Phenotypic variation in cold tolerance of an invasive insect (*Epiphyas postvittana* Walker): Implications for forecasting risk

A Dissertation

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Dedication

This dissertation is dedicated to *Epiphyas postvittana* (Walker); without you, this work would not exist.

Abstract

Predicting the spread and potential impacts of biological invasions is vital to management and policy decision-making. For much of North America, cold temperatures dictate portions of the geographic boundaries of ectotherm survival and are often included as a key component of risk models and maps for invasive insects. However, current risk assessments typically assume an invading species' response to cold and other environmental stressors is immutable over short to medium time horizons, in part due to a paucity of relevant biological data. Such assumptions may lead to misestimated probabilities of establishment and spread of invasive insects, such as a recent invader to the U.S.A, the light brown apple moth (*Epiphyas postvittana* Walker).

My research examined multiple sources of potential variation in cold tolerance of *E. postvittana*, a species that is predominantly freeze avoidant but also shows some amount of partial freeze tolerance in its overwintering stage. First, I evaluated the effects of larval host plant on direct tolerance to acute partial-freezing in late instars. There was a nearly ten-fold change in survival after partial freezing based on larval host, as well as changes in mean supercooling points. Importantly, the degree of cold tolerance could be predicted by the developmental suitability of a given host.

Second, I compared the fitness tradeoffs following exposure to two subzero temperatures (-10°C or the supercooling point) in late instars and pupae. Both lifestages could survive brief exposure to -10°C without significant fitness cost, and displayed small proportions of partial freeze tolerance. However, individuals that survived partial-freezing did so with a reproductive fitness cost.

Lastly, I conducted a long-term laboratory selection experiment to evaluate the potential for adaptation to partial freezing in late instars. While there did not appear to be sufficient additive genetic variance to see a consistent response to selection, there was considerable variability through time in survival after partial-freezing. Importantly, this variability cycled in a regular pattern (~six generations), driven by a yet unknown mechanism. The variation in *E. postvittana* cold response seen in this study resulted in different forecasts of geographic distribution, which can have important management and regulatory implications.

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CHAPTER 1:
Introduction of Relevant Concepts

Exotic, invasive species can cause significant economic and environmental harm. Predicting the spread and potential impacts of invasions is vital to management and policy decision-making. Ecological risk assessment is one way to systematically evaluate the likelihood and magnitude of impacts an invasive species may have on an area of concern.

For much of North America, cold temperatures dictate portions of the geographic boundaries of ectotherm survival and are often included as a key component of risk models for invasive insects. However, current assessments of risk typically assume an invading species' response to cold and other environmental stressors is immutable over short to medium time horizons, in part due to a paucity of relevant biological data. Such assumptions may lead to misestimated probabilities of establishment and spread of invasive insects, such as a recent invader to the U.S.A, the light brown apple moth (*Epiphyas postvittana* Walker). Empirical work that could aid in mitigating these uncertainties should characterize the variable nature of cold tolerance phenotypes for a given species, investigating multiple sources of variation through space and time.

This chapter will provide an overview of the main concepts that have framed my research on cold tolerance variation in the light brown apple moth. It will begin with basic information on the species, followed by relevant concepts in insect cold tolerance, invasion biology and ecological risk assessment, and sources of phenotypic variation. It will end with a summary of the aims and scope of my research.

1.1 Study Species: the Light Brown Apple Moth, *Epiphyas postvittana*

1.1.1 Nomenclature and Taxonomy

Epiphyas postvittana (Walker, 1863) is a member of the order Lepidoptera, family Tortricidae, tribe Archipini. It was originally described as *Teras postvittana*, with later authors placing it in the genera *Coecoecia*, *Tortrix,* and *Austrotortrix* (Brown et al.

2010). Common (1961) synonymized *Austrotortrix* with *Epiphyas* Turner, 1927; *Epiphyas* currently contains 40 species (including *postvittana*), all native to Australia (Brown et al. 2010). The common name "light brown apple moth" has been applied to any species of *Epiphyas*, and has also been used to refer to any leafroller attacking Australian orchard and horticultural crops (Geier and Springett 1976). For a list of species-level synonyms, see summary in Venette et al. (2003).

For a review of taxonomically-relevant morphology, see Brown et al. (2010). While *E. postvittana* is the only species of its genus known to occur in the United States, many other Archipini may co-occur with, and resemble, *E. postvittana* in the region. An online morphological and molecular identification tool has been developed to aid in distinguishing *E. postvittana* when examining specimens collected from the United States, particularly within California (Gilligan and Epstein 2009).

1.1.2 Geographic Distribution and Invasion History

Epiphyas postvittana is native to southeastern Australia (Danthanarayana 1975a). Export of many of its host plants from Australia has facilitated its spread into other regions and continents (Suckling and Brockerhoff 2010). Review of the updated invasion history of *E. postvittana* has been detailed in both Suckling and Brockerhoff (2010) and Brown et al. (2010). A summary is as follows: The species was first reported in New Zealand in 1891 (Varela et al. 2010), likely also occurring in Tasmania by that time, and was widespread across the South and North islands of New Zealand by the 1890s. It was collected in the Hawaiian Islands in 1896 and appeared throughout the archipelago by the 1920s. It was considered established in Cornwall, England in 1936 and has spread through much of the urban areas in England and Wales over the past few decades, arriving in southeast Ireland in the late 1990s. Spread into Western Australia was discovered in 1968.

In 2006, moths were caught in northern California (Alemeda Co, Berkeley) and in 2007, following additional surveys, *E. postvittana* was confirmed to be established in the contiguous United States (Marshall et al. 2007, Brown et al. 2010). Interceptions have also been reported from Sweden and the Netherlands, as well as New Caledonia and

Japan, but evidence of established populations in these areas remains to be thoroughly verified (Brown et al. 2010, Suckling and Brockerhoff 2010, He et al. 2012).

A study was recently undertaken to track the relationships between geographic populations of *E. postvittana* from Australia, New Zealand, Hawaii, and California using mitochondrial DNA haplotypes (Rubinoff et al. 2011). The authors concluded that Hawaii was likely invaded by Australia or New Zealand founders approximately 100 years ago, and that a recent introduction (or series of small introductions) from New Zealand or Australia constituted the California invasion, with no contribution from Hawaii.

1.1.3 Biology

Additional components of *E. postvittana* life history/biology are summarized in others sections, including its cold tolerance (*1.3.3*), pest risk assessments (*1.2.4*), and phenotypic variation (*1.4.3*).

Life cycle. Female moths lay eggs in tectiform (or "roof-like) masses on smooth surfaces of their host plants, typically along the midrib of the upper surface of leaves (Geier and Briese 1980a, Powell and Common 1985). Temperature and larval host plant can significantly affect the fecundity of females (Danthanarayana 1975b, 1983), but female moths have an average lifetime fecundity of 115-460 eggs, typically laying 20-50 eggs per mass (reviewed in Venette et al. 2003).

Neonates disperse actively by crawling, or passively through ballooning (Geier and Briese 1980a). *Epiphyas postvittana* is one of several thousand species of moths belonging to the Tortricidae family, named so for their propensity to roll or "twist" (Latin *tortus*, *tortrix*) leaf tissue into a shelter with silk as larvae. Larvae feed within and around this shelter, often leaving to form new chambers on new feeding surfaces as they grow (Geier and Briese 1980a). Larvae that are feeding on deciduous plants will drop to the ground as plants senescence and begin to feed on leaf litter; late instars can also survive without feeding for up to two months (Geier and Briese 1980a, reviewed in Venette et al. 2003). No life stages diapause in this species, but development is slowed during cold temperatures (Chapman 1973, Geier and Briese 1980a). Larvae are generally considered to be the predominant overwintering stage (Danthanarayana 1975a, Geier and Briese

1980a). Initial reports from a two-year survey at multiple California locations suggested that 4th, 5th, and 6th instars were the only lifestage found during winter (Bürgi and Mills 2010). However, further study revealed all developmental stages were present in varying proportions throughout the year, including adults (Buergi et al. 2011)

Instar number is variable in this species, with five or six being typical, but four to seven also observed (Dumbleton 1932, Danthanarayana 1975a). Pupation occurs within the larval chamber, with pupae appearing green initially and then hardening to a reddish-brown. Female pupae weigh more than males (Danthanarayana 1975a).

Moth forewing color and patterns are sexually dimorphic and can vary within sex, especially among males (Brown et al. 2010). Female moths tend to be larger than males and have a pre-oviposition period of 2-7 d (Danthanarayana 1975a). Females oviposit at dusk and lay most of their eggs within 10-14 d of emerging (Danthanarayana 1975a, reviewed in Venette et al. 2003).

Development. Populations of *E. postvittana* perform best in areas with mild summers (mean annual temperature of 13.5°C) and high relative humidity (~70%) and rainfall (Danthanarayana et al. 1995, Wang et al. 2012). Multiple developmental parameters for *E. postvittana* have been calculated, predominantly for insects from Victoria and New South Wales, Australia (Danthanarayana 1975a, Geier and Briese 1981). Venette et al. (2003) provides a summary table of developmental thresholds and degree-day (DD) estimates published before 2000. Gutierrez et al. (2010) reanalyzed data from Danthanarayana (1975a), refining the estimates slightly: lower and upper developmental thresholds of 6.8 and 31.3°C for eggs and 6.8 and 31.5°C for larvae and pupae. Using a 6.8°C lower threshold, the physiological time required for egg development is 120.8 DD, and 335 DD and142 DD for larval and pupal development, respectively. From egg to egg, 594DD are needed, and from egg to 50% oviposition requires 646 DD (Gutierrez et al. 2010). The modelled estimate of 646 DD matches the phenology of coastal California populations well, except for the overwintering generations which require roughly 100-300 additional degree-days (Buergi et al. 2011). The extended development time is potentially due to a facultative response to a reduction in photoperiod, as has also been recorded from Australian populations (Buergi et al. 2011). Extended development may also result from sub-optimal high temperatures

(Danthanarayana 1975a). Larval development time can vary with the type and stage of the host plant, but the pupal duration (and the lower development threshold) do not appear to vary with host (Danthanarayana 1975a).

Voltinism. *Epiphyas postvittana* is multivoltine throughout its current range (see section 1.1.3 for native and invaded range), with 2-4 generations generally recorded (Brown et al. 2010). The annual number of generations varies with temperature and latitude (Buergi et al. 2011, CABI 2015). There are 3-4 generations/year in Australia, with three being most common and nearly five being suggested in some regions (Danthanarayana 1983, Mo et al. 2006, CABI 2015). New Zealand typically has 2-4 generations, with the warmer northern regions having four and southern areas limited to two (Varela et al. 2010, CABI 2015). Two are most commonly seen in Britain (Suckling and Brockerhoff 2010). In California, 2-4 generations have been reported, with 3-4 most frequent (Bürgi and Mills 2010, Buergi et al. 2011).

Due to the absence of a synchronizing event in *E. postvittana*, such as diapause (Chapman 1973, Danthanarayana 1983), there is considerable overlap between generations within a given area, making clear generational distinctions difficult (Buergi et al. 2011).

Host plants. Though its adapted affinity for cultivated apples (*Malus domestica*) gave rise to the common name of the light brown apple moth, *E. postvittana* has been associated with an extensive list of other plants, with the current count spanning >500 species in 363 genera and 121 families (Suckling and Brockerhoff 2010). Notable is the preponderance of exotic host species that the larvae will feed on, within and outside their native geographic range (Danthanarayana et al. 1995, Suckling and Brockerhoff 2010). Various authors have compiled lists of the native and exotic host species range (e.g., (Danthanarayana 1975a, Venette et al. 2003, Brown et al. 2010, CABI 2015) which include a diversity of herbaceous and woody dicotyledonous plants, monocotyledons, and conifers. Danthanarayana (1975a) reported that the dominant host families were from Rosacae (Rosales), Leguminosae (Fabales), and Compositae (Asterales), while a more recent assessment suggests Rosales, Fablaes, Vitales, and Saxifragales (Suckling and Brockerhoff 2010). The California Department of Food and Agriculture (CDFA) provides a list of notable host plants of economic importance that *E. postvittana* may feed

on in the region, including, but not limited to: apple, pear, peach citrus, persimmon, almond, oak, poplar, Monterey pine, eucalyptus, grape, berries, beans, alfalfa, jasmine, and chrysanthemum (CDFA 2008a).

The ancestral host of *E. postvittana* within Australia is generally thought to be native evergreens, such as acacia (Danthanarayana 1975a). Due to differences in developmental and reproductive suitability, Danthanarayana (1995) suggested the species actually originated on herbaceous plants and then evolved to feed on woody species.

Pest Status. *Epiphyas postvittana* feeds on numerous economically-valued trees, shrubs, and cultivated crops. In Australia and New Zealand, most economic loss has been associated with pome fruits (e.g., apples, pears), berries, grapes, citrus, and ornamentals (Danthanarayana 1975a, Varela et al. 2010). Untreated populations have caused 70%-85% losses in yield (Danthanarayana 1975a, Suckling and Brockerhoff 2010).

Larvae are predominantly foliage feeders, favoring new growth, leaves, and flowers, but will occasionally feed on fruit. Fruit damage is generally superficial but can increase through the season as fruits and leaves increase in contact (Lo et al. 2000). Most economic damage, however, comes from injury to fruit surfaces as either direct blemish to the product or as secondary fungal infection at the feeding site (reviewed in Venette et al. 2003). Additional economic loss can result from trade restrictions with other regions and countries (Varela et al. 2008, 2010).

Despite past issues with insecticide resistance (e.g., organophosphates, Suckling et al. 1990), relatively recent efforts in New Zealand to manage *E. postvittana* using integrated pest management techniques (e.g., biological control programs and threshold-based, selective insecticide use) have yielded great success (Varela et al. 2010). Additional management tools are reviewed in Suckling and Brockerhoff (2010) and Varela (2010).

Interestingly, *E. postvittana* has never been considered a major pest in Hawaii and its distribution on the islands seems to be receding; reasons for this decline remain unknown (Rubinoff et al. 2011). See section *1.1.4* for the pest status of *E. postvittana* in the continental U.S.

1.1.4 Epiphyas postvittana in the Continental US

Prior to its arrival in California, *E. postvittana* was considered a pest of concern in the United States for decades (reviewed in Brown et al. 2010). Multiple trapping surveys for *E. postvittana* were conducted before 2006, the most extensive of which began in the 1980s by the federally-funded Cooperative Agriculture Pest Survey (CAPS) and covered multiple apple-producing states and regions. The CAPS program also produced a risk assessment for *E. postvittana* in 2003 (see section 1.2.4). No surveys revealed positive moth catches (Brown et al. 2010). However, the trapping regimes used were likely inadequate to rigorously ascertain the absolute presence or absence of *E. postvittana* (Berenbaum et al. 2009).

Once officially confirmed in 2007, *E. postvittana* was classified as an "actionable quarantine-significant pest" by the United States Department of Agriculture Animal and Plant Health Inspection Service (USDA-APHIS) and as a "Class A pest" by the California Department of Food and Agriculture (CDFA). Such classification gave both agencies authority to implement quarantine restrictions and eradication efforts to limit the intra- and interstate spread of the insect (Marshall et al. 2007). Trade restrictions and quarantines were immediately put in place, with an initial Federal Order limiting the movement of potentially infested materials from eight California counties and all Hawaiian counties (the suspected source of infestation) (USDA-APHIS 2007a). Simultaneously, measures were begun to eradicate populations using aerially-applied mating disruption pheromones as the predominate tactic (TWG 2007). What followed was a public relations debacle mixed with poorly-mediated scientific disagreement, largely stemming from the use of aerial sprays to attempt eradication (Carey and Harder 2013, Lindeman 2013, Liebhold 2014).

Petitions to reclassify *E. postvittana* as a non-quarantine pest were submitted by citizen stakeholders in 2008 and 2009 (USDA-APHIS 2014). The USDA-APHIS response to the petition was reviewed by the National Research Council (Berenbaum et al. 2009) for its regulatory validity and sound evidence. While the NRC found USDA-APHIS was within its authority to classify *E. postvittana* as they had, they felt USDA-APHIS had done so with unclear and poorly communicated justification and, in some cases, unsubstantiated scientific evidence (Berenbaum et al. 2009).

Following NRC review, USDA-APHIS revised its initial response to petitioners but did not reclassify *E. postvittana* (USDA-APHIS 2010b). Quarantined areas and materials continue to be updated (with expansion and contraction in both lists) in cooperation with the CDFA (e.g., USDA-APHIS 2015a, 2015b) and surveys are ongoing. At the time of this dissertation, the most recent published stance of USDA-APHIS regarding *E. postvittana* reflects a change in focus away from eradication efforts towards suppression and management, but maintains caution in removing a tight regulatory framework for this insect (USDA-APHIS 2014, 2015c). Moths have been intercepted in southern Oregon (Polk Co.) (DeBruyckere 2011, CDFA/USDA-APHIS 2015), but surveys suggest they are not yet from resident populations in Oregon (OISC blog 2011).

Figure 1.1 shows the most recent map of quarantined areas due to the presence of *E. postvittana* for the contiguous U.S. (thus far, limited to California. Twenty-one California counties and all Hawaiian counties are currently included (USDA-APHIS 2015d) with the largest numbers of moths being found in the Central Coast and San Francisco Bay regions.

Populations in California are most abundant in urban landscapes on ornamental plants, such as manzanita (*Archtosaphylos densiflora*), the Mexican orange (*Choisya dumosa*), a Mediterranean myrtle (*Myrtus communis*), Japaneese cheesewood (*Pittosporum tobira*) and Australian tea tree (*Leptospermum laevigatum*) (Wang et al. 2012). The actual impact of *E. postvittana* since its arrival in California has yet to be fully quantified or assessed.

While direct applicability of successful IPM tactics used in other countries to California requires caveats (CDFA 2008b), the effectiveness of native generalist natural enemies (predominantly parasitoids) in achieving suppression of California populations is being acknowledged, and biological control remains an active area of research (Wang et al. 2012, Hogg et al. 2013, Bürgi and Mills 2014, CDFA/USDA-APHIS 2015)

1.2 Invasive Species Risk Assessment

1.2.1 Invasive Species

The spread of a species into an environment where it has not historically occurred (i.e., species that are "exotic", "non-indigenous", "non-native", or "alien" to a given area) can be mediated by natural processes (e.g., climate) or the intentional (e.g., domestication, classical biological control) or unintentional (e.g., trade, globalization) actions of humans. The perceived effect(s) of this movement can also vary, with assignment of benefit, harm, neutrality, or some gradient therein, often being highly value-laden and mutable (ISAC 2006, Simberloff et al. 2013). When a species is perceived to be harmful – economically, environmentally, or to human health – to a new environment into which it spreads, it is considered "invasive" (Alpert et al. 2000). Though some authors use the term 'invasive' to describe both the exotic nature of a species and its ability to spread and cause harm (e.g., ISAC 2006), others argue for more effective communication by referring to a species' location of origin (e.g., exotic) separate from its ability to spread and cause harm (i.e., invasive), given that not all exotic species are harmful and not all invasive species are exotic (Alpert et al. 2000, Lodge and Shrader-Frechette 2002). Still others call for a more extensive overhaul in language and shift away from the potentially detrimental "militarization" of the field of invasion biology, generally (Larson 2005). For my purposes, I will use conventional terminology and distinguish both the location of origin and ability to cause harm.

Similar to the terminology used to describe invasive species, the process of invasion varies in how it is conceptualized. Invasion is characterized by discrete stages, with most authors using three stages, such as in the seminal work of Williamson (1996): arrival, establishment, and spread; or four stages: arrival, establishment, integration, and spread (e.g., Venette and Carey 1998). However, some authors use as many as six stages (Henderson et al. 2006) and as few as two (Davis 2009).

Though my work is applicable to any of these models, the schemes most apropos to the questions I address are those involving an explicit "integration/adaptation/naturalization" stage (e.g., Vermeij 1996, Venette and Carey 1998, Richardson et al. 2000). This phase generally describes when a species is adapting

to the biotic and abiotic environment in parallel to when the native community is adjusting to its presence. Importantly, despite the demarcation implied by stages, the true continuous nature of the invasion process is widely acknowledged (Davis 2009).

1.2.1 Ecological Risk Assessment (ERA)

Given the vast evidence of negative effects that exotic invasive species can impart on economies, natural environments, and human health (Pimentel et al. 2000, 2005, Mooney and Cleland 2001, ISAC 2006, NISC 2008, Gallardo et al. 2015, but see also Vilà et al. 2011), having a decision-making tool to systematically evaluate the likelihood and magnitude of effects from a species in a given area is crucial to mitigate adverse effects. Such is the goal of ecological risk assessment (ERA), a component of the larger, tripartite process of risk analysis, which also includes risk management and risk communication (U.S. EPA 1998).

Discussion of the variations in both national and international protocols for assessing the risk of invasive species has been reviewed by others (e.g., see reviews in Andersen et al. 2004, USDA-APHIS-PERAL-CPHST 2012; Flint et al. 2012 for invasive genotypes; van Lenteren et al. 2006 for exotic biological control agents). For my purposes, I will use the framework detailed in the most recent guidelines by the Environmental Protection Agency (EPA) for ecological risk assessment (U.S. EPA 1998; Figure 1.2). The EPA framework has historically been directed at the risk of physical and chemical environmental stressors to human health, but has broadened to include biological stressors and risks to the environment. While this framework's utility in evaluating risk of biological stressors, such as invasive species, remains tenuous for some (Alpert et al. 2000), the basic principles are sufficient for my applications.

The EPA risk assessment framework has three main phases: problem formulation, analysis (not to be confused with the broader process of risk analysis), and risk characterization (Figure 1.2). In problem formulation, information is gathered to determine the nature of the problem and identify potential ecological entities at risk. Important expansions of this phase to include explicit stakeholder input to aid in determining the scope of the ERA have been proposed (Nelson and Banker 2007). The analysis phase follows, where data are used to determine the likelihood of exposure to a

stressor (e.g., an invasive species) and the magnitude and type of ecological effects given exposure occurs. An additional model for considering exposure and effects for invasive species is presented by Orr (2003); exposure analysis can follow the steps of invasion and effects analysis should consider economic and social consequences, in addition to environmental effects (Table 1.1). Risk characterization integrates the exposure and effects analysis to interpret the risk and describe sources of uncertainty. Critically, though these phases are presented sequentially, ERA is an iterative process (U.S. EPA 1998).

Within the ERA framework presented, my dissertation will predominately address the "exposure" element of risk assessment. Specifically, it will help to characterize the potential for integration/adaptation of *E. postvittana* to temperate regions of the U.S.A.

1.2.3 Pest Risk Mapping

To estimate the risk a given species may pose to a given area in space and/or time, mechanistic and phenomenological risk models can be developed to assess the likelihood of successful invasion into a given area (exposure) and the of magnitude of the subsequent harm (effect) (Venette et al. 2010). Mechanistic models are those that forecast a relationship between variables, where the relationship is defined in terms of the biological process(es) thought to give rise to the data variables. Phenomenological, or statistical models, forecast a relationship between variables where the relationship seeks to best describe the data without reference to the mechanism of the relationship (Hiblorn and Mangel 1997).

Pest risk maps are powerful tools used to visually represent the output of pest risk models and are integral to effective risk communication and risk management decisions (Venette et al. 2010). Venette et al.(2010) provide a detailed outline for developing a pest risk map, which is a process similarly iterative to ERA (U.S. EPA 1998) and contains parallel components of problem formulation, analysis (here, model selection and validation), and risk characterization (Figure 1.3). The authors note that individual pest risk maps may contribute components to an overall assessment of pest risk (e.g., likelihood of establishment, potential habitat suitability due to specific factors) and not necessarily depict comprehensive risk.

A current challenge in pest risk modelling and mapping lies in addressing uncertainty (Venette et al. 2010), which can impede the development of risk assessments that are biologically-meaningful over space and time. Uncertainty is inherent in any model, and can stem from multiple sources (reviewed in Elith et al. 2002, Elith and Leathwick 2009, Uusitalo et al. 2015). For mechanistic (or deductive) models in particular, significant epistemic uncertainty often arises from a lack of biological data (Venette et al. 2010). Additionally, simplifying assumptions are made concerning the biology of modelled organisms, in part because of the aforementioned paucity of relevant empirical data. A notable example is the treatment of species as "homogenous and immutable entities" over space and time (Lee 2002). Models rarely account for the plasticity of traits or the potential for adaptation to new environments (Hoffmann and Sgrò 2011, Bebber 2015, but see Kearney et al. 2009) even though invasive species tend to have high levels of phenotypic plasticity (Davidson et al. 2011) and invasions frequently involve rapid evolutionary events (Reznick and Ghalambor 2001). Models and maps that ignore these sources of variation could be particularly misleading regarding their estimates of the likelihood and extent of establishment and spread, and the subsequent effect an invasive species could have in an area. Furthermore, incorporating climate change into risk models could further confound forecasts because of the interaction of climate change with both phenotypic plasticity and evolutionary potential (Davis et al. 2005, Chown et al. 2007).

However, the question of how trait variation and adaptation will impact the overall outcome of complex mechanistic and phenomenological models for invasive species remains to be adequately explored. More empirical research using invasive species, designed for this application, is needed for such exploration to meaningfully proceed.

1.2.4 Risk Assessments of Epiphyas postvittana

Various risk assessments have been produced to evaluate aspects of *E. postvittana* damage (e.g., in fruit, (Fowler et al. 2009); in wine grapes, (USDA-APHIS-PERAL-CPHST 2015); in cut flowers (USDA-APHIS-PERAL-CPHST 2015b) and control (e.g., California eradication program, (USDA-APHIS 2008); isolated population eradication,

(USDA-APHIS 2007b); sterile insect technique, (USDA-APHIS 2009, 2011); mating pheromone disruption, (USDA-APHIS 2010a) in the U.S.A.). Only one (Venette et al. 2003), however, was produced to evaluate the risk of invasion to the U.S prior to the moth's arrival in California. The "mini" risk assessment was published for the CAPS program, and concluded the overall risk of *E. postvittana* to the U.S. was medium, based on qualitative estimates of low arrival potential, high establishment potential, and high economic damage potential.

Multiple authors have forecasted the potential geographic distribution of *E. postvittana* in the U.S. using pest risk maps. Venette *et al.* (2003) used a climate-matching tool to compare the biomes found in the previous range of *E. postvittana* with those in the U.S.; around 80% of the continental U.S. was found to be potentially suitable. Fowler *et al.* (2009) used a threshold model based on extreme cold and estimated that most of the western and southern U.S. were at risk, but the northern and eastern regions were not. Gutierrez *et al.* (2010) used a climate-driven demographic model to forecast risk in California and Arizona. They concluded that coastal regions and the northern Central Valley of California would be most at risk. He *et al.* (2012) used an ecological niche model that incorporated population growth and stress indices. This forecasted a limited risk to the U.S., with suitable habitat occurring only along the west coast and part of the southeastern coast and interior. Though the methodologies in each forecast were quite different, in most cases some measure of cold stress was shown or assumed to be an important parameter for defining the boundaries of risk.

As is often the case for exotic invasive species, *E. postvittana* assessments and maps have been constructed with relatively limited available biological and ecological data. More detailed characterizations of this species' response to environmental stress will improve estimates. Cold stress response will be an especially useful predictor for temperate regions of North America.

