially important species trait to predict vulnerability to warming is the amount of (pre-feeding) development to be completed after winter. Some insects overwinter in stages that have significant amounts of development to complete before the resumption of feeding, for example late-instar larvae, pre-pupae, or pupae (in species where most development occurs post-winter) for holometabolous insects. Conversely, other insects overwinter in stages that can begin feeding immediately upon the return of favourable temperatures (should suitable host plant be available), for example early-instar larvae or nymphs, pharate adults, or adults. I predict that the amount of post-winter, pre-feeding development required will correlate positively with vulnerability to warming (Table 6.1).

Increased diet breadth will provide species with more flexibility to match their phenology with changing climatic conditions in both the autumn and the spring, without being constrained by host plant phenology (Diamond et al. 2011). Adaptive changes in phenology have considerable power to increase energy storage and buffer insects from the

negative effects of winter warming (Section 6.2, Chapter 5). Diet breadth can be estimated by the number of host plants potentially used by immature stages (Diamond et al. 2011); or in this case simplified to mono- (one primary host plant at any given point in the range), oligo- (several possible host plants at any point in the range), or poly-phagous (more than three possible host plants at any given point in the range). I predict a negative correlation between diet breadth and vulnerability to warming (Table 6.1).

6.3.3 **The utility of the proposed framework**

Insect species for which data on vulnerability to winter warming exist are compiled in Table 6.2, with the aim of providing an example of how species can be classified under the proposed framework. For a study to be included in this initial survey, I required that the levels of warming be ecologically relevant. Most climate change scenarios predict increases in global mean temperature of 4-7 °C by 2100 (IPCC 2007), and winter temperatures will warm faster than global averages. I thus included laboratory studies that applied warming treatments of up to 12 °C, and that included a control group (against which to assess fitness correlates) that was kept at an above-zero temperature that did not induce mortality or energetic drain due to energetic costs of cold resistance. Where a species could be assigned to more than one level of a category, where possible I took the modal level (e.g. *Ips typographus* may overwinter in the larval, pupal, or adult stage, but most individuals overwinter as adults), so as to best represent population-level responses. If that was not possible (e.g. for extent of climatic range, described below), I took a conservative approach and assigned the level of highest vulnerability for a variable where there were two possible levels that could be assigned.

There are too few data to make phylogenetically-informed conclusions as to the predictive value of the traits. No one trait predicted vulnerability perfectly, but for the two genera for which there are species with a range of vulnerability, the traits outlined here would predict the difference in vulnerability in the experimentally determined direction (Table 6.2). *Papiliio troilus* is predicted to be more vulnerable than *P. glaucus* by the proposed scoring system (Table 6.2), while I did not find experimental evidence of increased vulnerability in *P. troilus* compared to *P. glaucus* (Chapter 3). *Hyphantria*

cunea was predicted to have low or moderate vulnerablility in most (4/6) traits, in concordance with my findings of insensitivity to winter warming (Chapter 4). *Erynnis propertius* was predicted to have moderate - high vulnerability in 5/6 traits, also in concordance with laboratory estimates of sensitivity to increased energetic demand over winter (Chapters 3 and 5). However, this is a somewhat circular test of the criteria, as the studies used to test the criteria in some cases contributed to the formation of the hypotheses. Data that were not used to formulate the hypotheses are required to test them, and representatives from many more taxonomic levels, with a range of species traits, need to be empirically examined for susceptibility to winter warming, before any conclusions on traits that have predictive values can be reached. I hope this will prove a useful starting point to guide the methodology and choice of study species for researchers examining overwintering energy use in insects.

A similar broad-scale approach has been used to identify species traits that predispose Lepidoptera to phenological shifts (Diamond et al. 2011). Species with narrower larval diet breadths, more advanced overwintering stages, and smaller range sizes have experienced greater shifts in phenology during recent climate change (Diamond et al. 2011). Thackeray et al. (2010) found that lower trophic levels exhibit the greatest phenological shifts across taxa in the U.K.. These studies show that species traits can be useful predictors of responses to climate change, although large data sets are required to do so. I hope that the existence of this framework will prompt increased investigations into the effects of winter warming on the energetics of overwintering insects, and other ectotherms. As energy limitations can set range limits; increased overwintering energetic demands could have important effects on insect populations. The evidence to date indicates that species differ considerably in their vulnerability to energetic drain resulting from winter warming, thus a framework to predict the patterns of vulnerability to warming will be an asset in attempts to forecast changes in species distributions and population dynamics.