1.3 Insect Cold Tolerance

1.3.1 Paradigm

Cold tolerance (or hardiness) is a complex, multi-faceted trait. It describes how an organism responds to and survives cold temperature exposure, where cold is a term relative to the species and lifestage in question (Lee 1991, 2010). It is typically assessed by using a subset of individuals, but discussed as a species or population-level phenomenon. The semantics and characterization of insect cold tolerance have generated extensive discussion (e.g., Zachariassen 1985, Lee 1989, Bale 1996, 2002, Sinclair 1999, Nedved 2000, Hawes and Bale 2007, Chown et al. 2008, Hawes 2008). Broadly, though, evolutionary and geographic relationships distinguish between groups that withstand freezing of their bodies and those that do not (Addo-Bediako et al. 2000, Vernon and Vannier 2002, Sinclair et al. 2003b). Insects that cannot tolerate freezing, either due to mechanisms of freeze injury (freeze intolerance) or injury from near- or sub-zero, non-freezing temperatures (chilling intolerance) use the strategy of *freeze avoidance*. Insects that can tolerate freezing under certain conditions employ the alternative strategy of *freeze tolerance* (Lee 2010). In practice, the dichotomy of strategies is deceptively simple, which has led some to argue for categories that reflect more graded responses (e.g., Bale 1996, Sinclair et al. 2003).

For discussions of how freezing and non-freezing temperatures cause injury to insects, see Lee (1991, 2010 and refs therein) and MacMillan and Sinclair (2011). In brief, injury from chilling (i.e., non-freezing temperatures) depends on the length of exposure and generally seems to be the result of damage to cell membranes and consequent loss of ion homeostasis and selective permeability (Koštál et al. 2004, Koštál 2010, Lee 2010). Chilling injury can be repaired in some instances, or may accumulate to cause death (termed, pre-freeze mortality) (Koštál et al. 2007). Freezing can cause mechanical damage (e.g., physical injury to cells from the expanding ice lattice, recrystallization) or physiological damage (e.g., changes in solute concentrations and cell volume, protein destabilization, oxidative stress), though the latter is much less understood (Lee 2010, Storey and Storey 2010).

For discussions of how insects avoid and protect themselves from the deleterious effects of cold, see also reviews in Lee (1991, 2010), Storey and Storey (1991), Leather et al. (1993), Sømme (1999), Clark and Worland (2008), and Duman et al. (2010). In brief, regulation of freezing is important for both freeze avoidant and tolerant insects; it represents death for the former and a major change in physiological state for the latter. All insects have some ability to maintain the liquidity of their body fluids below the freezing point/melting point equilibrium temperature (~0°C). This is termed supercooling, which continues until the *supercooling point* (i.e., the temperature at which internal ice formation begins) is reached (Figure 1.4). A number of factors can increase or decrease the capacity of supercooling, such as ice nucleators, anti-freeze proteins, inoculative freezing, and the accumulation of low molecular weight molecules (Lee 1991). To prevent freezing, freeze avoidant insects can depress their supercooling point by removing incidental ice nucleators, such as food in their gut, or avoiding moist overwintering habitats that could cause inoculative ice nucleation. Conversely, freeze tolerant insects accumulate ice nucleators to initiate controlled freezing at relatively high (e.g., -2 to -5°C) subzero temperatures (Zachariassen and Hammel 1976).

Most notable among freeze avoidant species is their ability to enhance greatly supercooling capacity (sometimes to -40°C and below) by accumulating low molecular weight polyols and sugars, such as glycerol (most common), sorbitol, glucose, trehalose, and fructose (Lee 1991). These molecules colligatively depress the freezing point of fluids, meaning the number of molecules rather than the chemical identity causes the change (Storey and Storey 1991). Proteins (also termed anti-freeze proteins or thermal hysteresis proteins) can similarly depress the supercooling point, but through non-colligative means; they prevent inoculative freezing and inhibit potential ice nucleators (Duman et al. 2010). Freeze tolerant insects also contain cryoprotective proteins, in some cases to serve as ice nucleators, and in others to stabilize membranes and protect against recrystallization or osmotic stress, (Lee 1991, 2010). As an aside, the term "cryoprotectant" most often refers to polyols and sugars, though it has also been used to encompass any of the aforementioned molecules that enhance cold or freezing tolerance (Lee 2010).

Lastly, insects can also respond to cold temperatures through behavioral modifications, such as seasonally migrating to warmer latitudes (e.g. Oberhauser et al. 2015) or seeking microclimates that significantly buffer ambient temperatures (Danks 1991, Duman et al. 1991a).

Numerous factors can influence the measurement of an individual's response, and, therefore, categorization of cold tolerance strategy. Such factors include, but are not limited to: intensity and duration of exposure (Payne 1926, Salt 1936), number of exposure events (Marshall and Sinclair 2012), life stage (e.g., Boychuk et al. 2015), nutritional and feeding status (Lavy and Verhoef 1997, see also Chapter 2), seasonality/thermal history (e.g., diapause (Tauber and Tauber 1976, Denlinger 1991), acclimation (e.g., Rako and Hoffmann 2006), rapid cold hardening (Lee et al. 1987)), additional physiological stress (Holmstrup et al. 2010, Sinclair et al. 2013), and cross-generational effects of cold exposure (Watson and Hoffmann 1996, Magiafoglou and Hoffmann 2003).

Insect cold tolerance can be experimentally characterized in myriad ways. As evidenced from the conceptual framework above, freezing has historically been a focus of laboratory assessments. Measuring the supercooling points of individuals is regularly done, determined using contact thermocouple thermometry; a thermocouple is attached to the surface of an insect and records its body temperature as it is cooled below 0°C. Temperatures are then digitally graphed to visualize the supercooling point, seen as the lowest point reached before an exotherm, or abrupt increase in temperature (e.g., Carrillo et al. 2004) (Figure 1.4). Though a logistically simple assay, supercooling points by themselves can inaccurately represent the cold tolerance of an insect (or population) if they experience high pre-freeze mortality, or are freeze tolerant. Supercooling point data must be contextualized with the ecology of the insect, as well as with additional laboratory assessments of mortality and response to non-freezing temperatures (Renault et al. 2002, van Lenteren et al. 2006, Terblanche et al. 2011).

Other common laboratory cold tolerance assays include "lower lethal temperature", which measures the intensity of temperature that causes death to some portion of individuals, and "lower lethal time", which measures the duration of time at a given temperature that causes death to some portion of individuals (reviewed in Morey

2010). Less extreme exposure assays are also used, such as “chill coma recovery” which measures the time until recovery after an insect is immobilized by cold and has been linked to the geographic distribution of Drosophila (e.g., Gibert et al. 2001, Macdonald et al. 2004). Also common is the quantification of various biochemical changes (e.g. cryoprotectants, water content, membrane lipids, proteins) in individuals after cold exposure (reviewed in Denlinger and Lee 2010).

Many authors have emphasized the importance of methodological rigor (and transparency) and caution in drawing ecological conclusions from laboratory-collected data (e.g., Baust and Rojas 1985, Bale 1987, 1991, 2010, Block et al. 1990, Renault et al. 2002, Sinclair et al. 2003, Terblanche et al. 2011, Morey et al. 2012). Since cold tolerance assays overwhelming seek to define survival following cold exposure, one particular point of concern centers on the variable and often arbitrary way “survival” is assessed in the laboratory. As Baust and Rojas (1985) state, “survival estimates have become a function of experimental convenience rather than a measure of evolutionary consequence.” Typically, survival is assessed within a few hours or days after cold exposure (e.g., Nedvěd et al. 1998), defined by visible response to a stimuli (e.g. (Khodayari et al. 2013) or obvious necrosis. Not only do variable post-exposure conditions, recovery intervals, and measures of qualitative behavior potentially confound estimates of the immediate mortality from cold injuries, mortality could occur at a later point in the lifecycle (Hawes and Wharton 2011). Moreover, sublethal effects from cold on development and reproduction can have important fitness consequences (see Chapter 3).

Attempts to standardize methods and assess ecologically-relevant measures should continue to be sought (Baust and Rojas 1985, Danks 1996). However, given the myriad logistical constraints of experiments and variation in insect systems, it is most imperative for researchers to be transparent and thorough in describing whatever cold tolerance methodologies they employ.

1.3.2 Partial Freeze Tolerance

As stated, freeze avoidance and freeze tolerance likely each contain a continuum of responses. Within freeze tolerance, one such response is partial freeze tolerance

(Sinclair et al. 2003b). A poorly characterized phenomenon, partial freeze tolerance is most consistently mentioned as the ability to survive formation of ice within the body, but to a much more limited extent than traditionally recognized freeze tolerant species (i.e., those that survive maximal ice formation at a given temperature). In reference to Figure 1.4, when an insect is cooled to a point beyond their supercooling point but then rewarmed before reaching the equilibrium freezing point, they are considered to have partial freeze tolerance if they survive (Block et al. 1988, Sinclair et al. 2003b, Hawes and Wharton 2010). Baust and Rojas (1985) similarly references this condition in relation to tolerance of variable ice formation, but one that extends to temperatures beyond the equilibrium freezing point. Given that ice formation is a time-temperature dependent event, the authors suggest that if multiple species have an equivalent supercooling point and die at different temperatures below that point, the species differ in their ability to tolerate the physiological changes that accompany ice formation progression (Baust and Rojas 1985). Hawes and Wharton (2010) also consider partial freeze tolerance as a population phenomenon, in addition to an individual phenotype. Here, individuals are able to survive equilibrium freezing, but they only constitute a proportion of a population that otherwise cannot survive equilibrium freezing (purportedly as in Todd and Block 1995).

Partial freeze tolerance has received very slight discussion in the literature in comparison to other insect cold tolerance phenotypes and even less directed empirical investigation. Though the immediate ecological relevance of partial freeze tolerance is questionable, it may be more germane to evolutionary questions of insect cold tolerance; the ability to briefly tolerate small amounts of ice formation could be an evolutionary intermediate between no tolerance and more extensive tolerance to freezing (Sinclair 1999, Hawes and Wharton 2010).

1.3.3 Cold Tolerance of Epiphyas postvittana

The cold tolerance of *E. postvittana* has been previously investigated, but largely from the prospective of commodity disinfestation, where cold is used as an insecticide to apply to plant products and nursery materials before importation/exportation. Many of these studies look at the combination of prolonged cold exposure with other

environmental conditions (namely, controlled atmospheres) to maximize the efficacy of postharvest treatments (Waddell et al. 1990, Whiting and Hoy 1997, Chervin et al. 1999, Tabatabai et al. 2000) and are less relevant to ecological applications of cold tolerance. However, some evaluate the effect of cold alone, especially prolonged exposures to subzero, non-freezing temperatures. These studies are summarized in Table 1.2, in addition to the more recent work conducted by Bürgi and Mills (2010).

Bürgi and Mills (2010) currently provide the most complete assessment of *E. postvittana* cold tolerance, conducting multiple laboratory assays using late instars (4^{th}-6^{th}). For each instar, they measured the supercooling points and calculated mortality estimates (LT_{50} and LT_{99}) at four different low temperatures. The authors conclude that *E. postvittana* late instar larvae are chill-susceptible (*sensu* Bale 1996) due to substantial mortality experienced at temperatures above the mean supercooling points. They also compared their *E. postvittana* estimates to those of a native, non-diapausing tortricid restricted to the western coast of the U.S.A. (*Argyrotaenia franciscana*). Given the relatively poorer cold tolerance of *E. postvittana* to the native species, the authors suggest that *E. postvittana* will be substantially limited in its further U.S. spread.

With the exception of one study (van Den Broek 1975), all the current literature on *E. postvittana* cold tolerance relates to the direct effects of low temperature on mortality, where mortality is short-term and loosely defined (e.g., response to stimuli 24h after exposure, progression to next developmental stage). van Den Broek (1975) measured the effects of various lengths of time at 3°C on fertility and found no detectable differences. However, no additional work has investigated fitness costs to similar or different cold exposures, and no studies have explored the capacity of this species to adapt to low temperatures.

Preliminary investigation within my graduate laboratory found that a proportion of *E. postvittana* larvae appeared to display partial freeze tolerance (i.e., individuals that survived brief exposure to some internal ice formation). This observation served as the basis for much for my research and is explored in more detail in the subsequent chapters.

1.4 Sources of Phenotypic Variation: the Environment and Genetic Variation

1.4.1 Definitions, and Relevance to Invasion Biology and Insect Cold Tolerance

Definitions. Environments vary; through time and space, biotically and abiotically, from extrinsic and intrinsic forces. As a consequence, individuals and populations may respond to environmental variation via variation in their response within and between generations (Meyers and Bull 2002, Whitman and Agrawal 2009). Within-generation variation occurs in individuals, does not involve a genotypic change, and may allow individuals to adjust to current environmental variation (Whitman and Agrawal 2009, though see also Ghalambor et al. 2007 and Morris and Rogers 2013 for discussions of non-adaptive plasticity). Between-generation variation refers, in part, to the genetic changes that result from microevolution, such as natural selection acting on heritable variation within a population. Such change in allele frequencies brought about by selective environmental pressures can produce a long-term adaptive response for a population. Framed another way, organisms can respond to changes in their environment through phenotypic plasticity (see below) or with genetic adaptation from selection pressures (also genetic accommodation (West-Eberhard 2003), genetic assimilation, or canalization (Pigliucci 2006)). Importantly, these mechanisms of variation are not mutually exclusive, and the role of the environment in shaping both is increasingly argued as critical (West-Eberhard 2003, Pigliucci 2006, Whitman and Agrawal 2009). Whatever the source, variation in the phenotype of a given trait (or traits) is required for a population to persist in a novel environment.

Phenotypic variation (V_P), expressed as statistical variance (mean of the squared deviations around the population mean), results from a combination of sources, partitioned as follows:

$$V_p = V_G + V_E + V_{GxE} + V_{error},$$

where V_G is the proportion of phenotypic variation attributable to genes (genetic variance); V_E is the proportion attributable to the environment (phenotypic plasticity); V_{GxE} is the genetic variation for phenotypic plasticity (genotype x environment

interaction); and V_{error} is any unexplained variation, such as measurement error, developmental noise, etc. (Pigliucci 2001, Whitman and Agrawal 2009). The focus of my subsequent chapters will be on describing V_E within, and resulting from, certain cold tolerance phenotypes, and measuring a component of V_G using quantitative genetic methods.

Phenotypic plasticity (V_E) is commonly defined as the property of a genotype to produce different phenotypes in different environments (Pigliucci 2001, Whitman and Agrawal 2009), though a variety of alternative terms (Forsman 2015) and definitions (Whitman and Agrawal 2009) have been used. Phenotypic plasticity can be adaptive or non-adaptive, and behavioral, biochemical, physiological, or developmental traits may show plasticity (Pigliucci 2006, Ghalambor et al. 2007, Whitman and Agrawal 2009). Typically, phenotypic plasticity is visualized using reaction norms, which are graphical comparisons of trait values under different environments for different genotypes (e.g., Schlichting and Pigliucci 1998, Whitman and Agrawal 2009, Fusco and Minelli 2010). Phenotypic plasticity provides an obvious benefit for quickly responding to variable environments, but it can also carry evolutionary and ecological trade-offs and limitations (DeWitt et al. 1998, Agrawal 2001)

Genetic variance (V_G) can be partitioned further, expanding the previous equation as:

$$V_p = (V_A + V_D + V_I) + V_E + V_{GxE} + V_{error},$$

where V_A is the proportion of the genetic variance due to the additive effects of alleles (additive genetic variance); V_D is the proportion of the genetic variance due to the non-additive effects of dominance (dominance variance); and V_I is the proportion of genetic variance due to the non-additive effects of epistatic interactions between loci (interaction variance) (Falconer and Mackay 1996). The adaptive evolutionary potential of a population depends on the genetic architecture of the trait(s) in question. In particular, the extent of additive genetic variance is often considered the strongest indicator of overall variation (Falconer and Mackay 1996), though see Hill et al. (2008) for additional discussion.

Individual components of phenotypic variance can be estimated by comparing the resemblance between relatives. For example, narrow-sense heritability (h^2) is the ratio of additive genetic variance to phenotypic variance of trait in a given environment (i.e., V_A/V_P) (Falconer and Mackay 1996). Heritability is calculated using various experimental breeding designs, often by the regression of offspring phenotypic values on those of their parents (Falconer and Mackay 1996). In my subsequent work, I will specifically use the regression of offspring values on mid-parent values (the average value of the two parents); the slope of this regression yields h^2.

Invasions and cold tolerance. In biological invasions, species often undergo rapid environmental transitions and must quickly respond to new biotic and abiotic stresses (Lee 2002, Gilchrist and Lee 2007). Successful establishment and spread has been attributed to numerous traits, but the thermal tolerance of the invading populations is especially important for insects (e.g., Nyamukondiwa et al. 2010, Zerebecki and Sorte 2011).

As reviewed in section 1.3, insects exhibit extensive phenotypic variation in their response to cold, both between and within species. Separating the influence of environmental versus genetic factors on cold tolerance can be difficult, as is evident from the continued discussion of the role of phenotypic plasticity and genetic variation in thermal adaptation, and consequently, invasion success (e.g., Hoffmann and Hercus 2000, Price et al. 2003, Butin et al. 2005, Dybdahl and Kane 2005, Richards et al. 2006, Chown et al. 2007, Ghalambor et al. 2007, Gienapp et al. 2008, Hoffmann and Sgrò 2011). Some studies indicate trade-offs in the extent of plasticity due to basal cold tolerance (Nyamukondiwa et al. 2011, Gerken et al. 2015). However, given that phenotypic plasticity can also create the conditions that result in an adaptive genetic response, and that phenotypic plasticity is itself under genetic control, a mix of mechanisms seems conceivable (Lee 2002, Pigliucci 2006, Ghalambor et al. 2007).

Much of the current work that quantifies the genetic variation of cold tolerance in insects uses *Drosophila* spp. (see Chapter 4), though the few studies that have actually linked cold tolerance phenotypes with variation in a gene have used butterflies or a beetle (see Appendix 1). Most studies point to there being substantial genetic variation for cold

stress resistance within widely-distributed populations, and little such variation for species with restricted distributions (Nyamukondiwa et al. 2010, Davis et al. 2014). How much this pattern extends to other insect species in various cold environments remains to be shown (Overgaard et al. 2010).

Further review of the roles and mechanisms of phenotypic plasticity and genetic variation in evolution is beyond the scope of this review and is summarized elsewhere (e.g., Via et al. 1995, Davis and Shaw 2001, Sgrò and Hoffmann 2004, Gienapp et al. 2008, Hoffmann and Sgrò 2011, Franke et al. 2012, Davis et al. 2014). My interest in these concepts, here, relates to their use as complimentary tools to more rigorously characterize the immediate and future phenotypic response of an invasive insect population to cold temperature. For my work, I will be focusing on phenotypic plasticity (V_E) (Chapters 2-4) and additive genetic variance (V_A) (Chapter 4) in extreme cold tolerance in *E. postvittana.*

1.4.2 *Adaptability and Climate Change*

To accurately forecast the dynamics of an invasive species' distribution, having empirically-derived estimates of phenotypic and genetic variation can add greatly to the robustness of mechanistic models. This becomes particularly important when incorporating climate change into forecasts, though it is rarely done (Hoffmann and Sgrò 2011). Here, I provide a very brief overview of discussions surrounding climate change and invasive species, and the impact of climate change on insect cold tolerance.

There is general concern that climate change will intensify the likelihood and effects of biological invasions (e.g., Stachowicz et al. 2002, Hellmann et al. 2008, Walther et al. 2009, Burgiel and Muir 2010) with the predicted general warming and drying (Easterling et al. 2000) thought to favor invasive species over indigenous ones (Dukes and Mooney 1999). However, mechanisms of synergy between climate change and invasions are not well understood, especially for terrestrial species (Chown et al. 2007). The importance of migration, phenotypic plasticity, and genetic adaptation through selection have all been proposed as drivers of a species persistence during climate change (Price et al. 2003, Davis et al. 2005, Chown et al. 2007), with phenotypic plasticity generally considered to contribute more (Hoffmann and Sgrò 2011).

Given that most insects are phytophagous (Grimaldi and Engel 2005), one important consequence of climate change to insects is loss of synchrony with host phenology (e.g., Visser and Holleman 2001). Sufficient genetic variation and strong selection pressures could overcome this potential asynchrony (Bradshaw and Holzapfel 2001) or, alternatively, plasticity in host range could allow for host-switching without a change to the herbivore phenology (van Asch et al. 2007). Climate warming is expected to increase fitness in most insect species occurring in temperate regions and northern latitudes (Deutsch et al. 2008). This is due to warming proceeding faster with increasing latitudes and more in winter than in summer months (IPCC 2007), essentially alleviating cold stress without inflicting much heat stress (Bradshaw and Holzapfel 2006, but see also Overgaard et al. 2014). Additionally, the decrease of extreme winter temperature events (Easterling et al. 2000) may support range expansions of some species, however other species that have evolved reliance on certain temperature cues during development (e.g., diapause termination) may be at a disadvantage (Bradshaw and Holzapfel 2010a). The seasonal synchronization of many insects with photoperiodic cues leads some authors to conclude that the primary target of selection from climate change in temperate regions will be on timing of seasonal events, rather than changing thermal tolerances and optima (Bradshaw and Holzapfel 2001, 2010a).

1.4.3 Variation in Epiphyas postvittana

In its native range, *E. postvittana* has been described for decades as an insect with notable intraspecific variability in numerous physiological, morphological, and behavioral traits (Danthanarayana 1975a, 1976a, 1976b, Geier and Springett 1976, Geier and Briese 1980b, Suckling et al. 1990). To investigate the genetic underpinnings of the species' purported adaptability, Gu and Danthanarayana (2000) compared the genetic variance and covariance of several life history traits between two field-collected Australian populations. Each population was reared under two temperature regimes (23°C and 28°C) to also detect any genotype by environment interactions for each trait measured. In sum, the authors conclude that *E. postvittana* is plastic in many life-history traits and shows significant genetic variance in some traits (development time and adult body weight) for both populations. There appeared to be strong genotype by environment

(temperature) interactions in both populations and the authors suggest that differences seen between the populations may be a result of adaptation to local thermal environments (Gu and Danthanarayana 2000).

In the U.S.A., recent mtDNA analysis of the *E. postvittana* California population suggests high genetic diversity has been maintained in this area, likely from recent repeated introductions or a single large introduction (Rubinoff et al. 2011). The authors remark that the lack of low genetic diversity more typical of founder events may help the moth to adjust to adverse conditions in its newly invaded range.

Given that environmental temperature and host plant appear to impart substantial influence on *E. postvittana* life-history variation (Danthanarayana et al. 1995, Gu and Danthanarayana 2000) these factors will be the focus of my dissertation. Furthermore, the genetic variation previously observed in thermal tolerance of Australian populations (Gu and Danthanarayana 2000) may suggest the opportunity for selection in other temperature environments, such as the extreme cold used in my research.

1.5 Aims and Scope of Dissertation

1.5.1 Aims

The pieces of my dissertation are unified by assessing variation in cold tolerance phenotypes within laboratory populations of *E. postvittana*, from direct effects within individuals, to indirect fitness tradeoffs on individuals, to long-term effects on populations. Specifically, I look at these processes as they relate to acute, extreme cold exposure. My studies add to the currently sparse literature on how cold tolerance varies across multiple scales in a non-drosophilid insect. In addition, this work is designed to be informative to models and maps of pest risk; it can aid in addressing sources of uncertainty, specifically those surrounding how a species may vary in its response to present and future thermal environments.

The aforementioned aims will be explored in four subsequent chapters:

1. In Chapter 2, I evaluate the effects different host plants can have on direct tolerance to acute partial freezing in individuals. I also test a previous

hypothesis (Voituron et al. 2002) that posits insect cold tolerance strategy may be associated with the fitness of individuals during periods of ideal temperature conditions.

2. In Chapter 3, I compare the fitness tradeoffs in individuals following exposure to two subzero temperatures (-10°C or the supercooling point). Using developmental and reproductive measures, I can estimate the cross-generational effects of cold. Using additional data from the experiment in Chapter 2, I also measure the indirect effects of host plant on acute cold tolerance.
3. Chapter 4 measures cold tolerance variation on the broadest scale of my work; populations through time. I conduct a multi-year laboratory selection experiment to evaluate the long-term effects of tolerance to acute partial freezing and use quantitative genetic measures in attempt to disentangle phenotypic variation from potential genetic adaptation in this response.
4. Finally, in Chapter 5, I apply a snapshot of the data collected in Chapter 4 to a simplified pest risk mapping exercise. Using a threshold-based model, I show areas at risk in North America to potential *E. postvittana* exposure based on historical minimum annual temperatures. The point of the exercise is to show how variation in cold response, whether due to phenotypic plasticity or adaptation, can alter estimates of geographic risk.

1.5.2 Scope

The topics touched on in this dissertation stem from a variety of disciplines, and could themselves each be the focus of a research program. As such, there are obviously limits to the depth into which I went with any single area. I feel it is important to acknowledge, here, the general scope of my work and be transparent in its limitation, thus, allowing adequate comparisons with relevant current and future work.

The data collected within were from a single species maintained for almost a decade in constant laboratory conditions, with no known out-crossing with wild individuals. Cold exposure was predominately limited to acute, partial freezing in late instars, though other subzero exposure was evaluated in Chapter 3. No acclimation (e.g.,

changes in temperature, photoperiod, or humidity) was applied to approximate seasonal changes.

The following non-exhaustive list reflects areas of research that were beyond the scope of this dissertation, but that I feel would be important (in no particular order) future directions with regards to cold tolerance variation in *E. postvittana*:

- *Field validation* – Validating the results of my laboratory cold tolerance assays with natural populations of *E. postvittana* would be especially important for making robust contributions to a risk model/map. Similarly, collecting samples from multiple locations within the invaded and natural range may reveal interesting comparisons and additional variation.
- *Risk model comparisons* – The extension of my data into model construction has been minimal here and restricted to simple threshold models. In addition to applying the data to more complex risk models (e.g., those that incorporate growth indices and additional abiotic stresses, those that forecast under climate change scenarios, etc.), using multiple modelling platforms for comparisons will be equally, if not more, important to see how my data interact under different assumptions and parameter emphasis (Venette et al. 2010).
- *Additional cold tolerance characterization* – The cold tolerance assays here all involved extreme, acute cold exposures. As stated earlier, cold tolerance is a complex trait that is best characterized with an array of measurements. My experiments mostly focused on a single measure for logistical feasibility and consistency. However, further studies involving factors such as chronic exposure, acclimation, and fluctuating temperatures could provide a more comprehensive assessment of cold tolerance in this insect. Especially with regards to the time-series resulting from the selection study, showing a similar periodicity in additional measures of cold tolerance would bolster the hypothesis that the cycle of survival after partial freezing has ecological relevance.

- *Molecular/genetic characterization* – As noted by Lee (2010), recent advances in molecular and cell biology have allowed for deeper and more refined characterization of insect cold tolerance. Appendix 1 of this dissertation provides preliminary investigation of a study to link enzyme variation with cold tolerance variation. Continued application of molecular techniques to compliment more traditional whole organism assays will shed light on the underlying mechanisms of this species' response to cold temperature.
- *Alternative selection pressure(s) and response(s)* – While the selection study in Chapter 4 did not show a consistent response to selection, a different cold selection pressure could yield a different result. Less extreme temperature exposure (e.g., as in Anderson et al. 2005) could show a divergence in response. Alternatively, assessing a developmental or reproductive proxy to fitness (e.g., pupal weight, fecundity), rather than survival itself, may reveal a different pattern in response

Tables and Figures

Table 1.1: A complimentary model for considering the exposure (probability of establishment) and effects (consequences of establishment) analysis in the Environmental Protection Agency ecological risk assessment framework (U.S. EPA 1998). Risk model presented by Orr (2003) as part of a larger Generic Nonindigenous Organism Risk Analysis Review Process for aquatic species.

Probability of Establishment[*] (P)	**Consequences of Establishment (C)**
$P = X_A+X_E+X_I+X_S$	$C = X+Y+Z$
X_A = Arrival potential	X = Economic impact potential
X_E = Establishment potential	Y = Environmental impact potential
X_I = Integration potential	Z = Perceived impact (Socio-
X_S = Spread potential	political influences)

[*]The stages of establishment have been modified to fit the invasion framework of Venette and Carey (1998).

Table 1.2: Summary of previous cold tolerance research involving *Epiphyas postvittana*. Data here only represent studies, or portions of studies, that evaluated the singular effect(s) of cold, either instantaneously or through time.

Lifestage Exposed	Temp-erature	Time	Host	Other Specified Conditions	Mortality Caused[a]	Total Insects Tested	Other Response(s) Measured	Reference
Egg	3°C	Observations every 7d, for 70d	Obtained from lab colony	23°C following exposure	*(hatch)* 82% @ 7d 100% from 35-70d	384	---	*(f)*
Larva[b]	-10.0°C -16.0°C	1d	---	---	30% @ -10°C 100% @ -16°C			*(g)*
3-4th instars[c]	3.0°C	Observations every 7d, for 70d	Artificial medium	Reared from 1st instar at 21°C for 14d and then exposed; 23°C after exposure	*(adult emergence)* 40% @ 7d 68% @ 35d 72% @ 70d	250	% pupation, fertility & fecundity	*(f)*
4th instars	-14.5 to -16.0°C[e]	S*upercooling points*	Artificial medium	Unacclimated and acclimated[d]	Not measured	40	---	*(h)*
4th instars	3.0°C	10 intervals between ~300-1400h	Artificial medium	Held at 21°C for 24h after exposure	*(response to probe)* 50% @ 775h 99% @ 1864h	56	---	*(h)*
4th/5th/6th instars	-0.9°C	10 intervals between ~90-500h	Artificial medium	Held at 21°C for 24h after exposure	*(response to probe)* 50% @ 197.6h 99% @ 464.1h	52	---	*(h)*
4th/5th/6th instars	-6.5°C	16 intervals between ~1-65h	Artificial medium	Held at 21°C for 24h after exposure	*(response to probe)* 50% @ 40.9h 99% @ 76.1h	46	---	*(h)*

4th/5th/6th instars	-10.5°C	11 intervals between 1-6h	Artificial medium	Held at 21°C for 24h after exposure	*(response to probe)* 50% @ 2.5h 99% @ 5.07h	52	---------	*(h)*
5th instars	-13.0 to -14.7°C[e]	*Supercooling points*	Artificial medium	Unacclimated and acclimated[d]	Not measured	40	---------	*(h)*
5th instars	0°C	76d	Reared on artificial diet, assayed on persimmons	Held at 20°C for 3d after exposure before assessment	*(response to probe)* 95%	~800, but unclear	Mortality of heat w/cold; mortality of heat alone	*(i)*
5th/6th instars	3°C	10 intervals between ~300-1400h	Artificial medium	Held at 21°C for 24h after exposure	*(response to probe)* 50% @ 1029h 99% @ 2391h	56	---------	*(h)*
6th instars	-12.5 to -14.1°C[e]	*Supercooling points*	Artificial medium	Unacclimated and acclimated[d]	Not measured	40	---------	*(h)*
Pupa	3°C	Observations every 7d, for 70d	Coles dwarf broad beans	23°C after exposure	*(adult emergence)* 25-17% @ 7d 17% @ 35d 75% @ 42d 100% from 49-70d	132	Fertility	*(f)*
	0°C	Weekly, for 5 wks	Artificial medium	19°C after exposure	*(adult emergence)* 2% @ 0 weeks 0% @ 1 wk 39% @ 2wks 73% @ 3 wks 90% @ 4 wks 96% @ 5wks	45 per time trt	Mortality and fertility of combined stresses (controlled atmosphere, cold, rearing conditions)	*(j)*

[a] select results presented

[b] instar not specified

[c] instar not specified; allowed 210 degree-days after becoming 1st instars before cold exposure

[d] larvae were tested as acclimated (10°C for 7d and starvation 72h prior to being supercooled) and unacclimated (supercooled directly from 21°C). No difference in supercooling points was detected with acclimation.

[e] estimated range of mean values from Figure 1 in Bürgi and Mills (2010).

(f) Van Den Broek (1975)

(g) Gutierrez et al. (2010)

(h) Bürgi and Mills (2010)

(i) Dentener et al. (1997)

(j) Tabatabai et al. (2000)

Figure 1.1: Map of current state and federal quarantine locations due to the presence of *E. postvittana* in the contiguous United States [source: California Dept. of Agriculture, USDA-APHIS-PPQ].

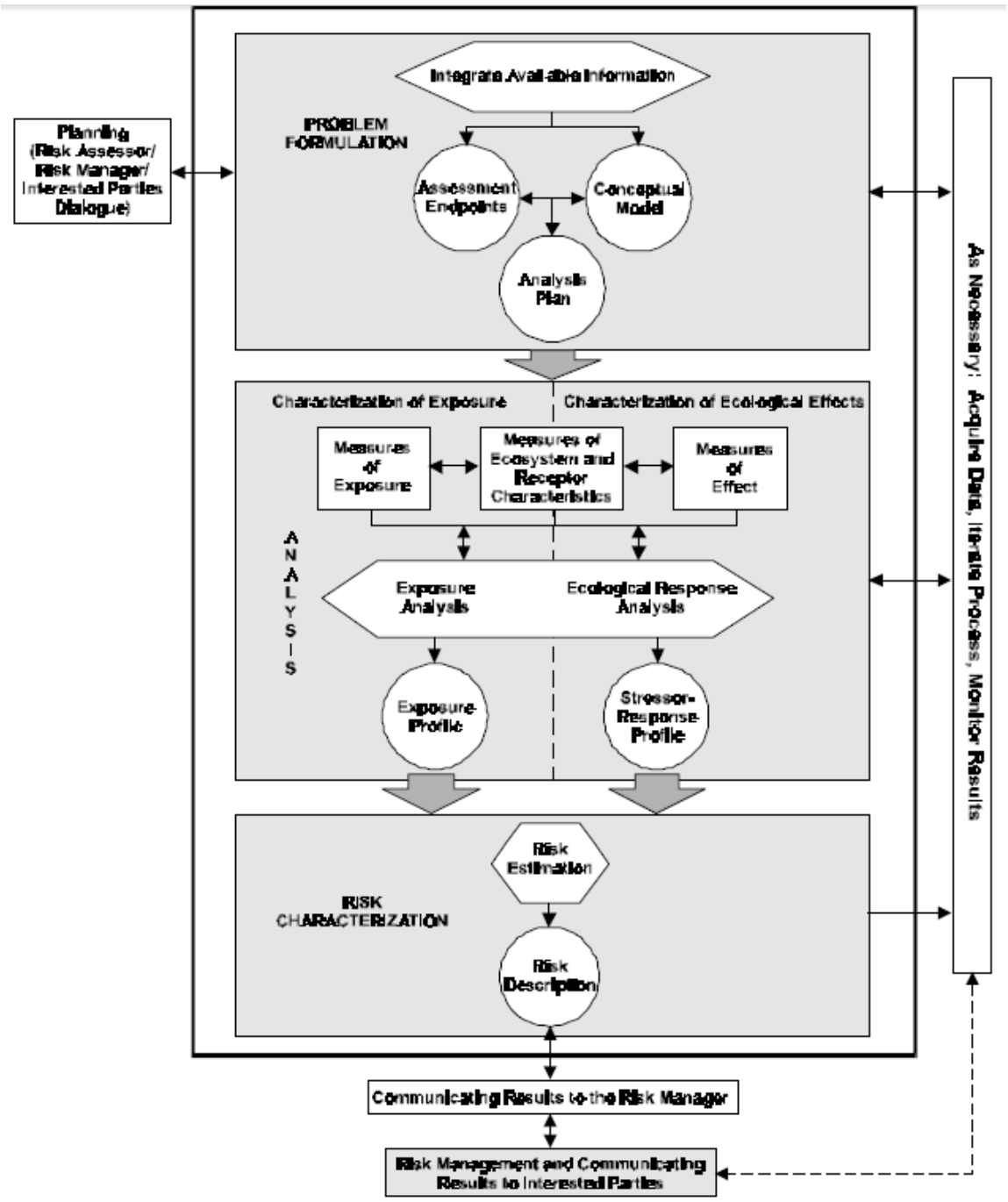


Figure 1.2: A detailed view of the ecological risk assessment framework presented by the U.S. Environmental Protection Agency in their 1998 document entitled, *Guidelines for Ecological Risk Assessment* (U.S. EPA 1998). The research within this dissertation primarily relates to the Characterization of Exposure stage.

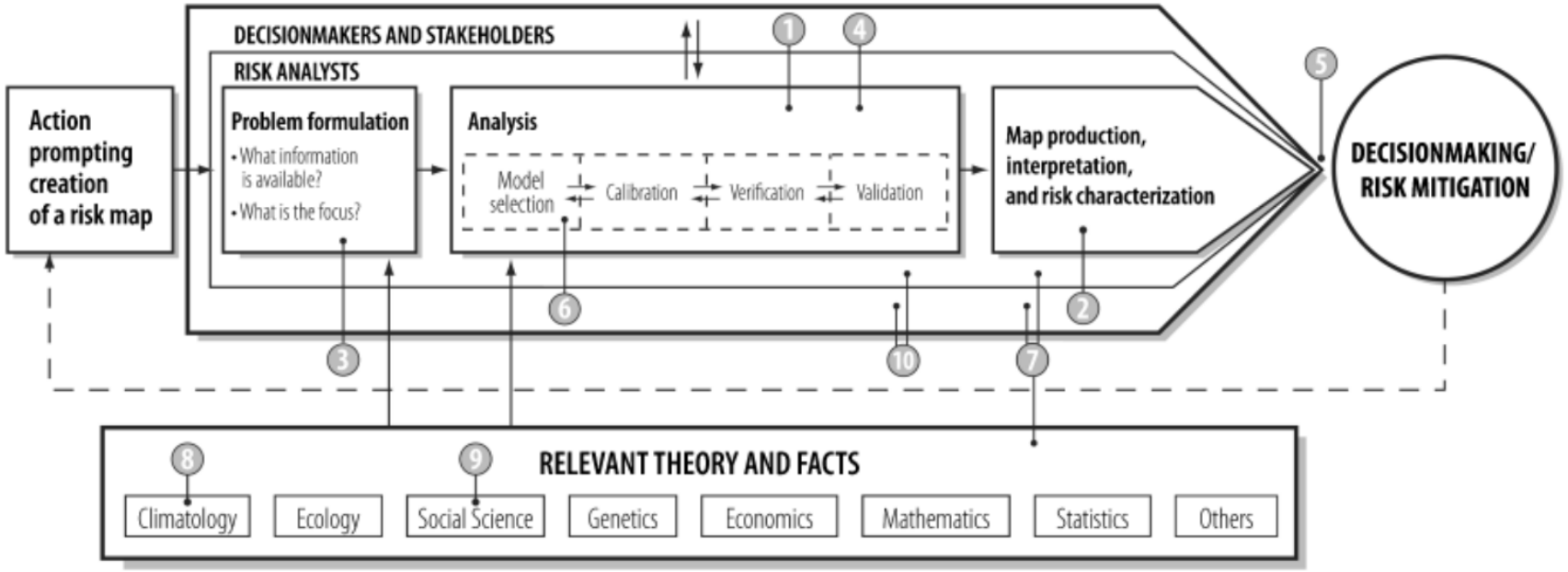


Figure 1.3: Conceptual map of the development of a pest risk map for exotic invasive species proposed by Venette et al. (2010). Numbers correspond to recommendations for improvement provided within the original full article.

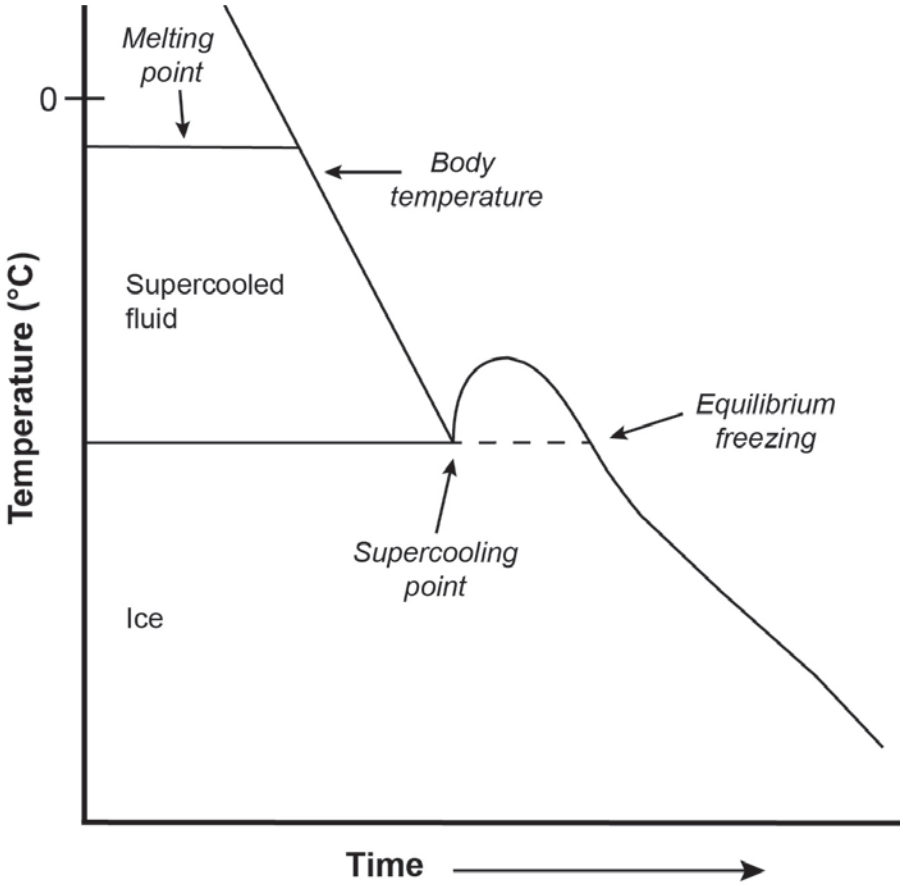


Figure 1.4: Insect body temperature during cooling at a constant rate to subzero temperatures. Below its melting point, insect hemolymph remains in a supercooled state until reaching its supercooling point (or temperature of crystallization). Heat (heat of crystallization) is released as body fluids transition from a liquid to solid. Equilibrium freezing is reached once the body temperature returns to the supercooling point temperature [Modified from Lee 2010].

CHAPTER 2:
Host-mediated shift in the cold tolerance of an invasive insect

2.1 Summary

While most insects cannot survive when ice begins to form within their bodies, a few species have been identified that can tolerate partial, but not complete, internal ice formation. These species may be evolutionary intermediates between freeze intolerant and freeze tolerant species. For phytophagous insects, host may affect cold tolerance strategy, but the evolutionary impacts of insect-plant interactions have rarely been considered. Here, we provide the first empirical test of a theorized mechanism for the evolution of insect cold tolerance that relates fitness in ideal temperature conditions with cold tolerance strategy. We used the overwintering stage of an invasive moth *Epiphyas postvittana* (Walker), which exhibits partial-freeze tolerance. This species showed a dramatic change in survival after partial freezing – from less than 8% to nearly 80% – based on the larval host, as well as changes to its supercooling point. Importantly, the degree of cold tolerance could be predicted by the developmental suitability of a given host; insects fed hosts that were less suitable in ideal conditions showed greater survival after freezing exposure compared to those fed a more suitable host. Our research offers an important contribution to understanding the underpinnings of insect cold tolerance evolution. Additionally, characterizing the effects of host plants on insect cold tolerance dynamics will enhance forecasts of invasive species dynamics, especially under climate change.

2.2 Introduction

In temperate environments, the inclement conditions of winter can be a strong selective force on insect life histories. In the face of sub-zero temperatures and the potentially lethal effects of ice formation, insects generally either avoid freezing (a strategy known as *freeze avoidance*, resulting in *freeze* or *chilling intolerance*) or they

tolerate it under certain conditions (*freeze tolerance*) (Lee 2010). Passionate discussion has surrounded the importance of gradients within and between these strategies, and how responses to cold could be further delineated (e.g, Bale 1996, Sinclair 1999, Nedvěd 2000, Chown et al. 2008). Broadly, though, evolutionary and geographic relationships distinguish groups that withstand freezing and those that do not (Addo-Bediako et al. 2000; Sinclair et al. 2003b), with evidence supporting freeze tolerance as the derived state relative to freeze avoidance in most circumstances (Vernon and Vannier 2002, Costanzo and Lee 2013), but see also (Chown and Sinclair 2010). Additionally, evolutionary intermediates are likely to exist between complete freeze avoidance and tolerance, such as individuals and/or populations displaying "partial-freeze tolerance" to varying levels of internal ice formation (Baust and Rojas 1985, Block et al. 1988, Sinclair 1999, Hawes and Wharton 2010). This type of response is exhibited by species that can survive some amount of ice formation within their bodies, but cannot survive complete freezing (Hawes and Wharton 2010). However, few insect examples exist in the literature. Identifying and characterizing such evolutionary intermediates may provide valuable insights into how species adapt and evolve to withstand low temperatures (Sinclair 1999, Voituron et al. 2002).

Freeze tolerance has evolved independently multiple times among insects, and many factors may drive the change, such as climatic predictability (Sinclair et al. 2003a), behavioral and physiological mechanisms that affect ice nucleation (Zachariassen 1985, Duman et al. 1991a, Lee and Costanzo 1998, Strachan et al. 2011), energy conservation from altered metabolic rates (Irwin and Lee 2002), and cross tolerance with other environmental stressors (e.g., Holmstrup et al. 2010, Sinclair et al. 2013). Of note, Voituron et al. (2002) developed a formalized mathematical model that predicts the conditions under which a particular cold tolerance strategy should be optimal, based on an energetic definition of fitness. Freeze avoidance is energetically costly, so the authors concluded that freeze tolerance would be favored in conditions where the initial fitness level (at the onset of winter) is low, whereas freeze avoidance would be favored where initial fitness is high. However, no empirical evidence currently exists to support this hypothesis.

For phytophagous insects, especially those that are polyphagous, fitness may vary significantly depending on host plant (hereafter, 'host') (e.g., Awmack and Leather 2002). We previously documented partial-freeze tolerance in the light brown apple moth, *Epiphyas postvittana* (Walker) (Morey et al. 2013), a recent invader to the continental United States (USDA-APHIS 2007c) with the capacity to feed on over 360 plant genera (Suckling and Brockerhoff 2010). This species predominantly overwinters as late-instar larvae, which are generally considered freeze avoidant (Bürgi and Mills 2010, Buergi et al. 2011). We observed that a small proportion of late instars from a laboratory population could survive the initial formation of ice within their bodies. Moreover, many of the survivors continued development and eclosed as reproductively-successful adults (Morey et al. 2013). Given this insect's partial-freeze tolerance response, and its extensive polyphagy, we sought to examine the extent to which host affected the cold tolerance strategy of *E. postvittana*.

Our study revealed that hosts can dramatically affect the cold response of this species. Across five hosts, we found a nearly ten-fold range in the survival of *E. postvittana* larvae after ice formation. These hosts also affected survivorship rates and development times in the absence of cold. Most importantly, we found that as fitness decreased, the extent of partial-freeze tolerance increased, thus supporting the hypothesis of Voituron et al. (2002) that low initial fitness may favor a shift towards freeze tolerance.

Accurate estimations of geographic distributions and population dynamics are crucial to both the mitigation of invasive species (Venette et al. 2010) and conservation of native species (Bosso et al. 2013). For invasive insects in temperate regions, characterizing how factors (i.e., host) influence and drive low temperature responses is foundational to the success of their management. Such research becomes particularly insightful in the midst of the complexities of climate change.

2.3 Materials and Methods

2.3.1 Insect colony and plant materials

Epiphyas postvittana eggs were obtained from a laboratory colony maintained by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) in Albany, CA, founded from wild California moths in 2007. All subsequent handling and experimentation was conducted in a Biosafety Level 2 Containment Facility in St. Paul, MN (APHIS permit P526P-14-03759). Eggs were surface-sterilized in a 1% bleach solution, and held at 23 ±2°C, 60-65% RH, 14:10 (L:D) inside a growth chamber (Percival Scientific, Perry, IA) until hatch.

Host treatments were selected from a list of documented North America hosts for *E. postvittana* (Venette et al. 2003), with priority given to those species and varieties that occur in temperate (Midwestern United States) climates. The following host plants were used during the experiment: *Vitis vinifera* L. (var. 'Frontenac'), *Malus domestica* Borkh. (var. 'Zestar!'), *Pinus banksiana* Lamb., and *Populus deltoides* ssp. *monilifera* (Aiton) Eckenwalder (var. 'Siouxland'). *Vitis vinifera* were potted as cuttings from the University of Minnesota Horticultural Research Center (Excelsior, MN). *Pinus banksiana* used in 2012 (blocks 1 and 2) were potted as seedlings from the Minnesota Department of Natural Resources Badoura State Forest Nursery (Akeley, MN). *Pinus banksiana* used in 2013 (block 3) were from mature trees planted on the University of Minnesota campus (St. Paul, MN). *Malus domestica* and *Po. deltoides* were planted as 2-3 year old nursery stock trees on the University of Minnesota campus (St. Paul, MN). All potted plants were housed in a greenhouse during winter months (Sept-May) and moved outdoors during summer months. No pesticides were applied. An artificial diet was used as control. The diet used dry *Phaseolus vulgaris* L (cv. Great Northern) and followed a modified formulation developed by Follet and Lower (2000) for *Cryptophlebia* spp. (Lepidoptera: Tortricidae).

Within 24 h of hatching, *E. postvittana* neonates were placed individually onto a randomly selected host using surface sterilized (with 70% ethanol in water) camel hair paintbrushes. Groups of three larvae were placed on a single excised leaf (or sprig, in the case of *Pinus*), which was contained in a Petri dish sealed with parafilm to prevent larval

escape. Leaf petioles were inserted into 1cm^3 of wet floral foam, which was re-wetted with de-ionized water every 2-3 d. Leaves were replaced on average every 3 d when discoloration was observed, or larvae consumed ~1/2 of the tissue area. Larvae were similarly applied to ~2cm^3 cubes of artificial diet in sealed plastic cups (29.5 mL P100 soufflés; Solo Cup Co., Lake Forest, IL). 20-26 dishes (or cups) were setup for each host treatment per block, except for block three of *Pi. banksiana* which required a total of 45 dishes due to poor initial viability of neonates.

2.3.2 Cold exposure

Instar was confirmed through head capsule measurement (Danthanarayana 1975a), and late instars (4th-6th) from each host were randomly assigned to one of two temperature treatments; cooled to the point of producing an exotherm (i.e., the supercooling point) or not cooled (i.e., temperature control). *Epiphyas postvittana* is known to have variable instar numbers (Dumbleton 1932, Danthanarayana 1975a). Therefore, we focused on "late instars", any of which could be the terminal instar before pupation and could overwinter. Larval feeding cessation prior to pupation (Nijhout 1994) could affect the supercooling point by altering potential ice nucleation sites in the gut (Sømme 1982). However, we did not determine feeding status of larvae at the time of cold exposure; large sample sizes and randomized specimen selection compensated for such potential effects. The number of larvae in each temperature treatment depended on the mortality of early instars from a given host. A total of 8-32 larvae were tested per temperature treatment (n=2) per host (n=5) per block (n=1-3). For the cold-exposed groups, specifically, the total number of late instars tested in each host, across all blocks, was: artificial diet, n= 64; *M. domestica*, n= 26; *Pi. banksiana*, n=48, *Po. deltoides*, n=38; *V. vinifera*, n=64.

All larvae were transferred to individual gelatin capsules (size 4; 14.3mm length, 5.1mm diameter). For the supercooling treatment, individuals were cooled at ~1°C/min to their supercooling point within calibrated styrofoam cubes inside a -80°C freezer, as per Carrillo *et al.* (2004). Temperatures were recorded once per second by using a coiled, copper-constantan thermocouple design (Hanson and Venette 2013), connected to a computer through a multichannel data logger (USB-TC, Measurement Computing,

Norton, MA). Each larva was immediately removed from the freezer and cube once they reached the peak of the exotherm, which could be observed in real-time as a plateau (typically lasting 15-25 s) following an abrupt spike in temperature. Larvae were then given fresh material of the host on which they were reared and returned to 23 ±2°C (60-65% RH, 14:10 (L:D)h) in a growth chamber to continue development. Temperature control individuals were left inside gel capsules at room temperature (~25°C) for approximately 1 h while cold-treated individuals were being chilled. Temperature controls were removed from the capsules and given fresh diet concomitantly with the supercooled larvae. Survival was monitored daily and was defined by successful eclosion as an adult. Some individuals were lost due to handling error following supercooling and were not included in subsequent observations.

2.3.3 Host suitability

To assess the developmental suitability of each host for *E. postvittana* without temperature stress, multiple developmental parameters were measured for individuals that had not been exposed to cold: three stage-specific survival proportions, pupal mass, and total developmental time. Proportion survival was assessed from neonate to late instar, from late instar to pupation, and from pupation to adult eclosion. Because temperature treatments were not assigned until larvae reached late-instars, the proportion of survival to late instar included all individuals initially reared on a given host (i.e., irrespective of future cold treatment). Once individuals were divided among temperature treatments as late instars, only control larvae were used to assess the effect of host on survival during the two subsequent developmental periods, pupal mass, and total development time. Pupae were weighed three to five days after pupation. Total developmental time (egg hatch to adult eclosion) was only calculated for those that survived to adult eclosion.

2.3.4 Analysis

Analyses were conducted in SAS® 9.4 (SAS Institute Inc., Cary, NC). The experiment followed an incomplete block design, with three total blocks occurring in February 2012, March 2012, and August 2013. Due to changes in availability of plant material, the same host species were not included during every block (Table 2.1). Sex

was not considered in any analyses because the sexes of larvae could not be differentiated; equal sex ratios could not be assumed at the outset of the experiment.

All continuous response variables (i.e., supercooling point, pupal mass, total developmental time) were analyzed using mixed-effects models (Proc GLIMMIX), with host treated as a fixed effect and block treated as a random intercept. For all variables, Levene's tests revealed unequal variances among some treatment groups, so treatment variances were grouped by block to account for heteroscedasticity (Littell et al. 2006). Variances were estimated using restricted maximum likelihood (REML) due to the unbalanced design, and degrees of freedom were approximated by the Kenward-Roger method (Spilke et al. 2005). Treatment means were estimated with least squares means (Spilke et al. 2005) and differences were separated by using Tukey-Kramer adjustments for multiple comparisons to maintain an overall $\alpha=0.05$ unless otherwise noted.

Survival measures were also analyzed using a mixed-effects model, but with a binomial distribution and logit link function (Proc GLIMMIX; events/trials syntax) to compare survival across hosts. Host was treated as a fixed effect, and block treated as a random effect. Degrees of freedom were estimated by using the Kenward-Roger method, and Tukey-Kramer groupings were used to compare differences in the least squares means (Littell et al. 2006). Before comparing host effects on freezing survival, an Abbott's correction (Rosenheim and Hoy 1989) was applied to the response to account for control mortality in each host treatment.

To evaluate relationships between host suitability and cold stress response, multiple-regression with backwards elimination was used to determine the explanatory power of each of the five developmental parameters measured to the response of proportion freezing survival (corrected for control mortality). Because we were interested in the broad impact of host suitability on partial-freeze tolerance, we did not distinguish between host species in this analysis. Instead, we treated the response from each host/block combination as a distinct host measure. Before model construction, potential correlations among candidate predictors were tested to ensure assumptions of regression were met. Of the five developmental parameters measured, mean pupal mass and mean total developmental duration were found to be negatively correlated ($P=0.016$, $F_{1,9}=8.68$). All other parameters were not significantly correlated with one another. So, two

regression models were constructed: the first initially included the three, stage-specific survival proportions and total development time, the second initially included the stage-specific survival proportions and pupal mass. Regressions were run as mixed-effect models (Proc GLIMMIX; events/trials syntax), with the developmental parameters treated as fixed effect, and block and block x host treated as random effects. Degrees of freedom were estimated by the Kenward-Roger method. The two reduced models included the same number of predictors, so a final model was selected based on best-fit (e.g., r^2).