Table 6.2 - An example of how the proposed framework may be used to assess the vulnerability of insects to winter warming based on species traits. A high score in any category is associated with hypothesised increased risk of vulnerability. Range: climatic variability of geographic range; Cold: degree of cold-adaptation; Exposure: degree of exposure of overwintering microhabitat to the environment; Resource: timing of resource acquisition; Development: amount of post-winter, pre-feeding development required; Diet: diet breadth (mono: monophagous; poly: polyphagous). See Table 6.1 for a description of scoring and categories.

	Vulnerability	**Range**	**Cold**	**Exposure**	**Resource**	**Development**	**Diet**	
Scale (1-3)	Low - high	diverse - restricted	none - complete	none - complete	income - capital	none - considerable	poly-mono	**Source**
Papilio troilus	1	2	2	3	2	3	2	1
P. glaucus	1	1	2	3	2	3	1	1, 2
P. canadensis	3	3	3	3	2	3	2	2
Hyphantria cunea	1	1	2	2	3	3	1	3
Erynnis propertius	3	3	1	2	2	3	3	1,4
Diplolepis variabilis	1	3	3	3	3	3	2	5
D. spinosa	3	3	3	3	3	3	3	5
Formica aquilonia	3	2	2	1	1	1	3	6
Ips typographus	3	3	3	1	1	1	2	7
Eurosta solidaginus	3	1	2	3	3	3	3	8

Sources: 1) Chapter 3; 2) Mercader and Scriber 2008; 3) Chapter 4; 4) Chapter 5; 5) Williams et al. 2003, 6) Sorvari et al. 2011; 7) Koštál et al. 2011; 8) Irwin and Lee 2000.

6.3.4 **Limitations and future directions**

In some cases, it was difficult to conclusively assign a score to some species, due to either a lack of information on the trait in question, or the presence of multiple trait values within a species. For example, in the absence of detailed information on species distributions, it was difficult in some cases to assess the climatic variability of a species' range. Some areas have multiple climate types within small geographic areas (e.g. south-western British Columbia is predominantly cold but has small pockets of arid and temperate regions), and descriptions of distributions of species inhabiting such areas (e.g. *Diplolepis* species; Shorthouse 2010) frequently do not indicate whether species ranges are contiguous or interrupted. For the *Diplolepis* species, I took a conservative approach and assumed that their distribution was restricted to cold climates. Including temperate and/or arid regions would result in their scores dropping from 3 (most vulnerable) to 2 in that category. Arbitrary decisions on whether a species range is contiguous over such an area, or whether they are confined to their dominant climate, can thus substantially influence estimates of climatic diversity. Incorrect estimates of this parameter may introduce noise into future models and reduce predictive power, but will not invalidate conclusions. For focal species whose vulnerability to warming is to be predicted based on such models, detailed distribution information is likely to be available. The degree to which a microhabitat was exposed to the elements was also sometimes ambiguous. Some species have intra-specific variability in microclimate choice (e.g. *Papilio* species may pupate above or below the snow layer), and intra-specific variability in microclimate choice can strongly influence energy use (and consequently fitness; Irwin and Lee 2003). I used a conservative approach, assuming the highest degree of vulnerability.