2.4 Results

2.4.1 Supercooling and partial freeze tolerance

Host affected the supercooling points of larvae ($P=0.0003$, $F_{4,9.54}=15.47$). Larvae fed *M. domestica* had significantly higher supercooling points than those fed artificial diet, and larvae fed *Pi. banksiana* had significantly higher supercooling points than those fed *Po. deltoides*. At $\alpha=0.05$, comparisons among the other hosts were logically inconsistent; larvae fed *V. vinifera*, *Pi. banksiana*, and *Po. deltoides* had statistically equivalent supercooling points. At $\alpha=0.06$, this inconsistency was resolved. Average supercooling points of larvae reared on *M. domestica, V. vinifera*, and *Pi. banksiana* were not different and occurred at -11.67°C ±0.47 (mean ±SEM), -11.74°C ±0.83, -11.84°C ±0.45, respectively. Supercooling points of larvae reared on artificial diet (-15.23°C ±0.30) and *Po. deltoides* (14.09°C ±0.50) were not different from one another, but were significantly lower than all other hosts (Figure 2.1).

Survival to adult eclosion after partial freezing (i.e., exposure to the peak of the supercooling point exotherm) was also affected by host ($P<0.010$, $F_{4,6}=9.07$). The proportion of late instars that survived to adult eclosion after partial freezing was lowest when reared on *Po. deltoides* and artificial diet (0.078 ±0.058 and 0.22 ±0.055, respectively). In contrast, larvae reared on *Pi. banksiana* survived in greatest proportion (0.78 ±0.067). Those fed *M. domestica* and *V. vinifera* showed intermediate, and

statistically no different, partial-freeze tolerance to the other hosts (0.51 ±0.11 and 0.47 ±0.069, respectively) (Figure 2.2).

2.4.2 Host suitability: stage-specific survival, total immature development time, and pupal mass without freezing stress.

Host had a significant impact on the proportion of larvae that survived to be late instars ($P<0.0001$, $F_{4,6}=60.55$). Nearly all larvae survived to late instars when reared on artificial diet (0.98 ±0.012), whereas those fed *Pi. banksiana* had the lowest survival (0.36 ±0.13). Larvae fed *M. domestica, Po. deltoides, V. vinifera* survived in statistically equivalent proportions (0.86 ±0.076, 0.83 ±0.089, and 0.88 ±0.066, respectively) (Table 2.2). In contrast, host did not affect the survival of late instars to pupae ($P=0.08$, $F_{4,6}=3.60$), nor of pupae to adult eclosion ($P=0.65$, $F_{4,4.7}=0.65$) (Table 2.2).

Host affected pupal mass ($P=0.0009$, $F_{4,16.1}=8.07$). Artificial diet produced, on average, the heaviest pupae (37.8 ±1.0mg) whereas *V. vinifera* produced significantly lighter pupae (24.4 ±2.2mg). Insects fed *Pi. banksiana, Po. deltoides,* and *M. domestica* showed no difference in pupal masses from one another, or from the other two hosts (Table 2.2).

Host also affected the time from egg hatch to adult eclosion of *E. postvittana* ($P<0.0001$, $F_{4, 218}=49.68$). Insects fed *V. vinifera*, *Pi. banksiana* took an average of nearly 50 d to develop, whereas those fed artificial diet and *Po. deltoides* developed within an average of less than 37 d. *Malus domestica*-fed larvae developed for an intermediate duration of 39.13 (±4.05) d, which was not statistically different from either extreme group (Table 2.2).

2.4.3 Relationship between host suitability and cold stress response

The proportion of individuals that survived the onset of ice formation was positively correlated with mean total developmental time ($P=0.023$, $F_{1,9}=7.45$), and was negatively correlated with the proportion of larvae that survived from hatch to late instar ($P=0.055$, $F_{1,8.94}=4.89$). Host suitability, and by definition ecological fitness, decreased as developmental time increased, or larval survivorship decreased. Variation in the extent of

partial-freeze tolerance was better explained by variation in total developmental time (r^2 =0.49) (Figure 2.3) than larval survivorship (r^2 = 0.38).

2.5 Discussion

We documented a nearly ten-fold difference in the survival of *E. postvittana* larvae following exposure to freezing due to larval host (Figure 2.2), and most notably, the host-mediated effect followed predictions that link the fitness expressed in ideal temperature conditions to the likelihood of exhibiting one cold tolerance strategy over another (Voituron et al. 2002) (Figure 2.3). Our study involved one of the few existing examples of a partially freeze-tolerant species, a putative evolutionary intermediate between freeze avoidance and freeze tolerance (e.g., Hawes and Wharton 2010). When pulled at the peak of their supercooling point exotherm, –thereby exposing them to some, but not complete, ice formation – a proportion of *E. postvittana* larvae successfully go on to develop into reproductive adults (Morey et al. 2013, see Chapter 5). We observed this phenomenon infrequently in a laboratory population of *E. postvittana* reared on an artificial diet (Morey et al. 2013, see Chapter 5). However, the present study showed that partial-freeze tolerance is not restricted to an artificially formulated diet; partial-freeze tolerance markedly increased in nearly all natural host treatments compared to the control. The extent of partial-freeze tolerance greatly increased among certain host plants, specifically those hosts that induced the longest developmental time and allowed fewer larvae to reach late instars. Supercooling points also changed with host (Figure 2.1), and interestingly, changed in a direction expected if a shift towards freeze tolerance were to occur; generally, freeze tolerant species supercool at warmer temperatures than freeze avoidant species (Sømme 1982, Lee 2010). The least partially-freeze tolerant larvae, those reared on artificial diet and *Po. deltoides*, had the lowest mean supercooling points. Moreover, larvae fed *Pi. banksiana* had higher supercooling points and a significant increase in freezing survival.

Several studies have documented myriad effects of host plant quality and nutrition on the fitness of phytophagous insects (e.g., Mattson 1980, Awmack and Leather 2002,

Röder et al. 2008). In *E. postvittana*, specifically, host species is known to affect immature survival, developmental time, and pupal weight (Danthanarayana 1975b, Tomkins et al. 1989), which are common proxies of ecological fitness (Via 1990). However, little investigation extends to effects of host plant on insect cold tolerance. Here, we provide strong evidence that differences in developmental fitness afforded by diverse host plants relate to insect cold tolerance strategy in a predictable way. The counter-intuitive relationship we observed, where hosts of poorer developmental quality produced larvae with a higher tolerance to freezing, is likely driven by the metabolic costs associated with different cold tolerance strategies (Voituron et al. 2002). However, this does not preclude involvement of additional or alternative mechanisms. The nutritional content of the host plant may cause a direct impact on the cold tolerance response, in addition to indirect effects mediated through energy metabolism. Many of the biochemical mechanisms that protect insects from the damaging effects of cold depend upon the synthesis and accumulation of low molecular weight polyols (Storey and Storey 1991), sugars (Bale 2002), fatty acids (Koštál 2010), or proteins (Duman et al. 1991b). These products, or their precursors, are often accumulated as a result of the type and quality of the food being consumed. For phytophagous insects, especially those with a wide host range, cold tolerance could therefore differ substantially among individuals or populations, depending on the host consumed. The few studies that have addressed dietary effects on cold tolerance mechanisms predominantly focus on artificially augmenting specific components of insect nutrition, such as amino acids (Koštál et al. 2012), proteins (Andersen et al. 2010), cholesterol (Shreve et al. 2007), sugar (Colinet et al. 2012), and nitrogen (Lavy and Verhoef 1997), or on host effects of indirect measures of cold tolerance, such as diapause (Hunter and McNeil 1997) and through tri-trophic interactions (Li et al. 2014).

Even fewer studies directly assess cold tolerance under different host plant environments. Changes in supercooling points and cryoprotectant levels have been observed due to the effects of host plant (Gash and Bale 1985, Liu et al. 2007, Verdú et al. 2010, but see also Rochefort et al. 2011, Kleynhans et al. 2014). Trudeau et al. (2010) examined the impact of parental host plant on offspring development and overwintering survival in *Malacosoma disstria* (Hübner) eggs, and concluded that seemingly poor

quality hosts yielded the highest overwintering success. Contrastingly, Liu et al. (2009) found poor developmental hosts produced low overwintering survival in diapausing *Helicoverpa armigera* (Hübner) pupae. However, none of these studies were designed to address host influences on cold tolerance strategy.

Future directions of this work should include investigation into the specific molecular mechanisms that operate to increase partial-freeze tolerance through the host plant. Additionally, sub-lethal effects of increased partial-freeze tolerance should be assessed, and as consequence, what the prolonged fitness costs may be over time.

Our study offers exciting implications for both theoretical and practical areas of ecology. By demonstrating that an increase in partial-freeze tolerance is favored when initial fitness is low, we give experimental support to the larger hypothesis that complete freeze tolerance may also be favored in such conditions. We also provide further evidence of partial-freeze tolerance in an insect, characterizing its tolerance through extended survival measures. Applications of these findings are especially relevant to forecasts of population distributions and dynamics. As ectotherms, insects have life histories that are intimately linked to their surrounding temperature environments. Thus, temperature tolerances, especially to cold, constitute a primary variable in risk assessments and models of insect distributions. However, these tools currently treat invading species as “homogenous and immutable entities” (Lee 2002), being particularly void of adaptive parameters and plant-insect interactions. Climate change is exerting a powerful influence on the distributions of insects (Deutsch et al. 2008, Huey et al. 2012) and plants (Kelly and Goulden 2008, Chown et al. 2012) and will continue to. Accurately forecasting the impacts of a changing climate on pest species demands a clear understanding of what drives their current temperature tolerances, and especially their adaptive capacities. For phytophagous insects, it is clear that host plants could substantially mediate both.

Tables and Figures

Table 2.1: Larval hosts of *E. postvittana* used in each experimental block.

	Block 1	Block 2	Block 3
Artificial diet	x	x	x
Malus domestica			x**
Pinus banksiana	x*	x*	x**
Populus deltoides			x**
Vitis vinifera	x*	x*	x*

*Material from potted seedlings or cuttings grown in a greenhouse

**Material from stock planted in St. Paul, MN

Table 2.2: Summary of metrics used to define the suitability of five larval hosts of *E. postvittana*. Data were collected from individuals that did not experience cold stress. Means (±SEM) are presented as least squares estimates to account for an unbalanced mixed-effect design. Numbers in parentheses indicate total sample size across all blocks. Cells within a column with the same letter are not significantly different (P> 0.05).

Host	Proportion Survival**			Mean pupal mass (mg)	Mean total developmental time (d)*
	Hatch to late-instar	Late-instar to pupation	Pupation to adult eclosion		
Artificial diet	0.98 ±0.001 (186)[a]	0.99 ±0.0075 (96)[a]	0.95 ±0.048 (95)[a]	37.8 ±1.0 (94)[a]	36.5 ±0.8 (90)[b]
Malus domestica	0.86 ±0.076 (75)[b]	0.95 ±0.060 (26)[a]	0.71 ±0.33 (21)[a]	30.8 ±6.1 (18)[ab]	39.1 ±4.1 (15)[ab]
Pinus banksiana	0.36 ±0.130 (254)[c]	0.98 ±0.025 (33)[a]	0.97 ±0.039 (32)[a]	31.9 ±1.7 (32)[ab]	49.6 ±2.5 (31)[a]
Populus deltoides	0.83 ±0.090 (75)[b]	0.95 ±0.065 (24)[a]	0.74 ±0.32 (19)[a]	31.0 ±2.3 (18)[ab]	34.6 ±1.5 (14)[b]
Vitis vinifera	0.88 ±0.066 (198)[b]	0.92 ±0.094 (100)[a]	0.87 ±0.11 (87)[a]	24.4 ±2.2 (74)[b]	49.9 ±0.2 (73)[a]

*Time from egg hatch to adult eclosion; only measured for those that survived to adult eclosion

**Sample size indicates the number of individuals going into a given developmental period

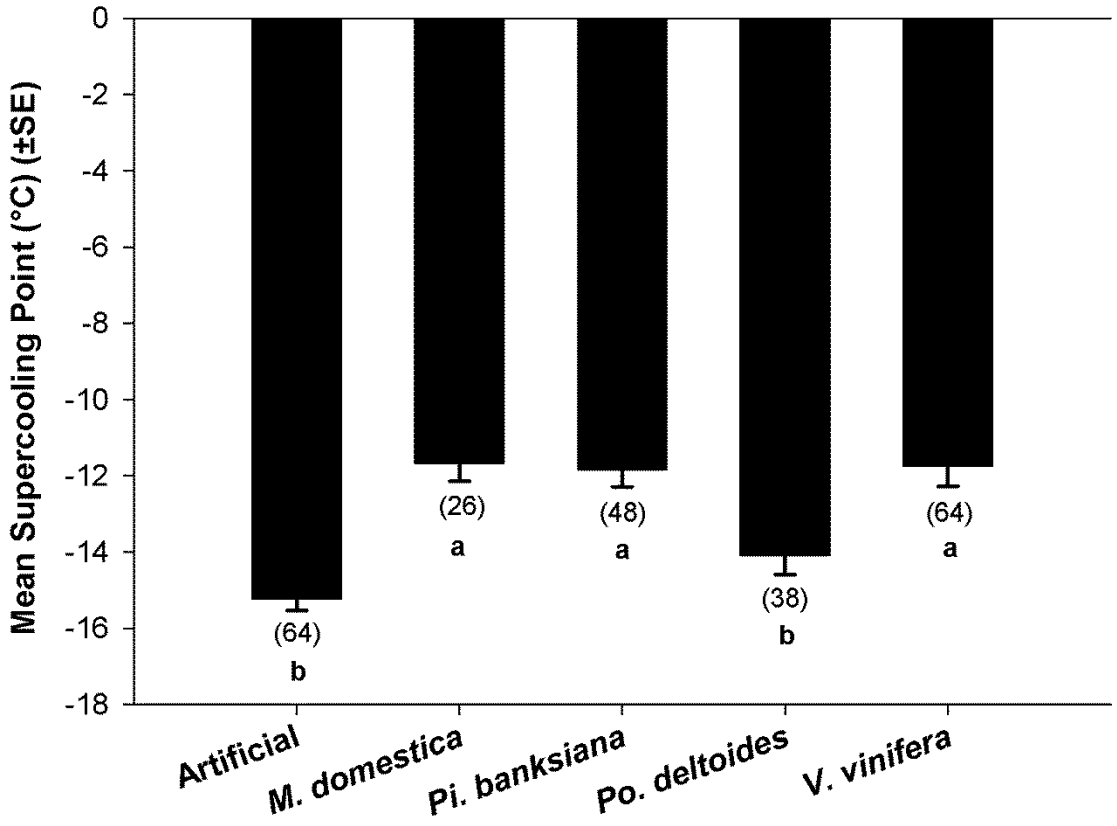


Figure 2.1: **Mean supercooling points (±SEM) of late instar *E. postvittana* reared on different hosts.** Least squares estimates are present to account for an unbalanced mixed-effects design. Bars with the same letter are not significantly different at α=0.06. Numbers in parentheses indicate the total sample size across all blocks.

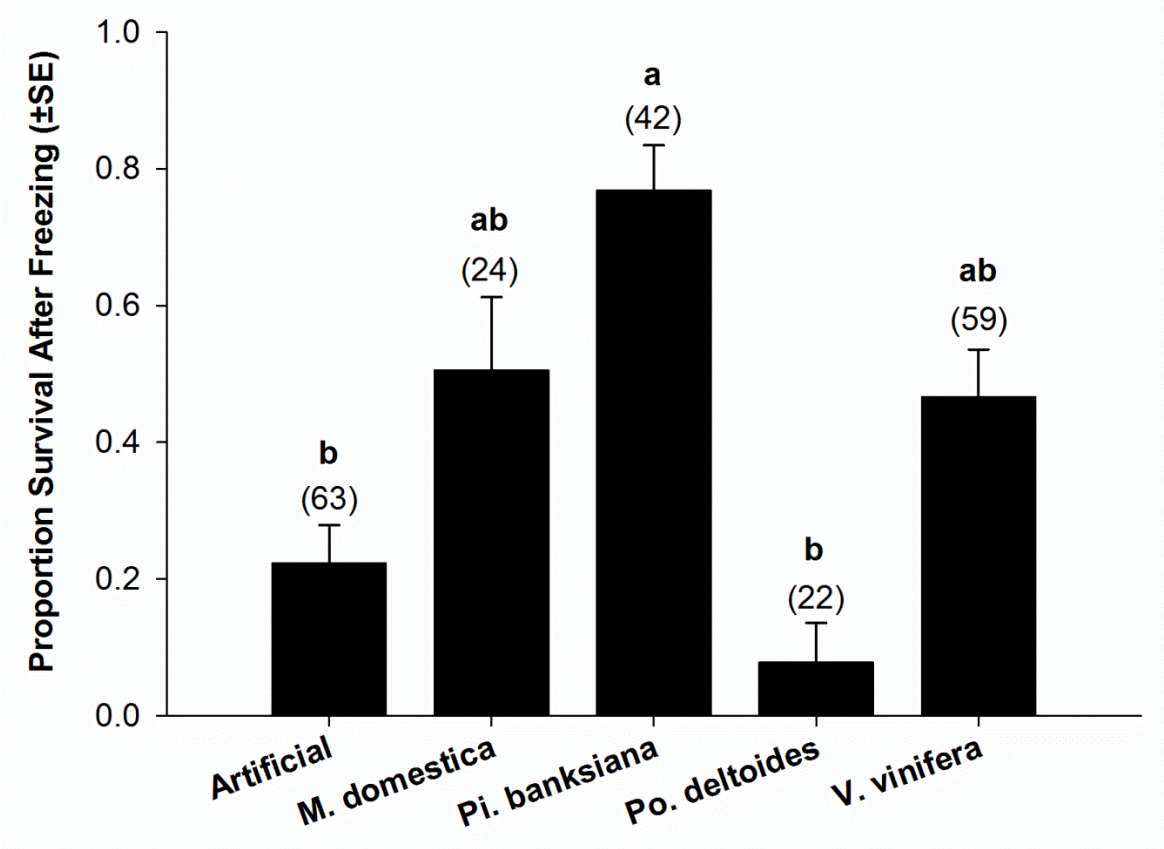


Figure 2.2: Mean proportion survival (±SEM) of late instar *E. postvittana* reared on different hosts following partial freezing. Survival was defined as successful adult eclosion. Bars with the same letter are not significantly different at α=0.05. Numbers in parentheses indicate the total sample size across all blocks.

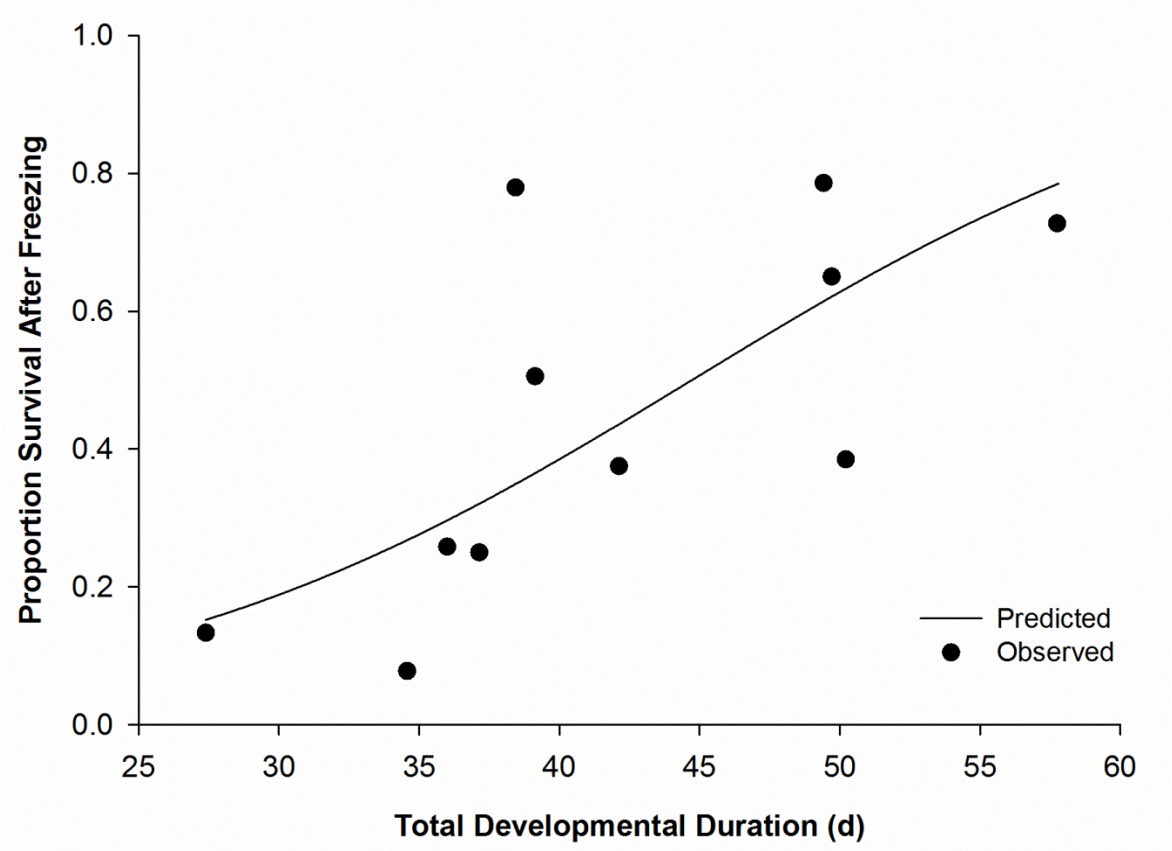


Figure 2.3: The effect of host quality (as represented by total developmental time for individuals without cold stress) on partial-freeze tolerance in late instars of *E. postvittana* (r^2 =0.49, $F_{1,9}$=7.45, P=0.023). The predicted line represents the results of a logistic regression. Points represent the proportion survival of each block within each host treatment.

CHAPTER 3:

Sublethal effects of subzero temperature on the light brown apple moth, *Epiphyas postvittana* (Walker): Fitness costs to surviving partial freezing

3.1 Summary

The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), a leafroller native to southern Australia, continues to invade new areas of California and Oregon in the U.S.A.. *Epiphyas postvittana* is highly polyphagous and threatens many agricultural and horticultural crops, with the potential to cause millions of dollars in damage annually. Damage estimates are sensitive to the final national geographic distribution of this pest. Current predictions assume the insect will not establish in areas with temperatures below -16°C, but do not account for sublethal effects of cold exposure. The potential for such effects could restrict the area of establishment or impact, but no prior studies have documented these effects in *E. postvittana*. We exposed late instars and pupae to cold temperatures and measured the direct and indirect effects of this exposure on subsequent survivorship, development, and reproduction. Cold exposure included -10°C (a low, but not immediately lethal temperature) and the supercooling point (the temperature at which internal fluids initiate freezing). Surviving insects were mated with untreated adults. We measured total immature development time, pupal mass, adult longevity, and fertility of treated females and mates of treated males. Nearly 20% of larvae and 8% of pupae were found to survive partial freezing and continue development to become reproductively viable adults. Importantly, there were significant fitness trade-offs to surviving partial freezing but not to exposure to -10°C; insects that survived partial freezing had lower fertility and shorter adult lifespans than either the -10°C or control group. Additionally, we measured the developmental effects (immature development time and pupal mass) of partial freezing in late instars reared on different host plants. Though we saw no clear sublethal effect that would correspond with the variable amount of survival after partial freezing seen across hosts, developmental measures may not be as well-suited for detecting fitness tradeoffs.

3.2 Introduction

In temperate regions, extreme low temperatures can be critical in defining the limits of an insect's geographic distribution (Overgaard et al. 2014). For exotic invasive species, risk-assessment and -management decisions often depend upon accurate characterizations of the effects of low temperature on a given species or population. Mechanistic models that forecast the distribution of exotic invasive species frequently rely on measures of direct mortality from low temperature. For example, distribution forecasts of the invasive moth, *Epiphyas postvittana* (Walker), define the impacts of extreme low temperature using only one or two short-term measures of mortality (e.g., the average supercooling point) (Fowler et al., 2009; Gutierrez et al., 2010). Importantly, though, effects on insects from low temperatures can be more subtle and manifest over time, as lethal and sublethal effects (Baust and Rojas 1985, Bale 1987, Lee 2010).

The current paradigm for the classification of insect cold tolerance describes the relationship between internal ice formation and mortality (Lee 2010). Most species are, broadly, *freeze-avoidant* because they die once ice forms within their bodies (i.e., are considered *freeze-intolerant*) or from the deleterious effects of non-freezing temperatures (i.e., are considered *chill-intolerant*). Other species can survive internal freezing under certain conditions (i.e., are considered *freeze-tolerant*). In addition, intermediate responses exist, such as individuals and/or populations displaying *partial-freeze tolerance* to varying levels of ice formation (Baust and Rojas 1985, Sinclair 1999, Hawes and Wharton 2010). This type of response includes individuals that cannot tolerate equilibrium freezing, but can survive some lesser degree of internal ice formation (Hawes and Wharton 2010).

Insect cold tolerance assessments are often based on laboratory measures of survival defined by direct mortality or lack of coordinated activity, assessed within hours or days of exposure (e.g., Nedvěd et al. 1998). Such designs may fail to capture mortality that occurs in later developmental stages or sublethal injury from cold that may more accurately reflect the impacts of cold exposure on a population (Baust and Rojas 1985, Hutchinson and Bale 1994, Renault et al. 2002). More recent work has evaluated sublethal effects of cold, particularly in the context of cold storage of biological control

agents and laboratory research stock. These studies reveal that numerous metrics of fitness may be affected by chronic (>24 h) cold stress, with sublethal effects typically increasing with decreasing temperature and/or increasing time (Chen et al. 2008, Ruan et al. 2012, Mockett and Matsumoto 2014; reviewed for parasitoids in Hance et al. 2007 and Colinet and Boivin 2011). Studies of more acute (<24 h), subzero exposure have seen similar impacts, such as reduced rates of development (McDonald et al. 1997), reduced fertility and reproduction (Coulson and Bale 1992, Hutchinson and Bale 1994), shorter adult lifespans, and increased incidence of adult malformations (Turnock et al. 1985). Reduced survivorship to subsequent developmental stages has also been cited as a sublethal effect (e.g., Turnock and Bodnaryk 1991, Yocum et al. 1994), though this could arguably be considered a lethal effect. Most studies evaluate sublethal effects of cold in freeze-avoidant insects and, with few exceptions (e.g., Fields and McNeil 1988, Layne and Blakeley 2002, Hawes and Wharton 2011), little is known about the fitness tradeoffs involved with tolerating internal ice formation.

Epiphyas postvittana is a highly polyphagous moth that is indigenous to southern Australia, and was documented in the contiguous United States in 2006 (Brown et al. 2010). Invasion of the U.S.A. by *E. postvittana* has potentially significant economic and ecological ramifications (Fowler et al. 2009), and early management responses were controversial (Lindeman 2013, Liebhold 2014). Thus, the potential distribution of this insect is of interest. The predominant overwintering stage in the U.S.A. is the late instar (Bürgi and Mills 2010), which slows development but does not enter diapause during winter (Geier and Briese 1981). However, more than one developmental stage may be present during the winter (Buergi et al. 2011). Bürgi and Mills (2010) classify late instars as “chill susceptible” (or chill intolerant) because larvae experience substantial mortality at temperatures above their supercooling point (Bale 1996). We have previously shown that a small proportion of late instars from a laboratory population exhibited partial freeze-tolerance, with some individuals surviving acute exposure to the onset of internal freezing and continuing to develop into reproductively-successful adults (Morey et al. 2013). Moreover, this phenomenon can vary greatly with larval host plant (Chapter 2). While partial-freeze tolerance affords an immediate fitness advantage over the freeze-

avoidant proportion of the population when temperatures are cold enough to initiate freezing, fitness tradeoffs for the survivors are currently unknown.

The purpose of our study was to examine the sublethal effects of acute low temperature exposure on *E. postvittana*. First, we compared the effects of brief, subzero temperature exposures on an ecologically-relevant measure of survival (i.e., adult eclosion), and on five measures of fitness (i.e., sublethal effects). Fitness was characterized by immature development times for larvae and/or pupae, pupal mass, adult longevity, and fertility in survivors. Low temperature was applied as either a subzero, but not immediately lethal temperature (-10°C, based on Morey et al. 2013 (Chapter 5)), or as a subzero temperature immediately lethal to the majority of the population (i.e., supercooling points). We examined effects when individuals were exposed as late instars and pupae, with pupae included as comparison of an alternate overwintering stage. Secondly, based on our previous findings that larval host could significantly alter the prevalence of partial-freeze tolerance in this species (see Chapter 2), we measured whether host also induced a concomitant change in the sublethal effects following variable freezing survival. We hypothesized that any change in a given measure across hosts would be a reduction in fitness for partially freeze-tolerant individuals.

3.3 Materials and Methods

Experiment 1: Sublethal effects of low temperature on larvae and pupae

3.3.1 Colony source

Epiphyas postvittana eggs were obtained from a laboratory colony maintained by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) in Albany, CA, founded from wild California moths in 2007. All subsequent handling and experimentation was conducted in a Biosafety Level 2 Containment Facility in St. Paul, MN (APHIS permit P526P-14-03759). Eggs were surface-sterilized in a 1% bleach solution, and held at 23 ±2°C, 60-65% RH, 14:10 (L:D) inside two environmental chambers (Percival Scientific, Perry, IA and Conviron, Winnipeg, Canada) until hatch.

Within 24 h of hatching, *E. postvittana* neonates were placed in groups of 3-4 onto ~2cm^3 cubes of artificial diet inside sealed containers (29.5 mL P100 soufflés; Solo Cup Co., Lake Forest, IL) and reared until late instars (4-6th) or pupae. The diet used great northern beans (*Phaseolus vulgaris* L.) and followed a modified formulation developed by Follet and Lower (2000) for *Cryptophlebia* spp. (Lepidoptera: Tortricidae).

3.3.2 Low temperature exposure

The experiment followed a randomized complete block design, with one block in March 2012 and another in June 2012. For larval-exposure treatments, instar was confirmed through head capsule measurement (Danthanarayana 1975a), and late instars (4th-6th) were randomly assigned to one of three temperature treatments; cooled to -10°C (across blocks, n=87), cooled to the production of an exotherm (i.e., the supercooling point, n=87), or held at room temperature (i.e., control, n=44). Sample sizes for controls were smaller than treatment groups due to specimen limitation.

Larvae were transferred to individual gelatin capsules (size 4; 14.3mm length, 5.1mm diameter) and cooled at ~1°C/min to the desired temperature within calibrated ploystyrene cubes inside a -80°C freezer, as per Carrillo et al. (2004). Temperatures were recorded once per second by using the coiled, copper-constantan thermocouple design of Hanson and Venette (2013) connected to a computer through a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Each larva was immediately removed from the freezer and cube once they reached either -10°C or the peak of their supercooling point exotherm; the exotherm peak was chosen because it was the point that guaranteed some ice formation, but could also be applied most consistently to all individuals. Larvae were then removed from their gelatin capsules and placed individually on a fresh cube of diet to continue development in a growth chamber (23 ±2°C, 60-65% RH, 14:10 (L:D)). Control individuals were left inside gel capsules at room temperature (~25°C) for approximately 1 h while cold-treated individuals were being chilled. Controls were removed from the capsules and given fresh diet concomitantly with the cold-treated larvae.

Individuals were monitored daily for pupation, adult eclosion, and/or death. Pupal mass was measured 3- 5 d after pupation to reduce damage to pupae from handling.

Survival was defined by successful eclosion as an adult. For survivors, larval duration (hatch to pupation), pupal duration (pupation to adult eclosion), adult longevity, and fertility were recorded. Sex of adults was determined from wing coloration and the presence (male) or absence (female) of a forewing costal fold (Brown et al. 2010). Fertility was measured as the total number of viable eggs produced as evidenced by the presence of a larval head-capsule within the egg (i.e., the presence of "black-heading") from a treatment-exposed female mated with an unexposed male, or from an unexposed female mated with an exposed male. Treatment-exposed individuals were provided with sugar-water (10% honey/water solution) within 1d after eclosion and placed with a mate within 2d after eclosion. Unexposed individuals used for mating were reared in tandem with the treatment insects, but were not handled until pupation. They were weighed as pupae and mated with an exposed individual within 2d after eclosion, and mated with an individual from one of the three treatment groups within 2d after eclosion.

For pupal-exposure treatments, pupae were weighed 3-5 d after pupation and exposed to -10°C (across blocks, n= 75), the supercooling point (n=76), or used as control (n=38) as described above for larvae. For survivors, pupal duration (pupation to adult eclosion), adult longevity, and fertility were measured. Larval duration and pupal mass were not analyzed because they could not be affected by cold exposure in this group.

3.3.3 Analysis

Statistical analysis was conducted in SAS® 9.4 (SAS Institute Inc., Cary, NC). All variables were analyzed using generalized linear mixed models (Proc GLIMMIX) with block treated as a random effect. When the response variable was supercooling point, pupal duration, or fertility, temperature treatment, sex, and stage of exposure (larva or pupa) were treated as fixed effects. For larval duration and pupal mass, only temperature treatment and sex were used as fixed effects; a stage effect was not included because these two measures were only analyzed for larval-exposed groups. Supercooling points and pupal mass were tested for homoscedasticity among treatment groups by using the Brown-Forsythe test (SAS/STAT® 9.22 User's Guide) to meet assumptions of ANOVA. Pupal masses were $\log_{10}$-transformed to achieve equal variances; means and

confidence intervals are presented as back-transformed values. Fertility counts were analyzed using a negative binomial distribution and log link (Stroup 2014). Immature developmental times and adult longevity were analyzed using a gamma distribution and log link (Manly 1989). Degrees of freedom were approximated by the Satterthwaite method (Spilke et al. 2005). Treatment estimates were obtained using the inverse link function (Littell et al. 2006) and differences were separated ($P<0.05$) by using Tukey-Kramer adjustments for multiple comparisons.

Survival was also analyzed using a generalized linear mixed model, but with a binomial distribution and logit link function (Proc GLIMMIX; events/trials syntax) to compare survival across temperature exposures. Temperature and stage of exposure were treated as fixed effects and block was treated as a random effect. Sex was not included as a fixed effect in this analysis because the sex of individuals that died before adult eclosion could not be determined. Degrees of freedom were estimated by the Satterthwaite method, and Tukey-Kramer groupings were used to compare differences in the least squares means. Treatment estimates were obtained using the inverse link function (Littell et al. 2006).

Experiment 2: Sublethal effects of freezing on larvae reared on different hosts

3.3.4 Insect colony and plant materials

Chapter 2 provides detailed descriptions of larval handling and plant materials. In brief, larvae of *E. postvittana* were reared from neonate to late instars on artificial diet, or leaves of *Vitis vinifera* L. (var. 'Frontenac'), *Malus domestica* Borkh. (var. 'Zestar!'), *Pinus banksiana* Lamb., or *Populus deltoides* ssp. *monilifera* (Aiton) Eckenwalder (var. 'Siouxland'). Individuals were only tested as late instars in this experiment.

3.3.5 Low temperature exposure

Experimental design and cold-exposure methods followed the description in Chapter 2. For each diet, late instars were exposed to their supercooling point or held at 23°C (control). Activity was monitored daily and survival was defined by successful adult eclosion. Survivors were sexed and immature development times (i.e., hatch to pupation and pupation to adult eclosion) and pupal mass (3-5 d after pupation) were

recorded. Adult longevity and fertility were not assessed due to logistical constraints (e.g., specimen and space limitations). Therefore, once moths emerged, sex was determined as described above, and the moths were disposed of. The number of larvae in each temperature treatment depended on the mortality of early instars from a given host. A total of 8-32 larvae were, therefore, tested per temperature treatment (n=2) per host (n=5) per block (n=3), but not all hosts were represented in each block (see Table 2.1 in Chapter 2). For the cold-exposed groups, specifically, the total number of late instars tested in each host, across all blocks, were: artificial diet, n= 64; *M. domestica*, n= 26; *Pi. banksiana*, n=48, *Po. deltoides*, n=38; *V. vinifera*, n=64.

3.3.6 Analysis

The effects of exposure to partial-freezing on pupal mass, immature development times, and survival were analyzed using generalized linear mixed models (Proc GLIMMIX) as in Experiment 1. Pupal masses were transformed with a natural log to achieve equal variances. The fixed effects in each model included temperature (supercooled or control), sex, and host (artificial diet, *M. domestica, V. vinifera, Pi. banksiana,* and *Po. deltoides*). Due to the unbalanced design, degrees of freedom were approximated by the Kenward-Roger method (Spilke et al. 2005).

3.4 Results

Experiment 1: Sublethal effects of low temperature on larvae and pupae

3.4.1 Survival and supercooling points

Temperature exposure had a significant impact on the proportion of insects that survived to adult eclosion ($P<0.0001$, $F_{2, 6}=65.84$), irrespective of which developmental stage was exposed (stage: $P=0.38$, $F_{1, 6}=0.90$; temp x stage: $P=0.49$, $F_{2, 6}=0.80$). The proportion of individuals that survived to adulthood was not different among insects exposed to -10°C (0.98 ±0.014; mean ± SEM) and those held at room temperature (0.98 ±0.018), but a significantly smaller proportion (0.10 ±0.04) survived exposure to their supercooling point (Figure 3.1a). The mean supercooling point for late instars was -14.13

(±0.35 SEM)°C, which was higher ($P<0.0001$, $F_{1, 139}=76.04$) than for pupae (-19.04 ±0.44°C) (Table 3.1).

3.4.2 Pupal mass (larval-exposed group only)

Exposure temperature did not affect the mass of pupae that survived to adult eclosion ($P=0.71$, $F_{2, 142}=0.34$). Females had significantly ($P<0.0001$, $F_{1, 142}=624.47$) greater mass (48.35 mg; 95% CI=46.77-49.99) that males (25.98 mg; 95% CI=25.07-26.92; Figure 3.1c). The interaction between temperature and sex was also significant ($P=0.03$, $F_{2, 142}=3.79$).

3.4.3 Immature developmental times

For individuals exposed to cold as larvae, exposure temperature did not affect the total time spent as a larva (egg hatch to pupation) ($P=0.58$, $F_{2, 139}=0.55$), typically lasting ~23 d. However, larval duration was different for males and females ($P<0.0001$, $F_{1, 139}=28.62$), with males taking an average of 22.3 d (±0.77 SEM) and females an average of 24.1 d (±0.84) (Figure 3.1b.). The interaction between exposure temperature and sex was not significant ($P=0.12$, $F_{2, 139}=2.13$).

Similarly, the time required for pupation in survivors also differed significantly between sexes ($P=0.0038$, $F_{1, 250.1}=8.56$) with males taking an average of 9.1 d (± 0.26) and females 8.6 d (± 0.29) (Figure 3.1d). There was no effect of exposure temperature ($P=0.23$, $F_{2, 250.2}=1.47$), stage of exposure ($P=0.62$, $F_{1, 250}=0.25$), or the interactions between main effects ($P>0.6$) on pupal duration.

3.4.4 Adult longevity

Exposure temperature affected adult longevity ($P<0.0001$, $F_{2, 243.4}=25.45$), irrespective of the stage at which exposure occurred (temp x stage: $P=0.20$, $F_{2, 243.2}=1.63$). Adult longevity was statistically the same for individuals exposed to -10°C (22.5 ±1.3 d) and the control (22.1 ±1.6 d), whereas individuals exposed to partial-freezing lived significantly fewer days as adults (14.4 ±2.1 d) than the other temperature groups (Figure 3.1e). The interaction of sex with temperature was marginally significant ($P=0.052$, $F_{2, 243.2}=2.98$), and suggested that male longevity may be more reduced than females

following supercooling point exposure (Figure 3.1e). Adult longevity was not affected by the stage of exposure ($P=0.27$, $F_{1,243}=0.27$) or sex alone ($P=0.20$, $F_{1, 243.2}=1.64$).

3.4.5 Fertility

Temperature exposure had a significant impact on the number of viable eggs produced when an exposed and unexposed individual were mated ($P=0.0007$, $F_{2, 185}=7.52$), irrespective of the sex of the exposed individual (temp x sex: $P=0.86$, $F_{2, 185}=0.15$) or the developmental stage at which they were exposed (temp x stage: $P=0.65$, $F_{2, 185}=0.44$) (Figure 3.1f). Pairs with one mate that was exposed to their supercooling point had the lowest average fertility (299.86 ±67.40 eggs). Pairs with one mate that was exposed to -10°C produced more viable eggs (546.43 ±44.0 eggs), which was not significantly different than the average fertility of control pairs (713.82 ±86.71 eggs) (Figure 3.1f). The production of viable eggs was not affected by the stage of exposure ($P=0.20$, $F_{1, 185}=1.67$) or sex of the exposed individual ($P=0.55$, $F_{1, 185}=0.36$).

Experiment 2: Sublethal effects of freezing on larvae reared on different hosts

3.4.6 Survival and supercooling points

A significantly smaller proportion of individuals survived exposure to the supercooling point than the controls for nearly all hosts ($P<0.0001$, $F_{1, 12}=51.64$). Larvae fed leaves of *M. domestica* showed a marginal reduction in survival ($P=0.069$, $F_{1, 12}=3.99$), whereas all other host treatments showed significantly less survival following the onset of freezing (Figure 3.2a). However, host also affected survival ($P=0.0053$, $F_{4, 12}=6.41$), and the proportion of individuals that survived supercooling point exposure (i.e., exhibited partial-freeze tolerance) and control conditions was highly variable across hosts (temperature x host; $P=0.011$, $F_{4, 12}=5.29$) (Figure 3.2a). Some larvae from each host were partially-freeze-tolerant, but larvae that had developed on *Pi. banksiana* showed the greatest proportion of partial-freeze tolerance and larvae fed *Po. deltoides* the lowest (Chapter 2). Refer to Chapter 2 for further details of partial-freeze tolerance among host groups. A summary of the supercooling points has been reprinted from Chapter 2 in Table 3.1.

3.4.2 Pupal mass

Partial-freezing had a significant effect on the pupal mass of insects that survived to adult eclosion within some host treatments, depending on the sex (host x temp x sex: P=0.0018, $F_{2, 251.4}$=6.46) (Figure 3.2c). Females fed artificial diet or *Pi. banksiana* as larvae weighed less as pupae after surviving partial-freezing than corresponding control females (P=0.015, F=5.95$_{1, 251.3}$ and P<0.0001, F=15.65$_{1, 251.7}$, respectively) (Figure 3.2c). An analysis of simple effects (Winer 1971) showed that these *Pi. banksiana*-fed females weighed significantly less than the artificial diet-fed females (P=0.008, t=3.01, df=251.6). No females survived to adult eclosion following partial-freezing in the *Po. deltoides* group and only one female survived partial-freezing in the *M. domestica* group (47.2 mg); these two groups were therefore not included in the analysis. Females fed *V. vinifera* as larvae showed no effect of temperature treatment on pupal mass (P=0.15, F=2.09$_{1, 251.4}$). Similar to females, male larvae fed artificial diet weighed less following partial-freezing than in the control (P=0.022, $F_{1, 251.9}$=5.30). Only one male survived partial-freezing in the *Po. deltoides* group (31.7 mg), so was not included in the analysis. There were no differences between the pupal masses of partially freezing tolerant males and control males within the remaining host groups (Figure 3.2c). All three main effects were significant (temperature: P=0.0008, $F_{1, 250.9}$=11.63; sex: P<0.0001, $F_{1, 252}$=215.75; host: P<0.0001, $F_{4, 195.5}$=24.25), but none of the 2-way interactions between main effects were (P>0.07).

3.4.8 Immature developmental times

Exposure temperature did not affect the total time spent as a larva (P=0.94, $F_{1, 269}$=0.00). However, host had a significant effect on larval duration (P<0.0001, $F_{4, 269.7}$=61.92) and females took significantly longer to pupate than males (P=0.0025, $F_{1, 269.1}$=9.32) (Figure 3.2b). Females reared on *V. vinifera* and *Pi. banksiana* took the longest to pupate (42.2 ±3.6 and 41.3 ±3.5 d, respectively), whereas those reared on artificial diet and *Po. deltoides* took the shortest time (24.7 ±2.1 and 23.2 ±3.1 d, respectively) (Figure 3.2b). Similarly, males reared on *V. vinifera* and *Pi. banksiana* took significantly longer to pupate (42.2 ±3.6 and 41.3 ±3.5 d, respectively; mean ± SEM), than those reared on *M. domestica*, *Po. deltoides*, and artificial diet (25.1 ±2.4, 22.7 ±2.7,

and 21.4 ±1.9, respectively) (Figure 3.2b). All two- and three-way interactions between temperature, host, and sex were not significant (P>0.3).

Similarly, the total time spent as pupae for insects that survived to adulthood also differed significantly between sexes (P=0.0098, $F_{1, 271}$=6.77) with males taking an average of 9.3 d (±0.3) and females 8.8 d (±0.3) (Figure 3.2d). Neither exposure temperature (P=0.96, $F_{2, 270.2}$=0.00), host (P=0.94, $F_{4, 268.1}$=0.20), nor the interactions between main effects (P>0.5) affected pupal duration.

3.5 Discussion

As noted by Hawes and Wharton (2011), the interplay of fitness with insect low temperature responses "has received wide recognition, but relatively less actual examination". Our work expands the otherwise limited body of empirical data that documents fitness tradeoffs associated with different insect cold tolerance responses. *Epiphyas postvittana* responds to subzero temperature in two ways; it is predominantly freeze-avoidant in that most insects succumb to temperatures near the supercooling point (< -10°C), but a small proportion is also partially freeze-tolerant and survives brief exposure to internal ice formation (i.e., the peak of their supercooling point exotherm) (Figure 3.1a). This apparent mixed response appeared in late instars and to a slightly lesser extent in pupae. Importantly, partial freeze-tolerance was defined by using an extended measure of survival, requiring successful adult emergence to occur. Previous work showed that if individuals exposed to their supercooling point could survive to adult eclosion, viable offspring could be produced (Morey et al. 2013). However, the relative reproductive output of a partially-freeze tolerant population to an unstressed population was unknown.

Given this putative mixed low temperature response, we were able to compare sublethal effects resulting from non-freezing, subzero temperatures to those resulting from partial-freezing. Brief exposure to a low, but not immediately lethal subzero temperature (-10°C) did not appear to affect *E. postvittana* late instars or pupae differently from control individuals, either in mortality or sublethal effects (Figure 3.1).

Other studies involving freeze-avoidant species have observed negative effects on subsequent development and/or reproduction following exposure to subzero, non-freezing temperatures (Turnock et al. 1985, Hutchinson and Bale 1994, Colinet and Boivin 2011). Since most of the *E. postvittana* population is freeze-avoidant, a possible explanation for the lack of sublethal effects seen in our study is that our design involved only acute exposure to -10°C; deleterious effects at this temperature may manifest with longer exposures.

In contrast to -10°C exposure, partial-freezing elicited fitness costs to survivors. For insects exposed as either late instars or pupae, these costs included shorter adult lifespans (Figure 3.1e) and a decrease in the production of viable offspring when mated with an untreated individual (Figure 3.1f). Adult longevity appeared to be more severely reduced for partially freeze-tolerant males than females, though this was only marginally significant. We structured our mating design to test for potential maternal- (Watson and Hoffmann 1996) or paternal-specific (Costanzo et al. 2015) effects of cold exposure on fertility, but the sex of the exposed individual did not appear to differentially impact fertility. Pupal mass and immature durations did not appear to be affected by either cold exposure, but did reflect the expected sexual dimorphism of *E. postvittana* in these developmental parameters (Figure 3.1b-d) (Danthanarayana 1975a). The sublethal consequences of freezing in insects are much less explored than those for freeze-avoidant species, with measures of reproductive costs being nearly absent from the literature (Hawes and Wharton 2011). While our study was not designed to explore the potential of complete freeze-tolerance in *E. postvittana*, demonstrating the presence of fitness trade-offs from partial freezing nonetheless underscores the importance of considering sublethal measures in cold tolerance studies. Even when assessments of lethality are based on more ecologically meaningful timescales (e.g., successful progression to the next developmental stage), the effect of cold on survival may not reflect the ultimate fitness of an individual (Renault 2011).

Partial freeze-tolerance was also observed in all insects reared on various natural host plant tissues (Figure 3.2a). Despite dramatic differences in partial freeze-tolerance due to host (see Chapter 2 for discussion), there was not a clear effect of partial freeze-tolerance on any of the three fitness parameters measured within a given host (Figure

3.2). Our expectation was that if an effect of partial freeze-tolerance on fitness (within a host) was observed, it would; (1) be a reduction in the fitness parameter relative to the control, and (2) the severity of reduction would be greater in hosts that showed more partial-freeze tolerance relative to hosts with less partial freeze- tolerance. This hypothesis assumed that if tolerating ice formation comes with an energetic cost (Voituron et al. 2002), there would be a tradeoff with other energy-demanding processes (Stearns 1989) such as those involved in subsequent development.

We observed a potential tradeoff in pupal mass with partial freeze-tolerance, though not consistently across hosts (Figure 3.2c). Partial freezing reduced the mass of females fed *P. banksiana* and both males and females fed artificial diet compared to their respective controls. Of note, within partially freeze-tolerant females, those fed *P. banksiana* (which was the host that exerted the highest proportion of partial freeze-tolerance) had a lower mean mass than partially freeze-tolerant females reared on artificial diet (which was the host that showed one of the lowest levels of partial-freeze tolerance). Unexpectedly, we did not observe an effect of partial freeze-tolerance on pupal mass in Experiment 1 for late instars, which would have been expected to mirror the responses associated with artificial diet treatment in Experiment 2. While the experiments were done at different times, there were no obvious differences in the methods or equipment to explain such a change in results. We suspect the discrepancy is likely an issue of sample size; the sample sizes within the supercooled treatments in both experiments were small and therefore may not have adequate power to consistently detect differences.

Partial freeze-tolerance showed no impact on immature developmental times for any host. The differences in larval and pupal durations among hosts (Figure 3.2b, d) reflect the variation in developmental suitability of a given plant, and also the expected differences in developmental times between males and females (Danthanarayana 1975a) as found in Experiment 1. Unfortunately, we were unable to include any direct measure of reproductive fitness (i.e., adult longevity and fertility) in Experiment 2. Based on the results in Experiment 1, reproductive fitness could be more likely than developmental parameters to reflect sublethal effects from partial-freeze tolerance. Pupal mass can be positively correlated with reproductive fitness in female insects (e.g., Moreau et al.

2006), so the loss of female pupal mass with partial-freezing in some hosts may suggest similar reproductive tradeoffs.

Current risk maps that exist for *E. postvittana* (e.g., Fowler et al. 2009, Gutierrez et al. 2010, Lozier and Mills 2011) acknowledge the importance of cold in shaping this species' potential U.S. range. However, the parameter(s) used to describe cold tolerance reflect short-term assessments of lethal responses to low temperatures and do not account for downstream attrition of fitness, including potential trade-offs in populations with variability in cold response. Further studies are needed to assess the extent and relevance of partial-freeze tolerance to *E. postvittana* in the field. If our laboratory findings reflect a natural population, even low levels of partial-freeze tolerance may have implications for the distribution limits of invading populations over time (Morey et al. 2013). However, we show here that partial-freeze tolerance may not be without consequence for organismal fitness. A more cold tolerant population may enable expansions of the invading population front during periods of cold stress (e.g., winter), but this potential could be limited by trade-offs in overall fitness (e.g., Blows and Hoffmann 1993, Watson and Hoffmann 1996), particularly if there is significant gene flow among populations during periods of non-cold stress (Jenkins and Hoffmann 1999). Including parameters that incorporate potential fitness costs of cold tolerance, especially for polyphagous species with a variable cold response, may be particularly useful to refine models of geographic spread and establishment of invasive pests, and thus increase accuracy of economic and ecological risk.

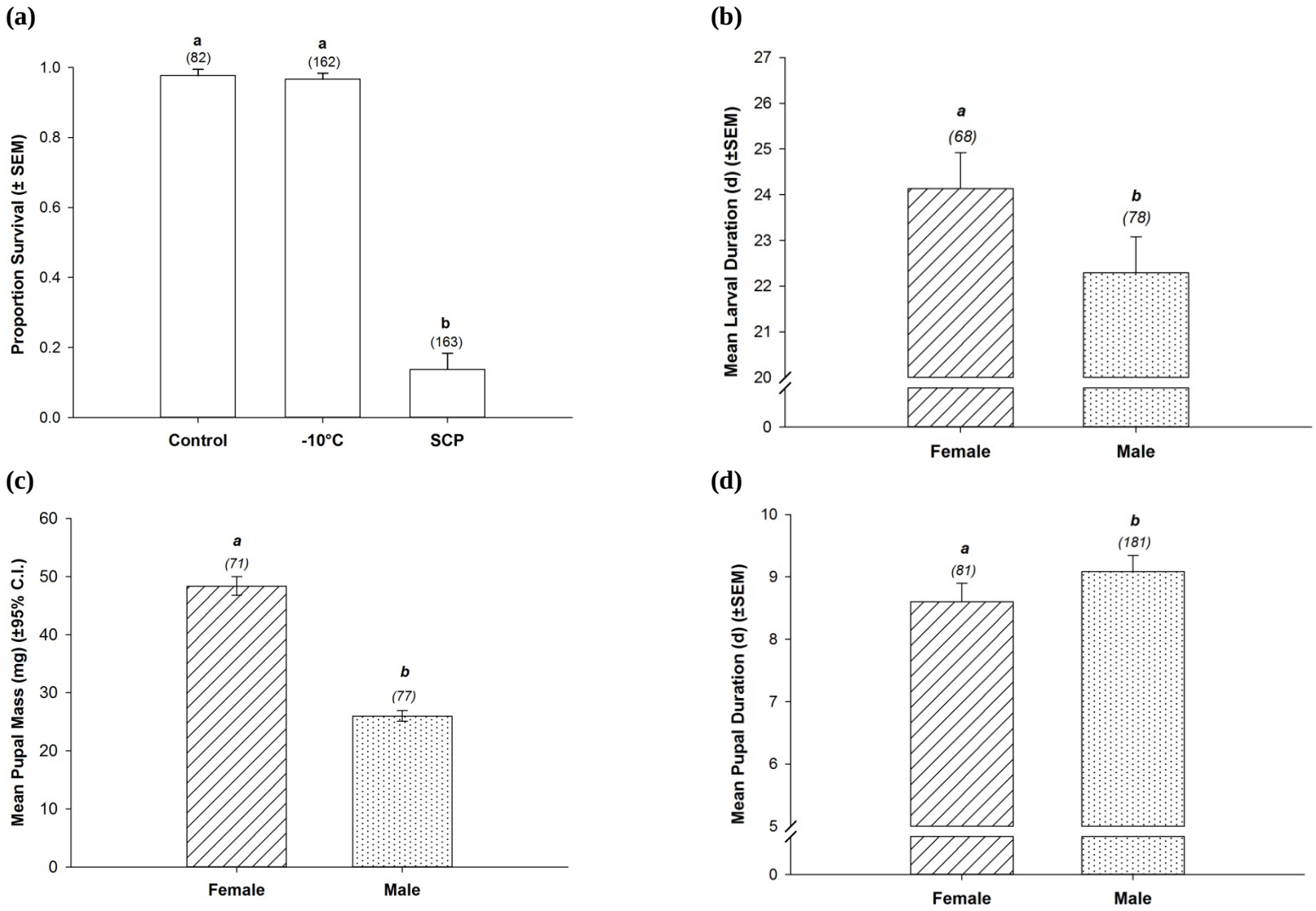
Tables and Figures

Table 3.1: Summary of supercooling points for *E. postvittana* used in both experiments. *n* is the total sample size across all blocks. Means and standard errors are presented as least squares. Means with different letters within an experiment are significantly different (GLMM; Tukey's). Values from Experiment 2 are reprinted from Chapter 2.