The predictions of vulnerability did not distinguish well between *D. variabilis* and *D. spinosa*, despite experimental evidence that *D. spinosa* is more vulnerable to energy drain than *D. variabilis* (Williams et al. 2003). The differential sensitivity to warming between these species was hypothesised to result from differences in cold adaptation (Williams et al. 2003). As the more sensitive species, *D. spinosa*, co-occurs with *D. variabilis* (Shorthouse 2010), no species-wide classification of cold tolerance will differentiate

between these species. The study specimens were however collected from areas with different climates. *D. spinosa* was collected from Sudbury, ON, which lies above the -11 °C isotherm for mean temperatures in January, February and March, while *D. variabilis* was collected from the Okanagan Valley, BC, which lies above the -1 °C isotherm. It is possible that local adaptation in *D. spinosa* populations resulted in the Sudbury population being more cold-adapted than the Okanagan Valley population would be, or alternatively that cold-adaptation does not account for the increased susceptibility to warming between the species. Local adaptation of populations in traits that influence susceptibility to warming will decrease the predictive power of models, but the magnitude of problem presented by intra-specific variation in species traits will not be known until more data are amassed. In this regard, more studies such as Chapter 4 and 5 are required to investigate intra-specific variability in overwintering energetics.

There are a number of additional variables that will need to be accounted for in future models utilising this approach. It will be desirable to calculate the temperature differential between warming treatment and control group in laboratory studies (mean warming treatment temperature - mean control group temperature), and include this as a variable in predictive models. This will allow for the control of effects stemming from differences in the degree of warming applied to each species. A variable indicating whether the experiments were conducted at stable or fluctuating temperatures, for example standard deviation of the temperature regimes used, will allow for the investigation of effects related to thermal variability. Another important variable to include as a covariate in analyses will be mass, as increased body size can be an indicator of improved starvation resistance (Stockhoff 1991, Chippendale et al. 1996), thus larger species may be less vulnerable to warming. This has been suggested as a possible factor underlying the decreased susceptibility of *P. glaucus* to winter warming, compared to *P. canadensis*, and the inclusion of a body mass covariate may better capture the difference in vulnerability between the two species. The effect of taxonomic grouping should also be investigated for their value as predictors of vulnerability, although the few data available to date suggest that even at the level of genera, there are considerable inter-specific differences in vulnerability to winter warming. In addition, habitat type can strongly influence

microclimate conditions, and may be an important variable to include in future models. Woodland habitats have reduced daily thermal range over winter compared to more open (heathland or grassland) habitats, due to reductions in radiative cooling from woodlands and an associated increase in daily thermal minima (Suggitt et al. 2011). I did not incorporate habitat type at this stage as other variables (e.g. exposure of overwintering site) may be sufficient to incorporate this potential source of variation in vulnerability to winter warming, and for eventual models to have utility the required number of parameters should be kept as small as possible. Once more empirical data are available, it will be possible to test the effect of habitat type, to refine the list of informative variables.

6.4 Summary and concluding remarks

The majority of species that I studied (*P. troilus*, *P. glaucus*, and *H. cunea*) did not show negative effects of moderate winter warming. In *H. cunea*, this insensitivity to winter warming was mediated by a suppression of metabolism in warm temperatures, and a plastic decrease in development time which shortened the period of dormancy and reduced energy demand. Northern populations of *H. cunea* also showed an increase in thermal sensitivity of metabolism, as predicted by metabolic cold adaptation theory. Even *E. propertius*, which did show energetic drain in response to increases in both mean and variance of temperature over winter, had physiological mechanisms to mitigate some of the negative consequences of increased energetic demand over winter. Rather than a general down-regulation of metabolic rate, *E. propertius* showed a decrease in thermal sensitivity of metabolism, which partially compensated for the increased energetic demands of a variable environment. The metabolic suppression exhibited by *H. cunea* and *E. propertius* is the first study of metabolic temperature compensation in an overwintering insect outside of the general phenomenon of diapause, but as a modulation of metabolic-rate temperature relationships was seen both facultatively and obligately in both of the species that I investigated, it seems likely that many overwintering insects have the capacity to compensate the metabolism. The range of metabolic compensation responses, and their underlying mechanisms, is a promising field for future study. The framework that I have outlined can be used to guide future research into the effects of

changes in overwintering temperatures on insects, with the end goal of identifying species traits that predict vulnerability to the negative effects of winter warming. Winter warming and changes in thermal variability are likely to reduce the fitness of some overwintering insects, but diverse physiological mechanisms exist to mitigate increased energetic demands.

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Appendix 1: Reprint permission from Journal of Insect Physiology (Chapter 2)

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J. Insect Physiology

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Regards,
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