	Mean (°C)	SEM	*n*
Exp.1*			
Late instars	-14.13[a]	0.37	86
Pupae	-19.04[b]	0.23	76
Exp.2**			
Artificial diet	-15.23[a]	0.30	64
Malus domestica	-11.67[b]	0.47	26
Pinus banksiana	-11.84[b]	0.45	48
Populus deltoides	-14.09[ab]	0.50	38
Vitis vinifera	-11.75[b]	0.53	64

* all insects were reared on artificial diet, but supercooled at two developmental stages

** all insects were supercooled as late instars, but reared on five different hosts.


(a)
Proportion Survival (± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
a
(82)
a
(162)
b
(163)
Control
-10°C
SCP
(b)
Mean Larval Duration (d) (±SEM)
27
26
25
24
23
22
21
20
0
a
(68)
b
(78)
Female
Male
(c)
Mean Pupal Mass (mg) (±95% C.I.)
60
50
40
30
20
10
0
a
(71)
b
(77)
Female
Male
(d)
Mean Pupal Duration (d) (±SEM)
10
9
8
7
6
5
0
a
(81)
b
(181)
Female
Male

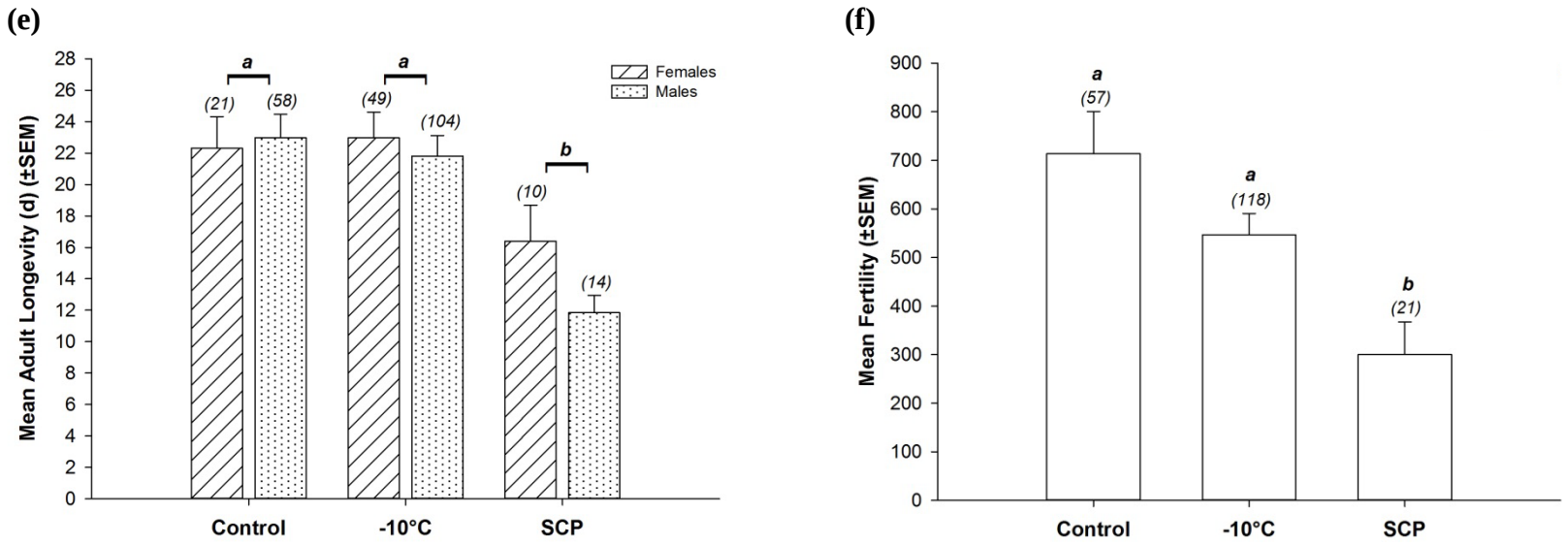
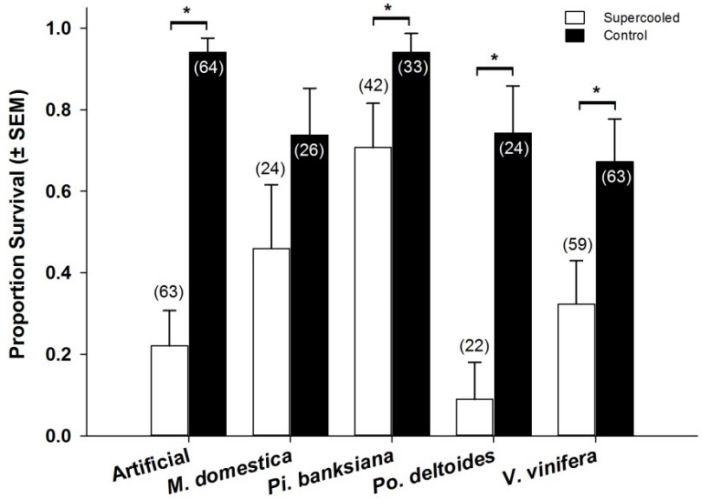
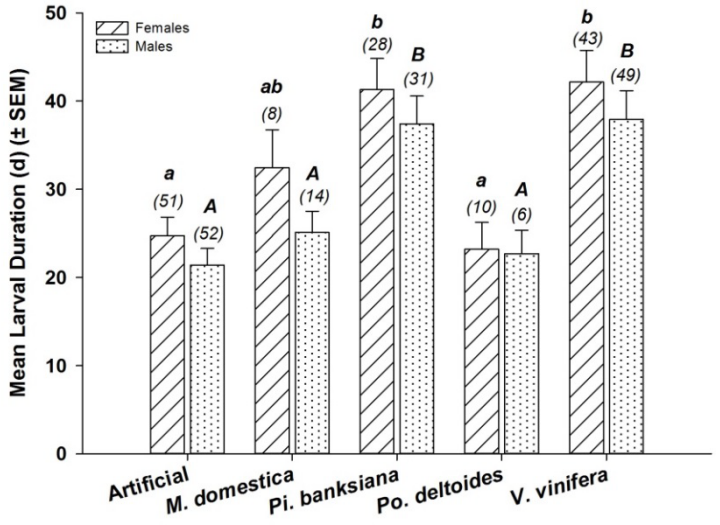


Figure 3.1: **(a)** Proportion survival, **(b)** pupal mass, **(c)** larval duration (hatch to pupation), **(d)** pupal duration (pupation to adult eclosion), **(e)** adult longevity, and **(f)** fertility following low temperature exposure of *E. postvittana* late instars (4-6th) and pupae to -10°C, their supercooling point (SCP), or contol conditions (23°C). For pupal mass, mean and confidence intervals are presented as back-transformed values. Estimates for sex and exposure stage within temperature groups are only displayed where significant main effects or interactions (P>0.05) occurred. Numbers in parentheses indicate sample size across all blocks. Different letters indicate significant differences across groups.

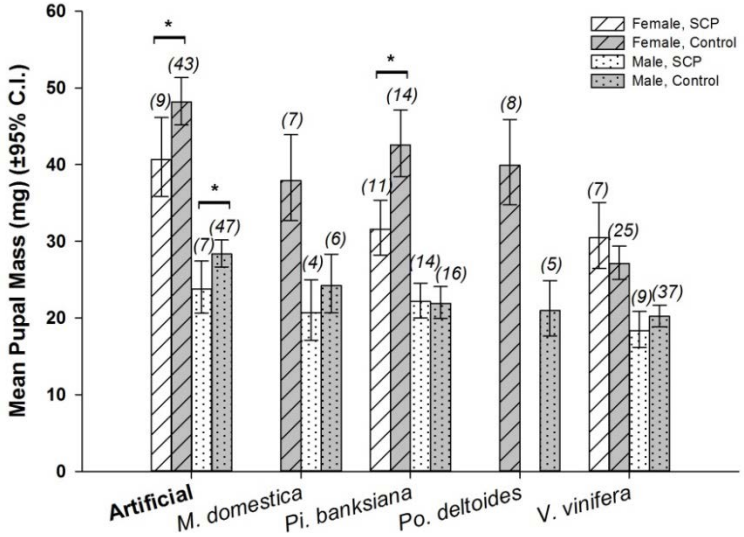
(a)


Proportion Survival (± SEM)
1.0
0.8
0.6
0.4
0.2
0.0
Supercooled
Control
*
(63)
(64)
(24)
(26)
(42)
(33)
(22)
(24)
(59)
(63)
Artificial
M. domestica
Pi. banksiana
Po. deltoides
V. vinifera

(b)


Mean Larval Duration (d) (± SEM)
50
40
30
20
10
0
Females
Males
a
(51)
A
(52)
ab
(8)
A
(14)
b
(28)
B
(31)
a
(10)
A
(6)
b
(43)
B
(49)
Artificial
M. domestica
Pi. banksiana
Po. deltoides
V. vinifera

(c)


Mean Pupal Mass (mg) (±95% C.I.)
60
50
40
30
20
10
0
Female, SCP
Female, Control
Male, SCP
Male, Control
*
(9)
(43)
*
(7)
(47)
(7)
(4)
(6)
*
(11)
(14)
(14)
(16)
(8)
(5)
(7)
(25)
(9)
(37)
Artificial
M. domestica
Pi. banksiana
Po. deltoides
V. vinifera

(d)

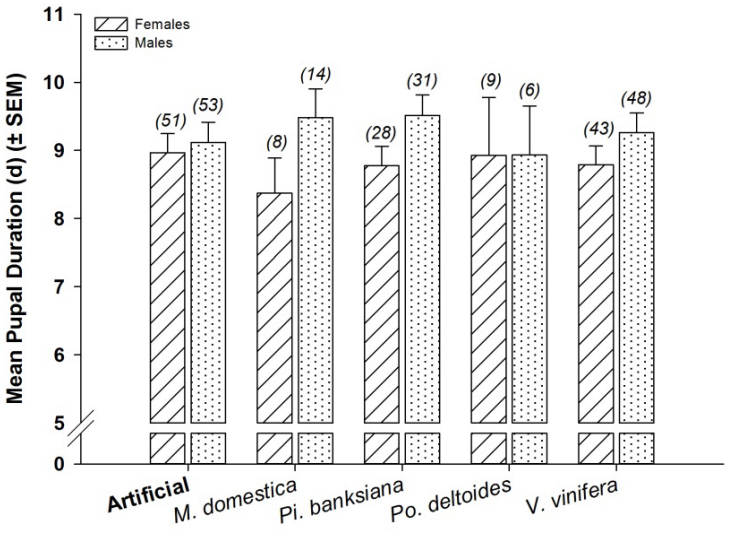

Mean Pupal Duration (d) (± SEM)
11
10
9
8
7
6
5
0
Females
Males
(51)
(53)
(8)
(14)
(28)
(31)
(9)
(6)
(43)
(48)
Artificial
M. domestica
Pi. banksiana
Po. deltoides
V. vinifera

Figure 3.2: **(a)** Mortality, **(b)** larval duration (hatch to pupation), **(c)** pupal mass, and, **(d)** pupal duration (pupation to adult eclosion) following low temperature exposure of *E. postvittana* late instars to their supercooling point (SCP) or contol conditions (~23°C) reared on different hosts. For pupal mass, mean and confidence intervals are presented as back-transformed values. Numbers in parentheses indicate sample size across all blocks. An astrisk indicates significant difference within a host, whereas different letters of the same case indicate significant differences across host groups (α=0.05). Data summaries for control groups are reprinted from Chapter 2.

CHAPTER 4:
Selection for increased survival after partial freezing in the invasive light brown apple moth, *Epiphyas postvittana* (Walker)

4.1 Summary

Adaptation to an invaded environment is often critical to the success of biological invasions by exotic insects. For temperate areas of North America, cold temperatures prevent invasions by many exotic species. Consequently, the effects of cold on populations of invading insects are often included in pest risk assessments. Current assessments of risk typically assume a species' response to cold is immutable over short to medium time horizons. In this study, we investigated how short-term cold exposures may impact the cold tolerance of an invasive light brown apple moth (*Epiphyas postvittana*) population over multiple generations. Late instars of *E. postvittana* exhibit partial freeze tolerance, such that a small proportion can survive exposure to their supercooling point and develop into reproductive adults. Therefore, we used freezing as a selection pressure; late instars were briefly exposed to their individual supercooling points, survivors were randomly mated as adults, and their progeny were then also supercooled as late instars. Selection was applied this way to two population replicates, continuing for 25 and 15 generations, respectively. There was no consistent response to selection in either population replicate. Heritability and genetic covariance measures suggest low genetic variance in supercooling points and whether they survive acute partial freezing. While we did not see a consistent response to selection through time, the degree of partial freeze tolerance was more variable over time than previously observed. Moreover, it cycled in a regular pattern, about every six generations, driven by a yet-unknown mechanism. The variability in cold tolerance observed in our long-term study was beyond what typical laboratory studies would capture. To forecast future risks posed by invasive insects, adequately characterizing the variability in current cold response may be equally, or more, important as estimating the rate of adaptation.

4.2 Introduction

Effective mitigation of the potential negative impacts of invasive species relies on accurate forecasts of their propensity to survive each stage of invasion. Considerable effort has been devoted to identify traits that reliably predict invasiveness across species (e.g., reviewed in Sakai et al. 2001, Lee and Gelembiuk 2008), with some researchers noting that success at different stages within the invasion process may require different traits (Kolar and Lodge 2001, Dietz and Edwards 2006). The ability to rapidly adapt to extreme abiotic stressors has been suggested as particularly prevalent among invasive species that successfully spread (Lee 2002, Butin et al. 2005, Whitney and Gabler 2008). However, adaptation is rarely considered in distribution forecasts and models of invasive species, and instead, species' traits are treated as static entities over space and time (Lee 2002, Whitney and Gabler 2008, Hoffmann and Sgrò 2011).

For ectotherms, such as insects, tolerance to extreme thermal events is linked to many species distributions (Addo-Bediako et al. 2000, Overgaard et al. 2014). In temperate regions, cold tolerance is an important adaptation of native species and may preclude the establishment of some invading species (Bale 2002). But the extent to which an invading population may rapidly evolve to tolerate novel cold environments remains relatively unexplored through empirical research.

The current paradigm for the classification of insect cold tolerance describes the relationship between internal ice formation and mortality (Lee 2010). Most species are, broadly, *freeze-avoidant* because they die once ice forms within their bodies (i.e., are considered *freeze-intolerant*) or from the deleterious effects of non-freezing temperatures (i.e., are considered *chill-intolerant*). Other species can survive internal freezing under certain conditions (i.e., are considered *freeze-tolerant*). In addition, intermediate responses exist, such as individuals and/or populations displaying *partial freeze tolerance* to varying levels of ice formation (Baust and Rojas 1985, Sinclair 1999, Hawes and Wharton 2010). A poorly characterized phenomenon, partial freeze tolerance is most consistently described as the ability to survive formation of ice within the body, but to a much more limited extent than traditionally recognized freeze tolerant species (i.e., those that survive maximal ice formation at a given temperature). For example, an individual is

considered to have partial freeze tolerance if they can survive exposure to a point beyond their supercooling point but only if rewarmed before reaching the equilibrium freezing point (Sinclair 1999, Hawes and Wharton 2010).

Insect cold tolerance studies largely explore the phenotypes of individuals, typically within a generation. However, to understand the adaptive evolutionary potential of a population, the underlying genetic variation of the cold tolerance phenotypes must be examined (Overgaard et al. 2010). Selection experiments can be powerful tools for estimating the quantitative genetic variation with regard to a specific trait or traits (Scheiner 2002, Huey and Rosenzweig 2009). Relatively few studies have investigated the role of genetic variability in insect cold tolerance traits using selection. Those that exist largely focus on *Drosophila* species, due in part to *Drosophila* being a model laboratory organism, but most generally suggest that substantial genetic variation for cold tolerance exists within many populations (Overgaard et al. 2010).

In artificial selection, individuals are selected and bred within lines by the experimenter for specific trait values, such as chill coma recovery time. For example, Anderson et al. (2005) exposed adult *D. melanogaster* every two generations for 30 generations to 0°C for 4 h (a non-lethal exposure) and the flies with the fastest recovery time (i.e., chill coma recovery) were used as parents in the next generation. The authors found that chill coma recovery was enhanced with directional selection, and selection also increased tolerance to survival at -2°C. Chill coma recovery has also been used to successfully select for increased cold resistance in adults of the nymphalid butterfly, *Bicyclus anynana* (Dierks et al. 2012b, 2012a, Franke et al. 2012).

Other studies have used more stressful conditions as a selection pressure in *Drosophila*, such as cold shock (e.g., the time at given temperature needed to cause a specific level of mortality) (e.g., Tucić 1979, Watson and Hoffmann 1996), or a combination of cold hardening followed by cold shock (e.g., Chen and Walker 1993). Mixed results are seen, with response and directionality of selection varying based on experimental design. For example, Chen and Walker (1993) observed an increase in cold shock resistance (i.e., an increase in time at -7°C needed to cause 50 and 90% mortality) with selection, most dramatically if flies were hardened prior to exposure. In contrast, Watson and Hoffmann (1996) saw a positive response to selection in cold shock

resistance (i.e., an increase in the time at -2°C needed to cause 50% mortality) in *Drosophila*, but only in the absence of prior hardening and if cross-generation effects were ameliorated by relaxed selection. MacMillan et al. (2009) also saw no change in the response to selection for cold shock survival (at -5°C for 1 h) with hardening.

Though less common, laboratory natural selection experiments have been used to study insect cold tolerance (Gilchrist et al. 1997, Kellermann et al. 2015). Here, population lines are held under different conditions for many generations and then evaluated at intervals for divergence in phenotypes; fitness, rather than the experimenter, acts as the selective agent (Scheiner 2002, Hoffmann et al. 2003, Kawecki et al. 2012). For example, *D. melanogaster* populations were maintained under three different temperature conditions for 100-175 generations, and then compared for differences in various thermal tolerance measures to explore the genetic potential for evolutionary change (Gilchrist et al. 1997). Correlated evolutionary effects of heat tolerance, but not cold tolerance (in eggs or adults), were seen with natural selection at different rearing temperatures.

Temperate and high-altitude populations have often been shown to show higher cold tolerance than tropical and low-altitude populations (e.g., Hoffmann et al. 2002, 2003, Karl et al. 2008). As such, an additional body of literature that explores the potential for adaptation to low temperature in insects are studies that compare field-collected populations of a species across climatic gradients. Such studies rear field-collected insects in a common-garden laboratory environment (or using reciprocal transplantation) and then compare some measure of cold tolerance (Overgaard et al. 2010). In some cases, any difference observed among populations is assumed to be due to genetic differentiation (e.g., Ayrinhac et al. 2004, Butin et al. 2005). Others provide estimates of quantitative genetic parameters, such as heritability (e.g., Jenkins and Hoffmann 1999, Kellermann et al. 2009, Davis et al. 2014, Ma et al. 2014). Some studies suggest the presence of significant genetic variation for cold tolerance in populations, and therefore, heritable clinal patterns of tolerance (Jenkins and Hoffmann 1999, Ma et al. 2014), whereas other do not (Hoffmann et al. 2005, Kellermann et al. 2009, Davis et al. 2014).

Here, we explore the potential for adaptation in an exotic, invasive insect, *Epiphyas postvittana* (Walker) to extreme cold temperatures through laboratory natural selection. *Epiphyas postvittana* is a highly polyphagous moth that is indigenous to southern Australia, and was documented in the contiguous United States in 2006 (Brown et al. 2010). Establishment and spread in the U.S.A. by *E. postvittana* has potentially significant economic and ecological ramifications (Fowler et al. 2009), and early management responses were controversial (Lindeman 2013, Liebhold 2014). Thus, the potential distribution of this insect is of interest. *E. postvittana* overwinter predominantly as late-instar larvae, which slow development but do not enter diapause during winter (Geier and Briese 1981). Late instars are generally considered freeze-avoidant (Bürgi and Mills 2010). However, our group has observed a small proportion of partial freeze-tolerance within late instars (and pupae; see Chapter 2), with some insects surviving acute exposure to the onset of internal freezing (i.e., the peak of their supercooling points) and continuing to develop into reproductively-successful adults. We previously reported a preliminary summary of the present study after nine generations of selection in a single line (Morey et al. 2013; see Chapter 5). Now, we present a more complete dataset, encompassing two replicated lines lasting 25 and 16 generations, respectively. Each selected line had a paired, unselected control, and the first selected replicate also included an assortative mating line (five lines total).

Using partial freezing as a selection pressure, we hypothesized that: (i) given sufficient genetic variation, tolerance to partial freezing would increase through time under directional selection; and (ii) based on the general trend that freeze tolerant species usually freeze at warmer temperatures than freeze avoidant species (Sømme 1982, Lee 2010), the population distribution of temperatures at which internal freezing begins (i.e., the supercooling point) would change through time, either as a change in mean and/or variance.

4.3 Materials and Methods

4.3.1 Colony source and maintenance

Epiphyas postvittana eggs were obtained in June 2011 (population replicate 1) and January 2012 (population replicate 2) from a laboratory colony maintained by the United States Department of Agriculture, Animal and Plant Health Inspection Service (USDA-APHIS) in Albany, CA, founded from wild California moths in 2007. All subsequent handling and experimentation was conducted in a Biosafety Level 2 Containment Facility in St. Paul, MN (APHIS permit P526P-14-03759). Upon arrival, eggs were surface-sterilized in a 1% bleach solution, and held at 23 ±3°C, 60-65% RH, 14:10 (L:D) inside two environmental chambers (Percival Scientific, Perry, IA and Conviron, Winnipeg, Canada) until hatch.

Within 24 h of hatching, we surface sterilized (with 70% ethanol in water) camel hair paintbrushes to place *E. postvittana* neonates in groups of 3-4 onto ~2cm^3 cubes of artificial diet inside sealed containers (29.5 mL P100 soufflés; Solo Cup Co., Lake Forest, IL). The diet used great northern beans (*Phaseolus vulgaris* L.) and followed a modified formulation developed by Follet and Lower (2000) for *Cryptophlebia* spp. (Lepidoptera: Tortricidae).

Insects were reared in constant conditions (23 ±3°C, 60-65% RH, 14:10 (L:D) h) and, in the first population replicate, randomly mated for one generation before the experiment began. The second population replicate was begun using the generation received from the USDA-APHIS colony (from the second egg mass shipment).

4.3.2 Selection protocol

We established the first population replicate (PR1) using larvae produced from 138 randomly mated adults. Neonates were applied to diet as described above and reared until late instars (4th -6th). *Epiphyas postvittana* has variable instar numbers (Danthanarayana, 1975a; Dumbleton, 1932). Therefore, we focused on “late instars”, any of which could be the terminal instar before pupation and could overwinter. Instar was confirmed through head capsule measurement (Danthanarayana, 1975a), and then larvae

were randomly assigned to either the selected line (n=127) or the unselected line (n=119) and transferred to individual gelatin capsules (size 4; 14.3mm length, 5.1mm diameter).

For the selected line, partial freezing served as the selection pressure. Individual larvae (within gel capsules) were cooled at ~1°C/min to their supercooling point within calibrated styrofoam cubes inside a -80°C freezer, as per Carrillo *et al.* (2004). Temperatures were recorded once per second by using a coiled, copper-constantan thermocouple design (Hanson and Venette 2013), connected to a computer through a multichannel data logger (USB-TC, Measurement Computing, Norton, MA). Each larva was immediately removed from the freezer and cube once they reached the peak of the exotherm, which could be observed in real-time as a plateau (typically lasting 15-25 s) following an abrupt spike in temperature. Removal at the exotherm peak allowed us to be consistent in ensuring larvae were exposed to the same relative point in internal ice formation. Larvae were taken from the capsules and placed in individual cups with fresh diet at 23 ±3°C (60-65% RH, 14:10 (L:D) h) in a growth chamber to continue development.

For the unselected line, individuals (i.e., "controls") were left inside gel capsules at room temperature (~25°C) for approximately 1 h while cold-treated individuals were being supercooled. Control larvae were removed from the capsules and returned to the growth chamber with fresh diet concomitant to the cold-exposed larvae.

Individuals from all lines were monitored daily for survival, which was defined by successful eclosion as an adult. Upon emergence, sex of adults was determined from wing coloration and the presence (male) or absence (female) of a forewing costal fold (Brown et al. 2010). Adults within a line were mated as described in the next section (see: "Mating design"). For those mating groups that produced viable offspring, approximately equal numbers of neonates (i.e., F1 individuals) were placed on diet from each mating group within a line (~20-40 cups, in the manner described above), when possible. Larvae were reared to late instars as before. Within a line, approximately equal numbers of late instars were randomly selected from each mating group, though, due to differences in viability and developmental timing, this was not always possible. These larvae were then exposed to the selection regime as previously outlined. This selection protocol was repeated for 25 total generations.

In all generations subsequent to F0, a cohort of the unselected line, representing as many parental lineages as possible, was supercooled in tandem with the selected line individuals. These larvae, (i.e., "checks") were not integrated into the unselected line if they survived cold-exposure. Rather, they served to monitor the supercooling points and survival after partial freezing in the unselected line through time for comparison with the selected line.

In the selected line, 137-307 larvae were supercooled in each generation. In the unselected line, between 102 and 221 larvae were supercooled as checks in each generation, with the exception of F2-F5 (Table 4.7). To maintain the unselected line, 60-145 control larvae were processed/handled (but not cold-exposed) in each generation and used as the breeding population.

The second population replicate (PR2) was established from an unknown number of randomly-mated individuals in the USDA-APHIS colony. F0 selection of PR2 began during selection of the F4 generation in the PR1, with 214 late instars supercooled to begin the PR2 selected line and 62 late instars processed to begin the unselected line. The protocol for PR2 was the same as in PR1, with both population replicates (four lines total) being maintained in tandem. Due to logistical constraints and apparent lack of selection response, the PR2 lines were only maintained through F15. In the PR2 selected line, 153-205 larvae were supercooled in each generation. In the PR2 unselected line, between 100 and 214 larvae were supercooled as checks in each generation. To maintain the PR2 unselected line, 62-160 control larvae were processed/handled (but not cold-exposed) in each generation, except for in F2 when only 27 individuals were available for use due to high early instar mortality.

4.3.3 Mating designs

Random mating. One to three individuals of each sex were randomly mated in 0.47L (16 fluid oz) clear plastic cups with lids. Details of the container design are provided in Figure 4.1. The parental history of moths placed in a given cup was tracked and, where possible, males and females within a cup were not from the same parent group to reduce inbreeding; within a sex, however, multiple individuals from the same parent group could exist. If appropriate mates were not immediately available, moths were given a piece of

cotton soaked with 10% honey solution (mass per volume) in de-ionized water within 24h of eclosion. Moths not included within a breeding group within six days of eclosion were not used in the breeding population. In some instances, if a moth died before sufficient eggs were collected from its breeding group, it was replaced with another moth of the same sex from the same parent group (or from any of the parent groups, if multiple included for a sex).

Moths were allowed to oviposit on the sides of the cup until their death or after ~two weeks. The range of oviposition times staggered subsequent larval development sufficiently to allow enough time to process large samples of individuals during the selection process. Viable eggs would hatch in ~7 d, so approximately every 4-6 d, reproducing moths would be transferred to a new cup. The maturing egg masses would be cut from the empty cup (as egg masses left attached to small pieces of the cup wall) and placed inside clean sealed cups (29.5 mL P100 soufflés; Solo Cup Co., Lake Forest, IL) to await hatch. The subsequent treatment of viable larvae was described in the previous section.

Assortative mating. In generation 19 of PR1, a random subset (n=54) of the selected larvae that survived supercooling were used to begin an assortative mating population. Here, a single male and single female comprised a mating group, and their pairing was based on their individual supercooling points. Though supercooling points varied through time (see: Results), an average of -15.5°C was estimated and this temperature was used to segregate mating pairs: moths with larval supercooling points > -15.5°C were paired together, and moths with larval supercooling points ≤ -15.5°C were paired together. This was done to explore the potential influence of parental supercooling points on progeny survival following partial freezing. We explored this relationship because insects that survive freezing often have higher supercooling points relative to those that do not survive freezing (Sømme 1982, Lee 2010), and cross-generation effects of cold stress have been observed in other species (e.g., Watson and Hoffmann 1996; Zhou et al. 2013).

4.3.4 Analysis

Statistical analysis was conducted in SAS® 9.4 (SAS Institute Inc., Cary, NC). To test for a statistical response to selection in either of our traits of interest (population supercooling points or proportion survival after partial freezing), we followed the methods of Wade et al. (1996) (*sensu* Muir 1986). For the response of supercooling points, the residuals from the regression of selected on unselected mean supercooling points were regressed on time measured in generations. For the response of survival after partial freezing, logit residuals from the logistic regression of selected on unselected proportion survival after partial freezing were regressed on time measured in generations. This was done for each of the three populations (i.e., PR1, PR2, assortative mating).

In addition to calculating overall variation in supercooling point and survival after partial freezing, we also examined within-generation variation. Within each generation for a given population replicate, the proportion survival after partial freezing was compared between the selected and unselected lines using the Z statistic (Kvanli 1988). The distribution of supercooling points within each generation were also compared; two-sample t-tests were used to detect differences in means between the selected and unselected lines and a Brown-Forsythe's test was used to detect any change in variance (Proc TTEST and Proc GLM, respectively). When unequal variances were present (α=0.05), the Satterthwaite approximation for degrees of freedom was used and when variances were equal, the pooled method was used (SAS/STAT® 9.2 User Guide, 2nd ed.).

To measure the degree to which any observed variation in cold tolerance phenotype was due to genetic variance, we calculated narrow-sense heritability (h^2) and genetic covariance estimates using offspring-midparent regressions (Falconer and Mackay 1996). For supercooling points through time, h^2 was estimated as the slope of the regression of offspring supercooling points on midparent supercooling points. Since the mating lineages were comprised of groups of adults rather than a single mating pair, "midparent" represents the average supercooling point for an entire mating group (~3-6 individuals). Initial investigation showed that generation and midparent supercooling points were significantly related (Proc GLM; df=4912, F=262.91, P>F=0.001). Therefore, h^2 estimates were calculated separately for each generation. Given the

frequent relationship between supercooling points and the ability to tolerate freezing (Lee 2010), the genetic covariance of parental supercooling points and offspring survival after partial freezing was also calculated using offspring-midparent regressions. Here, the slope of the logistic regression (Proc LOGISTIC) of offspring supercooling points on midparent survival after partial freezing estimated the genetic covariance between these two traits, with estimates calculated separately for each generation due to the aforementioned collinearity of generation and midparent supercooling points.

In addition to reporting the total census size of each generation's breeding population (in each line), we calculated the effective population size (N_e). To account for the unequal numbers of contributing males and females, the following equation was used: $N_e = (4*N_m*N_f)/(N_m + N_f)$, where N_m and N_f are the number of contributing males and females, respectfully (Falconer and Mackay 1996).

4.4 Results

4.4.1 Survival after partial freezing

The response of survival after partial freezing to selection, measured as the slope of residuals through time, was positive in PR1 and negative in PR2 and the assortative mating line (Table 4.1). However, in no case was the slope significantly different from zero, indicating that there was no difference in overall response between the selected and unselected lines through time. This is consistent with the close, nearly overlapping, plots of the raw proportions of survival after partial freezing in all lines (Figure 4.2).

Looking within generations, however, the selected and unselected lines differed significantly at various points in time, and in all cases, the selected line had greater survival (Tables 4.4-4.6). In PR1, the selected line had significantly higher proportion survival than the unselected line in F4, F15, and F23 (α=0.05), and marginally significantly higher survival in F9, F13, F17, F20, and F21 (α=0.1) (Table 4.4). In PR2, the selected line had significantly higher survival than the unselected line in F4 and F14 (α=0.05) (Table 4.5). The assortative mating line showed no significant difference in the

proportion survival after partial freezing between the selected and unselected lines at any point in time (Table 4.6).

While no consistent response to selection was observed, an apparent cyclical change in survival after partial freezing was seen in all lines, in both population replicates (Figure 4.2). Pooling across lines, the response ranged from 0.11 (±0.02 S.E.) to 0.60 (±0.04) proportion survival. The cycle, seen with the greatest regularity in the unselected line of PR1 (Figure 4.2; solid red line), seemed to follow a periodicity of ~six generations (or ~9-10 mos. in laboratory conditions) between minimum to minimum or maximum to maximum values.

Therefore, to explore the extent to which the apparent trend was non-random we used auto-correlation analysis (Proc AUTOREG), which can indicate when a time-series is significantly different from white noise and at which interval, or "lag", the ordinary regression residuals are correlated (Durbin-Watson statistic; SAS® 2010). This analysis was done for only PR1 since it was the longest time-series. The analysis was done after both 22 and 25 generations. After 22 generations, both the selected and unselected line showed a significant pattern (selected: df=1, t=2.76, Pr(t) = 0.012, unselected: df=1, t=2.97, Pr(t)=0.007). The unselected line showed a significant positive autocorrelation at lag one (Pr<DW = 0.042), 6 (Pr<DW = 0.011), and 12 (Pr<DW = 0.015). The selected line showed a significant positive autocorrelation at lag 6 (Pr<DW = 0.038). After 25 generations of selection, the pattern shifted slightly and the overall significance was lost in both lines (selected: df=1, t=0.53, Pr(t)=0.60, unselected: df=1, t-0.62, Pr(t)=0.54).

4.4.2 Supercooling points

The overall response of mean supercooling points to selection, measured as the slope of residuals through time, was negative in all lines (Table 4.2). However, in no case was the slope significantly different from zero, indicating that there was no difference in overall response between the selected and unselected lines through time. This is consistent with the general overlap of all lines in a plot of the raw means (Figure 4.3).

Looking within generations, the selected and unselected lines differed significantly at various points in time, both in mean differences and variance (Tables 4.7-4.9). Unlike in survival after partial freezing, the directionality of the difference between

selected and unselected lines was not consistent. In PR1, the selected line means were significantly (α=0.05) lower than the unselected means in F4, F7, F13 and F19 (marginally lower in F14 (α=0.1)), but significantly higher in F20 and F23 (Table 4.7). In PR2, the selected line means were significantly lower than unselected means in F6, but significantly higher in F2 and F5 and marginally higher in F13 and F14 (Table 4.8). In the assortative mating line, the mean supercooling point was significantly higher in the selected line compared to the unselected mean in F2 (corresponding to F21 in PR1), but was marginally lower than the unselected mean in F6 (Table 4.9). With respect to supercooling point variance, where there were significant differences within generations and the unselected lines tended to have higher variance than the corresponding selected lines. This occurred in F1 and marginally in F14 of PR1 (Table 4.7), and in F7 and F9 and marginally in F2 and F4 of PR2 (Table 4.8). Contrastingly, in F7 of PR1, the selected line had significantly higher variance than the unselected line, which also occurred with marginal significance in F4 (corresponding to F23 in PR1) of the assortative mating line (Tables 4.7 and 4.9).

Across all lines through time, the highest supercooling point for an individual was -8.59°C and the lowest was -24.87°C. Though initially supercooling point means across all lines oscillated together with a similar frequency to the 6-7 gen. cycle in survival after partial freezing, the range of oscillating mean values was much less extreme (~3°C difference) compared to the nearly 6-fold change in survival after partial freezing. Additionally, by about F15 of PR1 (F11 in PR2), the mean supercooling points across lines appeared to equilibrate around ~ -15.5°C (Figure 4.3).

4.4.3 Heritability and genetic covariance

The heritability of supercooling points and genetic covariance between supercooling points and survival after partial freezing were not consistent across generations, or across selected lines (Tables 4.10-4.12).

In the heritability estimates for PR1, two positive slope values (0.56 ±0.16 and 0.54 ±0.20) were significantly different from zero (α=0.05), and one positive (0.49 ±0.26) and two negative slope values (-0.47 ±0.27 and -0.38 ±0.21) were marginally significant (α=0.1) (Table 4.10). In the heritability estimates for PR2, one positive slope (0.58 ±0.15)

and one negative slope (-0.62 ±0.26) were significant (Table 4.11). In the assortative mating line, two positive slopes (0.23 ±0.10 and 0.32 ±0.12) were significant (Table 4.12). Therefore, in some instances within the random mating lines, ~50-60% of the variance in supercooling points was associated with additive genetic effects, while within the assortative mating line, ~20-30% was associated.

Similar to supercooling point heritability, the genetic covariance of supercooling point and survival after partial freezing was only apparent within a few generations within a line, and did not show obvious correlations across lines through time (Tables 4.13-4.15). In PR1, covariance slope estimates were significant in F3 and F21 (-1.39 ±0.48 and 0.39 ±0.20, respectively) (Table 4.13) and, in PR2, were significant in F5, F8, and F9 (0.43 ±0.18, 0.30 ±0.14, and -0.56 ±0.22, respectively) (Table 4.14). In the assortative mating line, the estimate in F5 was significant (0.69 ±0.34) (Table 4.15).

4.4.4 Assortative mating

The effect of assortative mating compared to the random mating population, on either cold tolerance response, appeared negligible (see previous results). Breeding moths based on their supercooling points also did not cause obvious change in supercooling point heritability or genetic covariance of supercooling points and survival after partial freezing.

While the response of the traits measured did not differ in assortative mating individuals, there did appear to be a greater fitness cost compared to the random mating regime. The effective population sizes (N_e) of the first three generations were comparable to the parallel random mating lines, but the assortative breeding populations began to rapidly decline in F3 (Table 4.3). This was due both to low overall survival after partial freezing (Figure 4.2 and Table 4.6) and then low viability among many mating groups. The F5 assortative breeding population had such low progeny viability that only one mating pair contributed progeny to the final round of selection in F6. This pair was comprised only of “low supercooling” individuals in that they both supercooled at ≤ -15.5°C as late instars. Three other low supercooling pairs and five pairs of “high supercooling” (supercooled at > -15.5°C as late instars) were bred, but produced no offspring that survived to late instar.

4.5 Discussion

4.5.1 Lack of consistent response to selection

Laboratory selection for increased tolerance to partial freezing in *E. postvittana* late instars did not show an overall response to selection, in either population replicate, for partial freeze tolerance or mean supercooling points (Figure 4.2 and 4.3; Table 4.1 and 4.2). Within some generations, there was a significant difference between the selected and unselected lines, and while there was no obvious pattern to when in time significance was occurring, all instances were in the direction hypothesized; the selected lines showing greater proportion survival after partial freezing than the unselected lines (Table 4.4 and 4.5).

Our first expected hypothesis (a response to selection would be seen) rested on there being sufficient additive genetic variance or covariance in the population for the traits of interest. Other studies have shown significant genetic variation in insect cold tolerance traits (e.g., summarized in Hoffmann et al. 2003 and Overgaard et al. 2010). Based on narrow-sense heritability (h^2) estimates of supercooling points (Tables 4.10 and 4.11) and genetic covariance estimates of progeny partial freeze tolerance and parental supercooling points (Tables 4.13 and 4.14), there does not appear to be heritable variation in either of these traits. Other explanations for a lack of expected response to selection have included inbreeding (Wade et al. 1996, Dierks et al. 2012a), trade-offs due to pleiotropy or linkage with other traits (Hoffmann 2013), or adaptation to laboratory conditions (Huey and Rosenzweig 2009, Kellermann et al. 2015). However, fitness-related traits are generally expected to have little or no genetic variation, since the alleles conferring high fitness would like go to fixation (but see Merilä and Sheldon 1999, Zhang 2012, Shaw and Shaw 2014 for additional discussion). Therefore, given that we were directly measuring survival, a lack of heritable variation may be the most likely explanation for our results.

Our second hypothesis, regarding changes in supercooling points with selection, was also not supported; neither mean supercooling points nor the variance of supercooling points were consistently different between the selected and unselected lines. While within generation differences could be seen between the selected and unselected

line at various points (in both the mean and variance of supercooling points), there was no obvious pattern to when differences were seen, nor in the directionality of the significance (Tables 4.7 and 4.8).

At generation 20 of PR1, we attempted to ameliorate possible cross-generational effects (e.g., Watson and Hoffmann 1996, Zhou et al. 2013) of covariance between survival after partial freezing and supercooling points (Sømme 1982, Lee 2010) in the event that this relationship may be confounding our ability to see a selection response. To do this, we used a subset of the PR1 selected individuals and rather than allow them to randomly mate as before, we assortatively mated them into two groups, based on whether their supercooling points were above or below -15.5°C. Assortative mating, however, had no effect on the consistency of trait responses (Tables 4.1, 4.2, 4.6, 4.9, 4.12, and 4.15). Uniquely, this population did suffer severe reduction in effective population size (Table 4.3) after three generations of selection, and would likely not have persisted beyond when the experiment was ended. Whether this loss of viability was due to genetic drift or some tradeoff unique to breeding based on supercooling points is unknown.

4.5.2 Plasticity of response through time

Despite the lack of apparent response to selection, there was a striking pattern to survival after partial freezing through time, in all selected and unselected lines (Figure 4.2). Statistical analysis indicated that the pattern may be more regular than random noise (see section 4.4.1) and that when a significant pattern is detected, a positive auto-correlation every six generations (or every ~nine months) was seen. The loss of a statistical pattern after generation 22 is likely due to sensitivity of the time series analysis to slight pattern deviations (e.g., a change in pattern periodicity from six to seven/eight generations) when using relatively small series of data points.

So what is going on? At the conclusion of this dissertation, I can only provide hypotheses based on correlation, not empirical evidence. A pattern that is consistent in all lines across time could be suggestive of exogenous influence(s), some biotic or abiotic factor regulating the response. Common factors that can produce a cyclical change in natural or laboratory populations include: seasonality, nutritional variation, density

dependent mechanisms (e.g., crowding, pathogens, mate availability), or changes in equipment, protocol, or researcher.

All of these explanations were initially rejected with relative confidence due to the following reasons: This experiment was conducted, continuously, in a highly environmentally-regulated quarantine facility, with insects being held inside environmental chambers when not being manipulated. There was no regular change in protocol (e.g., diet, handling, rearing conditions), equipment, or researcher. Insects were reared in small groups (or separately), and randomly-mated populations showed little change in effective size. Control mortality (non-cold-exposed individuals) was generally very low (<%5) in both population replicates, and in instances where greater than normal (e.g., F14 in PR1 and F15 in PR2), there was no obvious correlation with the variation in cold tolerance measures.

Given the lack of alternative explanations, we explored the possible role of seasonality in more detail, given that this seemed the most tenable option. Insect cold tolerance, like many life history traits of insects, is closely linked with seasonal changes in temperature, photoperiod, and moisture; cyclical changes in cold tolerance are often seen (e.g., Baust and Miller 1970, Bradshaw and Holzapfel 2010a, Morey 2010). While the insects in this study were in a highly controlled laboratory environment of constant temperature and photoperiod, we could not be as certain about the relative humidity; the conditions inside the quarantine facility may not be completely insulated from the oscillations in humidity during a Minnesota calendar year. However, humidity did not appear to consistently correlate with survival to partial-freeze tolerance (Figure 4.4).

A final hypothesis also relates to a seasonal response in *E. postvittana* cold tolerance, but one that has been incorporated into the endogenous organization of the population. Biological systems exhibit two major rhythms: a daily rhythm of about 24 h (circadian) and an annual rhythm of about 12 mos. (circannual) (Bradshaw and Holzapfel 2010b). The former enables organisms to track daily changes in their environment and the latter enable them to anticipate and prepare for seasonal changes in their environment (Saunders 2002, Bradshaw and Holzapfel 2010b). Of note, while these rhythms are endogenous (i.e., self-sustaining) they must be synchronized or “entrained” to the local environment by external cues to achieve the intended periodicity (Saunders 2002).

Without these cues, asynchronies will develop that mismatch the response from the environment (reviewed in Gwinner 1986).

This is relevant to my work as follows: The insects in this study were held in constant, ideal conditions (with the exception of the selection event) of 14:10 (L:D) h and ~23°C. In natural environments, they would experience much more variation in photoperiod and temperature, as well as other potential abiotic cues. The cyclical response we saw in partial-freeze tolerance did not cycle on a 12 mos. basis, but it was close (~nine mos.). Our results could, therefore, be an example of a de-synchronized circannual rhythm that resulted from insects being held in constant laboratory conditions for many generations. A challenge with this hypothesis, however, is that there are no instances of circannual rhythms in the literature that describe a cycle occurring over multiple generations through time. Documented examples of yearly cycles involve longer-lived organism (e.g., birds, uni-or bi-voltine insects), often in relation to their migration, gonadal development, diapause, or migration (e.g., Tauber and Tauber 1976, Gwinner 1986, Visser et al. 2010). To the best of my knowledge, a system where a biological rhythm oscillates over multiple generations in a population, where a signal is presumably passed transgenerationally, has not been described.

4.5.3 Implications for species distribution models and pest risk mapping

The degree to which phenotypic plasticity and genetic adaptation differentially influence the successful establishment and spread of invasive species is an active area of discussion (e.g., Dybdahl and Kane 2005, Chown et al. 2007, Hoffmann and Sgrò 2011). Some argue that phenotypic variation can result in an adaptive evolutionary response, even when it is environmentally-induced and non-heritable; plasticity may, in fact, facilitate/enhance the process of adaptive evolution (Ghalambor et al. 2007). In either case, evolutionary and plastic change in traits are currently absent from models of invasion species distributions and forecasts of species' response to climate change (Lee 2002, Hoffmann and Sgrò 2011).

Experimental populations are the units of replication to test evolutionary hypotheses (Kawecki et al. 2012). However, the direct applicability of laboratory natural selection to field settings should be made with caution (reviewed in Huey and

Rosenzweig 2009, Kellermann et al. 2015). Though we could not disentangle the influence of phenotypic plasticity from adaptive genetic potential in cold tolerance here, our results capture variability beyond what typical cold tolerance laboratory studies would. To forecast future risks posed by invasive insects, adequately characterizing the variability in current cold response may be just as important as estimating the rate of adaptation.

4.5.4 Future directions

Further physiological characterization of partial freeze tolerance in *E. postvittana* would provide useful insight in going forward. As mentioned, partial freeze tolerance is a poorly described phenomenon, with some inconsistencies in the fairly vague definitions that do exist (e.g., Sinclair 1999 versus Baust and Rojas 1985). Specifically, exploring the extent that freezing has occurred when individuals are pulled at the peak of the exotherm, and how the proportion of ice formation differs between those that survive and those that do not. This could help to better delineate where along the gradient of cold strategies partial freeze tolerance may fall evolutionarily. Also, comparing the metabolomic or proteomic profiles of survivors and non-survivors, perhaps also in larvae fed host species that induced high and low partial freeze tolerance (as in Chapter 2), may give insight to the molecular mechanisms behind the variability seen in my research.

While partial freeze tolerance does not appear to be a selectable trait, a less extreme cold tolerance response (e.g., acute non-lethal subzero temperature, chill coma recovery time) may show heritable variation in *E. postvittana*. Additionally, acclimation may be important in this species, and acclimation has been shown to affect the response to cold selection (e.g., Chen and Walker 1993). Though perhaps logistically challenging, comparing the cold tolerance response of multiple geographic populations of *E. postvittana* in a common garden experiment (Overgaard et al. 2010) would be useful for making inferences about the applicability of laboratory studies to field populations.

Lastly, further exploration of the cyclical pattern seen in partial-freezing survival could lead down interesting paths. A reasonable first step would be to rear populations under different temperature/photoperiod regimes (e.g., constant versus mimics of fluctuating seasonal change) and then compare their survival to partial freezing through

time. If a putative endogenous rhythm in this trait was present, supplying an appropriate entrainment source (e.g., seasonal change in temperature and/or photoperiod) would alter the pattern frequency (Gwinner 1986, Saunders 2002). Provided a change in rhythmicity was seen, a genetic analysis of specimens could reveal similar genetic mechanisms to what is known for lepidopteran and other insect biological clock genes (reviewed in Sandrelli et al. 2008).

Table and Figures

Table 4.1: Overall response of mean supercooling points of late instar *E. postvittana* to the selection regime. Linear regression of residuals for selected on unselected mean supercooling points on generation number within each population replicate (PR). The assortative mating line was compared with the corresponding unselected controls (F20-F25) of PR1.

Line	# of gen.	Slope	S.E.M.	*F*	Pr>*F*
PR1	25	0.0012	0.009	0.02	0.90
PR2	15	-0.0050	0.022	0.05	0.83
Asst. Mating	6	-0.0212	0.045	0.22	0.66

Table 4.2: Overall response of survival after partial freezing of late instar *E. postvittana* to the selection regime. Regression of logit residuals for selected on unselected line survival after partial freezing on generation number within each population replicate (PR). The assortative mating line was compared with the corresponding unselected controls (F20-F25) of PR1.

Line	# of gen.	Slope	S.E.M.	*F*	Pr>*F*
PR1	25	-0.0002	0.008	0.00	0.98
PR2	15	-0.0105	0.013	0.61	0.45
Asst. Mating	6	-0.0501	0.059	0.73	0.43

Table 4.3: Census and effective size (N_e) of breeding populations in the selected lines. Generation indicates the generation of the parental population. The three lines are arranged to reflect their correspondence with each other in time. Population sizes only include those individuals that were part of a mating group that produced offspring used in the subsequent selection event (e.*g*., in population replicate 1, 33 moths (census) from generation three produced the late instars that were used as selected individuals in generation four).

Population Rep 1			Population Rep 2			Assortative Mating		
Gen	**Census**	**N_e**	**Gen**	**Census**	**N_e**	**Gen**	**Census**	**N_e**
0	28	28						
1	44	44						
2	66	66						
3	33	31						
4	28	23	**0**	37	35			
5	35	34	**1**	33	33			
6	30	25	**2**	38	33			
7	37	36	**3**	50	49			
8	62	61	**4**	55	55			
9	43	40	**5**	36	36			
10	33	28	**6**	27	23			
11	23	22	**7**	22	21			
12	41	41	**8**	48	48			
13	35	34	**9**	30	29			
14	47	47	**10**	54	54			
15	44	44	**11**	42	42			
16	11	11	**12**	25	23			
17	27	27	**13**	52	52			
18	71	71	**14**	62	62			
19	24	24	**15**	--	--	**0**	34	34
20	58	58				**1**	47	47
21	41	41				**2**	52	52
22	28	27				**3**	18	18
23	28	27				**4**	11	11
24	25	25				**5**	2	2
25	--	--				**6**	--	--

$N_e = (4*N_f*N_m)/(N_f + N_m)$, where N_f and N_m are the number of contributing females and males, respectively (Falconer and Mackay 1996). It is rounded to the nearest whole individual.

Table 4.4: Within generation comparison of the proportion survival (±SEM) following partial freezing in late instar *E. postvittana* between the selected and unselected lines of population replicate 1. Comparisons between pairs of proportions were made using a Z-statistic to test the hypothesis H_o: $p_1 = p_2$. Using $\alpha=0.05$, H_o was rejected if $|Z^*| > 1.96$. Two-tailed p-values are presented. *n* = the total number of offspring supercooled in a given generation.

	Proportion survival after partial freezing[1]			
	Selected (*n*)	**Unselected** (*n*)	**|Z*|**	**Pr>|Z|**
F0	----	0.28 ±0.04 (112)	----	----
F1	0.28 ±0.03 (189)	0.30 ±0.08 (102)	0.24	0.81
F2	0.44 ±0.04 (137)	0.33 ±0.12 (16)	0.78	0.44
F3	0.23 ±0.02 (307)	0.16 ±0.05 (62)	1.21	0.23
F4	0.32 ±0.03 (202)	0.15 ±0.04 (93)	3.09	0.002*
F5	0.34 ±0.04 (160)	0.33 ±0.05 (86)	0.19	0.23
F6	0.40 ±0.04 (192)	0.35 ±0.05 (113)	0.73	0.47
F7	0.46 ±0.04 (173)	0.46 ±0.04 (143)	0.016	0.98
F8	0.50 ±0.04 (158)	0.53 ±0.04 (139)	0.50	0.62
F9	0.44 ±0.04 (168)	0.33 ±0.05 (110)	1.89	0.06
F10	0.23 ±0.03 (184)	0.24 ±0.03 (155)	0.23	0.82
F11	0.31 ±0.03 (228)	0.33 ±0.04 (143)	0.44	0.66
F12	0.35 ±0.03 (201)	0.34 ±0.04 (163)	0.32	0.73
F13	0.45 ±0.04 (157)	0.34 ±0.04 (131)	1.87	0.06
F14	0.49 ±0.04 (191)	0.47 ±0.05 (111)	0.40	0.69
F15	0.60 ±0.04 (187)	0.44 ±0.04 (185)	3.01	0.003*
F16	0.21 ±0.03 (150)	0.25 ±0.04 (122)	0.93	0.35
F17	0.58 ±0.04 (189)	0.49 ±0.04 (188)	1.80	0.07
F18	0.54 ±0.03 (236)	0.55 ±0.04 (186)	0.21	0.83
F19	0.55 ±0.04 (205)	0.58 ±0.04 (191)	0.59	0.56
F20	0.56 ±0.03 (232)	0.47 ±0.04 (186)	1.67	0.09
F21	0.48 ±0.03 (236)	0.51 ±0.04 (184)	0.46	0.65
F22	0.38 ±0.03 (232)	0.29 ±0.03 (215)	1.93	0.06
F23	0.23 ±0.03 (195)	0.11 ±0.02 (221)	3.20	0.001*
F24	0.17 ±0.03 (174)	0.18 ±0.03 (168)	0.29	0.77
F25	0.24 ±0.03 (164)	0.22 ±0.03 (166)	0.32	0.75

[1] proportion of late instars that survived acute exposure to partial freezing and eclosed as an adult.

Table 4.5: Within generation comparison of the proportion survival following partial freezing (±SEM) in late instar *E. postvittana* between the selected and unselected lines of population replicate 2. Comparisons between pairs of proportions were made using a Z-statistic to test the hypothesis H_o: $p_1 = p_2$. Using $\alpha=0.05$, H_o was rejected if $|Z^*| > 1.96$. Two-tailed p-values are presented. *n* = the total number of offspring supercooled in a given generation.

	Proportion survival after partial freezing[1]			
	Selected (*n*)	**Unselected** (*n*)	**|Z*|**	**Pr>|Z|**
F0	----	0.31 ±0.03 (214)	----	----
F1	0.31 ±0.03 (188)	0.23 ±0.03 (158)	1.54	0.12
F2	0.40 ±0.04 (171)	0.32 ±0.04 (119)	1.46	0.14
F3	0.55 ±0.04 (199)	0.53 ±0.04 (172)	0.36	0.72
F4	0.53 ±0.04 (166)	0.15 ±0.05 (93)	2.53	0.011*
F5	0.41 ±0.04 (177)	0.37 ±0.04 (100)	1.54	0.12
F6	0.41 ±0.04 (138)	0.36 ±0.04 (140)	0.84	0.40
F7	0.28 ±0.03 (229)	0.22 ±0.03 (142)	1.31	0.19
F8	0.39 ±0.03 (207)	0.40 ±0.04 (166)	0.34	0.73
F9	0.32 ±0.04 (158)	0.36 ±0.04 (133)	0.68	0.50
F10	0.53 ±0.04 (171)	0.46 ±0.05 (104)	1.13	0.25
F11	0.54 ±0.04 (190)	0.52 ±0.04 (186)	0.30	0.76
F12	0.22 ±0.03 (153)	0.21 ±0.03 (150)	0.19	0.85
F13	0.45 ±0.04 (189)	0.40 ±0.04 (190)	0.98	0.33
F14	0.55 ±0.03 (235)	0.42 ±0.04 (188)	2.74	0.006*
F15	0.43 ±0.03 (216)	0.50 ±0.04 (189)	1.45	0.15

[1] proportion of late instars that survived acute exposure to partial freezing and eclosed as an adult.

Table 4.6: Within generation comparison of the proportion survival following partial freezing(±SEM) in late instar *E. postvittana* between the selected and unselected lines of the assortative mating line (where F0 individuals came from the F19 selected population in population replicate 1). The assortative mating selected line was compared with corresponding unselected controls (F20-F25) of population replicate 1. Comparisons between pairs of proportions were made using a Z-statistic to test the hypothesis H_o: $p_1 = p_2$. Using α=0.05, H_o was rejected if $|Z^*| > 1.96$. Two-tailed p-values are presented. *n* = the total number of offspring supercooled in a given generation.

	Proportion survival after partial freezing[1]			
	Selected (*n*)	**Unselected** (*n*)	**|Z*|**	**Pr> |Z|**
F0	----	0.58 ±0.04 (191)	----	----
F1	0.45 ±0.03 (251)	0.47 ±0.04 (186)	0.39	0.70
F2	0.58 ±0.03 (232)	0.51 ±0.04 (184)	1.56	0.12
F3	0.35 ±0.03 (216)	0.29 ±0.03 (215)	1.31	0.19
F4	0.16 ±0.03 (142)	0.11 ±0.02 (221)	1.34	0.18
F5	0.15 ±0.03 (144)	0.19 ±0.03 (168)	0.74	0.46
F6	0.16 ±0.03 (123)	0.22 ±0.03 (166)	1.27	0.20

[1] proportion of late instars that survived acute exposure to partial freezing and eclosed as an adult.

Table 4.7: **Within generation comparison of the supercooling points of late instar *E. postvittana* between the selected and unselected lines in population replicate 1.** Means within a generation were compared using a two-sample T-test and variances were compared using the Brown-Forsythe test. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

	Mean Supercooling Points		**T-tests**[1]			**Equality of Variances**		
	Selected (*n*)	**Unselected** (*n*)	**df**	***t***	**Pr>*t***	**df**	***F***	**Pr>*F***
F0	----	-14.36 (127)						
F1	-14.83 (187)	-14.89 (34)	36.8	0.14	0.89	219	19.44	<0.001*
F2	-15.34 (142)	-15.73 (16)	156	0.82	0.41	156	1.15	0.29
F3	-15.99 (312)	-15.95 (63)	373	-0.14	0.89	373	1.47	0.23
F4	-16.06 (206)	-15.36 (93)	297	-2.59	0.01*	297	0.78	0.38
F5	-15.56 (164)	-15.49 (88)	250	-0.26	0.80	250	0.38	0.54
F6	-15.36 (194)	-15.22 (114)	306	-0.55	0.59	306	0.13	0.72
F7	-14.68 (173)	-14.23 (143)	306.9	-2.49	0.013*	314	7.23	0.008*
F8	-14.61 (169)	-14.47 (143)	310	-0.80	0.43	310	0.58	0.45
F9	-15.19 (173)	-15.29 (111)	282	0.39	0.70	282	0.96	0.33
F10	-16.07 (191)	-15.76 (157)	346	-1.22	0.22	346	2.34	0.13
F11	-16.15 (238)	-16.10 (156)	392	-0.24	0.81	392	0.68	0.41
F12	-16.22 (210)	-16.37 (166)	374	0.57	0.57	374	1.96	0.16
F13	-17.04 (156)	-16.28 (133)	287	-2.33	0.021*	287	0.00	0.97
F14	-15.81 (191)	-16.26 (112)	301	1.83	0.068	301	3.14	0.08
F15	-16.04 (190)	-16.02 (187)	375	-0.28	0.78	375	1.22	0.27
F16	-15.64 (157)	-15.24 (127)	282	-1.6	0.11	282	0.13	0.72
F17	-15.78 (191)	-15.69 (190)	379	-0.43	0.67	379	0.22	0.64
F18	-15.62 (237)	-15.38 (187)	422	-1.24	0.22	422	0.17	0.68
F19	-16.02 (205)	-15.58 (191)	394	-1.95	0.052*	394	0.64	0.42
F20	-15.77 (239)	-16.21 (189)	426	2.12	0.035*	426	0.02	0.89
F21	-15.84 (238)	-15.92 (191)	427	0.38	0.70	427	0.02	0.89
F22	-15.84 (235)	-16.03 (223)	456	0.95	0.35	456	0.33	0.56
F23	-15.44 (203)	-15.89 (238)	439	2.91	0.004*	439	1.49	0.22
F24	-15.97 (178)	-15.72 (176)	352	-1.33	0.19	352	0.06	0.81
F25	-15.44 (171)	-15.21 (172)	341	-1.41	0.16	341	0.03	0.86

[1] estimates are from a pooled test when variances were equal and from a Satterthwaite test when variances were unequal.

Table 4.8: **Within generation comparison of the supercooling points of late instar *E. postvittana* between the selected and unselected lines in population replicate 2.** Means within a generation were compared using a two-sample T-test and variances were compared using the Brown-Forsythe test. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

	Mean Supercooling Points		**T-tests****			**Equality of Variances**		
	Selected (*n*)	**Unselected** (*n*)	**df**	***t***	**Pr>*t***	**df**	***F***	**Pr>*F***
F0	----	-16.12 (204)	----	----	----	----	----	----
F1	-15.56 (191)	-15.74 (142)	331	0.99	0.32	331	1.30	0.26
F2	-14.79 (177)	-15.60 (120)	295	3.59	<0.001*	295	3.08	0.08
F3	-14.15 (203)	-14.31 (181)	382	1.01	0.31	382	0.09	0.76
F4	-14.62 (173)	-14.96 (110)	193.84	1.51	0.13	281	3.87	0.05*
F5	-15.17 (187)	-15.97 (157)	342	2.95	0.003*	342	0.09	0.76
F6	-16.06 (144)	-15.33 (142)	284	-2.47	0.014*	284	0.27	0.60
F7	-16.10 (239)	-16.43 (158)	295.77	1.24	0.25	395	5.41	0.02*
F8	-17.06 (211)	-16.67 (168)	377	-1.50	0.13	377	0.04	0.84
F9	-16.34 (163)	-16.47 (136)	269.82	0.44	0.66	297	4.87	0.03*
F10	-16.35 (174)	-15.96 (107)	279	-1.51	0.13	279	0.39	0.54
F11	-15.78 (192)	-15.97 (188)	378	0.76	0.45	378	1.22	0.27
F12	-15.70 (153)	-15.81 (157)	308	0.44	0.66	308	0.97	0.33
F13	-16.02 (191)	-16.43 (191)	380	1.71	0.089	380	1.57	0.21
F14	-15.17 (238)	-15.51 (192)	428	1.74	0.082	428	2.31	0.13
F15	-15.75 (222)	-15.95 (190)	410	0.98	0.33	410	0.45	0.50

[1] estimates are from a pooled test when variances were equal and from a Satterthwaite test when variances were unequal.

Table 4.9: **Within generation comparison of the supercooling points of late instar *E. postvittana* between the selected and unselected lines in the assortative mating population.** Means within a generation were compared using a two-sample T-test and variances were compared using the Brown-Forsythe test. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

	Mean Supercooling Points		**T-tests**[1]			**Equality of Variances**		
	Selected (*n*)	**Unselected** (*n*)	**df**	***t***	**Pr>*t***	**df**	***F***	**Pr>*F***
F0	----	-15.58 (191)	----	----	----	----	----	----
F1	-16.08 (256)	-16.22 (189)	443	0.63	0.53	443	0.08	0.78
F2	-15.32 (237)	-15.92 (191)	426	3.14	0.002*	426	2.15	0.14
F3	-15.97 (217)	-16.04 (223)	438	0.33	0.74	438	0.01	0.92
F4	-15.63 (144)	-15.89 (238)	380	1.47	0.14	380	3.23	0.07
F5	-15.82 (158)	-15.72 (176)	332	-0.51	0.61	332	0.01	0.94
F6	-15.55 (126)	-15.21 (172)	296	-1.77	0.08	296	0.31	0.58

[1] estimates are from a pooled test when variances were equal and from a Satterthwaite test when variances were unequal.

Table 4.10: Narrow-sense heritability (h^2) of supercooling points in the selected line of population replicate 1 (random mating regime). Heritability across a generation is represented by the estimated slope from the linear regression of offspring supercooling points on the average parental supercooling points. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**R-sq**	**Slope Estimate** (h^2)	**S.E.M.**	***t*-value**	**Pr>*t***
F0-**F1**	189	0.006	0.174	0.163	1.07	0.29
F1-**F2**	123	0.0001	-0.011	0.123	-0.09	0.93
F2-**F3**	313	0.006	-0.573	0.422	-1.36	0.18
F3-**F4**	205	0.055	0.559	0.163	3.44	0.001*
F4-**F5**	166	0.010	0.220	0.175	1.26	0.21
F5-**F6**	191	0.008	-0.278	0.223	-1.24	0.22
F6-**F7**	171	0.003	0.245	0.366	0.67	0.50
F7-**F8**	168	0.003	-0.080	0.109	-0.73	0.47
F8-**F9**	171	0.020	0.488	0.264	1.85	0.07
F9-**F10**	186	0.0003	-0.053	0.224	-0.24	0.81
F10-**F11**	234	0.0003	0.036	0.151	0.24	0.81
F11-**F12**	209	0.014	-0.472	0.273	-1.73	0.09
F12-**F13**	156	0.005	0.272	0.303	0.90	0.37
F13-**F14**	191	0.002	0.075	0.131	0.57	0.57
F14-**F15**	190	0.010	0.357	0.254	1.40	0.16
F15-**F16**	155	0.010	0.319	0.251	1.27	0.21
F16-**F17**	190	0.008	-0.218	0.182	-1.20	0.23
F17-**F18**	237	0.0001	0.034	0.241	0.14	0.89
F18-**F19**	205	0.016	-0.382	0.208	-1.84	0.08
F19-**F20**	237	0.001	0.168	0.320	0.52	0.60
F20-**F21**	238	0.028	0.535	0.204	2.62	0.009*
F21-**F22**	236	0.004	0.152	0.163	0.93	0.35
F22-**F23**	203	0.009	-0.240	0.178	-1.35	0.18
F23-**F24**	178	0.006	-0.093	0.094	-0.99	0.33
F24-**F25**	171	0.003	-0.035	0.047	-0.74	0.46

Table 4.11: Narrow-sense heritability (h^2) of supercooling points in the selected line of population replicate 2. Heritability across a generation is represented by the estimated slope from the linear regression of offspring supercooling points on the average parental supercooling points. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**R-sq**	**Slope Estimate** (h^2)	**S.E.M.**	***t*-value**	**Pr>*t***
F0-**F1**	191	0.010	-0.237	0.168	-1.40	0.16
F1-**F2**	177	0.004	0.192	0.231	0.83	0.41
F2-**F3**	203	0.008	-0.176	0.135	-1.30	0.20
F3-**F4**	173	0.008	-0.288	0.240	-1.20	0.23
F4-**F5**	187	0.002	-0.114	0.173	-0.66	0.51
F5-**F6**	144	0.009	0.328	0.284	1.16	0.25
F6-**F7**	239	0.001	-0.095	0.258	-0.37	0.71
F7-**F8**	210	0.063	0.577	0.154	3.75	0.0002*
F8-**F9**	163	0.003	-0.156	0.220	-0.71	0.48
F9-**F10**	175	0.004	0.202	0.250	0.81	0.42
F10-**F11**	192	0.006	-0.333	0.303	-1.10	0.27
F11-**F12**	153	0.035	-0.620	0.264	-2.35	0.020*
F12-**F13**	191	0.008	0.152	0.124	1.22	0.22
F13-**F14**	238	0.002	-0.088	0.135	-0.65	0.52
F14-**F15**	222	<0.001	0.006	0.195	1.03	0.98

Table 4.12: Narrow-sense heritability (h^2) of supercooling points in the selected line of population replicate 1 (assortative mating regime). Heritability across a generation is represented by the estimated slope from the linear regression of offspring supercooling points on the average parental supercooling points. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**R-sq**	**Slope Estimate** (h^2)	**S.E.M.**	***t*-value**	**Pr>*t***
F0-**F1**	256	0.022	0.227	0.095	2.39	0.018*
F1-**F2**	235	0.002	0.066	0.093	0.71	0.45
F2-**F3**	217	0.001	-0.053	0.109	-0.49	0.62
F3-**F4**	143	0.001	-0.043	0.138	-0.31	0.78
F4-**F5**	158	0.048	0.322	0.115	2.80	0.006*
F5-**F6**[1]	126	*n/a*	*n/a*	*n/a*	*n/a*	*n/a*

[1] only one mating group from F5 produced viable offspring, so covariance estimates could not be calculated

Table 4.13: Genetic covariance of parental supercooling points and offspring survival after partial freezing in the selected line of population replicate 1 (random mating regime). Covariance across a generation is represented by the estimated slope (analysis of maximum likelihood) from the logistic regression of offspring survival after partial freezing on the average parental supercooling point. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**Prop. Freeze Survival**[1]	**Slope Estimate**	**S.E.M.**	**Wald χ^2**	**Pr>χ^2**
F0-**F1**	180	0.28	0.139	0.315	0.20	0.66
F1-**F2**	119	0.44	0.111	0.137	0.66	0.42
F2-**F3**	307	0.23	-1.387	0.481	8.33	0.004*
F3-**F4**	195	0.32	0.108	0.163	0.44	0.51
F4-**F5**	162	0.34	0.0048	0.174	0.001	0.98
F5-**F6**	190	0.40	-0.368	0.233	2.50	0.11
F6-**F7**	171	0.46	0.554	0.398	1.94	0.16
F7-**F8**	158	0.50	0.075	0.127	0.35	0.55
F8-**F9**	166	0.44	0.152	0.244	0.39	0.53
F9-**F10**	179	0.23	0.143	0.217	0.43	0.51
F10-**F11**	225	0.31	-0.045	0.160	0.08	0.78
F11-**F12**	200	0.35	0.052	0.253	0.04	0.84
F12-**F13**	157	0.45	0.118	0.210	0.29	0.59
F13-**F14**	191	0.49	0.160	0.137	1.38	0.24
F14-**F15**	187	0.60	0.036	0.246	0.02	0.88
F15-**F16**	150	0.21	0.431	0.329	1.72	0.19
F16-**F17**	191	0.58	0.198	0.173	1.31	0.25
F17-**F18**	236	0.54	0.037	0.183	0.04	0.84
F18-**F19**	205	0.55	-0.030	0.178	0.03	0.87
F19-**F20**	230	0.56	0.349	0.309	1.28	0.26
F20-**F21**	236	0.48	0.386	0.200	3.72	0.05*
F21-**F22**	232	0.38	-0.019	0.158	0.02	0.90
F22-**F23**	196	0.23	-0.169	0.246	0.47	0.49
F23-**F24**	174	0.17	-0.026	0.144	0.03	0.86
F24-**F25**	164	0.24	-0.017	0.079	0.04	0.83

[1] proportion of late instars (offspring generation) that survived acute partial freezing and eclosed as adults.

Table 4.14: Genetic covariance of parental supercooling points and offspring survival after partial freezing in the selected line of population replicate 2.

Covariance across a generation is represented by the estimated slope (analysis of maximum likelihood) from the logistic regression of offspring survival after partial freezing on the average parental supercooling points. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**Prop. Freeze Survival**[1]	**Slope Estimate**	**S.E.M.**	**Wald χ^2**	**Pr>χ^2**
F0-**F1**	188	0.31	-0.0072	0.228	0.001	0.98
F1-**F2**	166	0.40	0.379	0.273	1.93	0.17
F2-**F3**	199	0.55	0.142	0.186	0.58	0.45
F3-**F4**	166	0.53	-0.267	0.318	0.70	0.40
F4-**F5**	177	0.41	0.430	0.175	6.03	0.01*
F5-**F6**	138	0.41	0.286	0.244	1.38	0.24
F6-**F7**	229	0.28	-0.363	0.240	2.29	0.13
F7-**F8**	209	0.39	0.296	0.136	4.74	0.03*
F8-**F9**	158	0.32	-0.563	0.220	6.55	0.01*
F9-**F10**	172	0.53	-0.170	0.236	0.52	0.47
F10-**F11**	190	0.54	0.118	0.253	0.22	0.64
F11-**F12**	157	0.22	-0.047	0.298	0.03	0.88
F12-**F13**	189	0.45	-0.091	0.115	0.63	0.43
F13-**F14**	235	0.55	-0.067	0.146	0.21	0.64
F14-**F15**	216	0.43	-0.014	0.188	0.01	0.94

[1] proportion of late instars (offspring generation) that survived acute partial freezing and eclosed as adults.

Table 4.15: Genetic covariance of parental supercooling points and offspring survival after partial freezing in the selected line of the assortative mating regime population (where F0 came from the F19 population in the random mating population of PR1). Covariance across a generation is represented by the estimated slope (analysis of maximum likelihood) from the logistic regression of offspring survival after partial freezing on the average parental supercooling points. *n* = the total number of offspring supercooled in a given generation. An ***** indicates significance at α=0.05.

Parent-**Offspring**	***n***	**Prop. Freeze Survival**[1]	**Slope Estimate**	**S.E.M.**	**Wald χ^2**	**Pr>χ^2**
F0-**F1**	251	0.45	-0.077	0.089	0.78	0.38
F1-**F2**	230	0.58	-0.090	0.11	0.67	0.41
F2-**F3**	216	0.35	-0.193	0.114	2.86	0.09
F3-**F4**	141	0.16	-0.403	0.232	3.02	0.08
F4-**F5**	115	0.15	0.687	0.341	4.06	0.04*
F5-**F6**[2]	123	0.16	*n/a*	*n/a*	*n/a*	*n/a*

[1] proportion of late instars (offspring generation) that survived acute partial freezing and eclosed as adults.

[2]only one mating group from F5 produced viable offspring so covariance estimates could not be calculated.

Figure 4.1: Mating container design. Containers were 0.47L (16 fluid oz) clear plastic cups with lids. The lids were perforated to allow air circulation. Cheesecloth was placed between the lid and the cup opening to deter females from ovipositing on the lids. To supply moths with water (10% honey/water solution), a cotton dental roll (1 1/2" x 3/8") was inserted into a hole cut into the bulb of a 5 mL disposable pipette. The tip of the pipette was also cut so that ~3/4" remained above the bulb. The pipette fit snugly into the straw-hole of the lid. The cotton was re-soaked every other day with honey solution using another pipette to slowly drip solution through the top opening of the cut pipette. Females oviposited on the sides and bottom of the cup

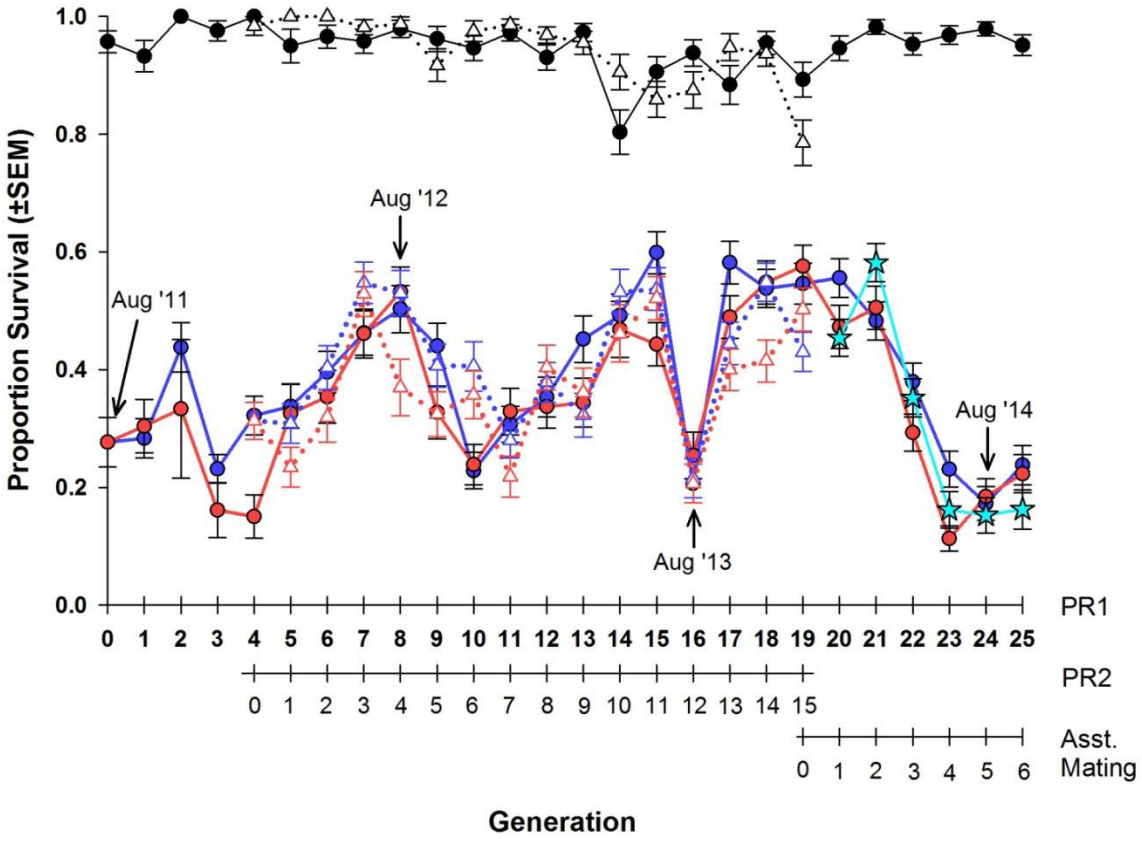


Figure 4.2: Survival (i.e., successful adult eclosion), through time, in laboratory populations of *E. postvittana* under various experimental conditions. Solids lines and filled circles or stars are experimental lines from the first population replicate (PR1; 25 gens). Dotted lines and open triangles represent experimental lines from the second population replicate (PR2; 15 gens). *Blue* lines indicate the proportion survival of late instars following partial freezing in selected lines. *Red* lines indicate the proportion survival of late instars following partial freezing in unselected lines. *Black* lines indicate the natural mortality (i.e., mortality unrelated, directly, to cold exposure) in the unselected lines. The *teal* lines and stars indicate the proportion survival of late instars after partial freezing in the assortative mating line (Asst. Mating; six gens). Approximate yearly intervals are marked for calendar date reference.

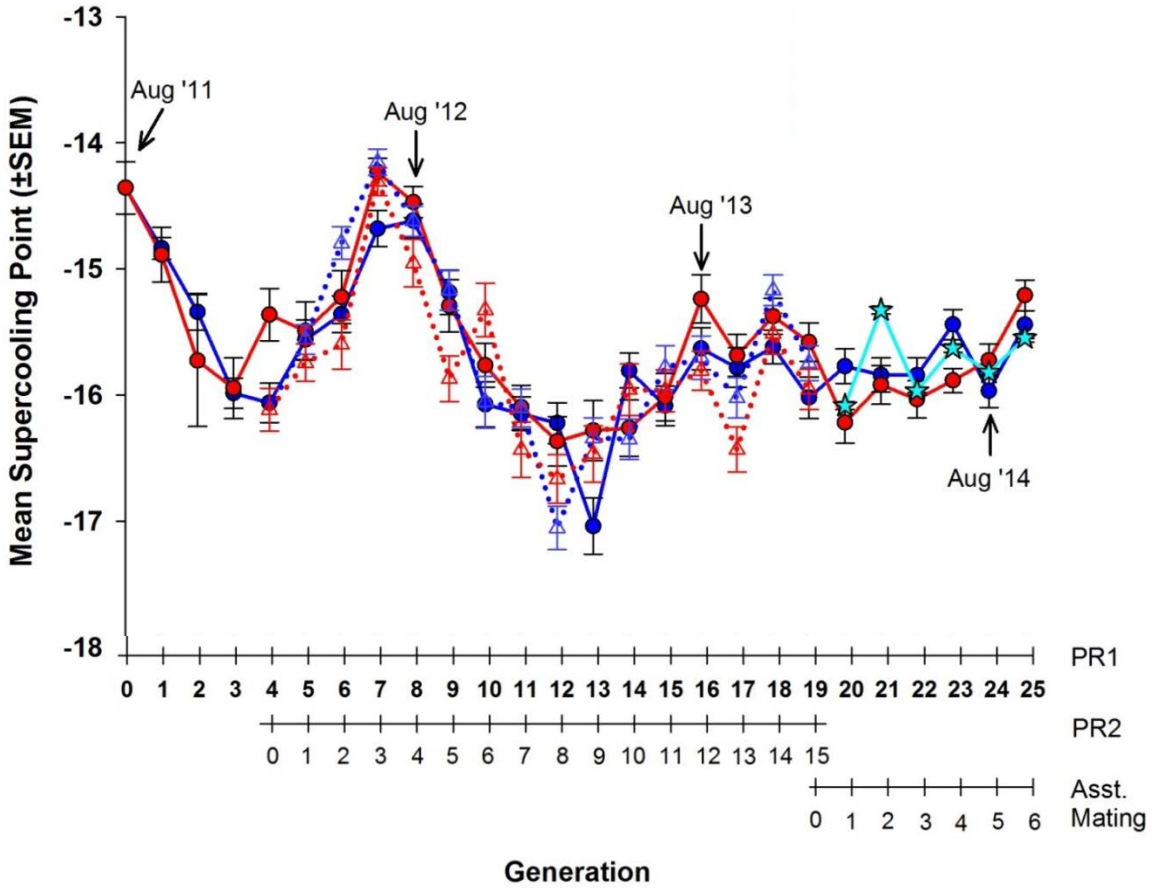


Figure 4.3: Mean supercooling points, through time, in laboratory populations of late instar *E. postvittana* under various experimental conditions. Solids lines connecting filled circles or stars are experimental lines from the first population replicate (25 gens). Dotted lines connecting open triangles represent experimental lines from the second population replicate (15 gens). *Blue* lines indicate the mean supercooling points of late instars in selected lines. *Red* lines indicate the mean supercooling points of late instars in unselected lines. The *teal* lines and stars indicate the mean supercooling points of late instars in the assortative mating line (six gens). Approximate yearly intervals are marked for calendar date reference.

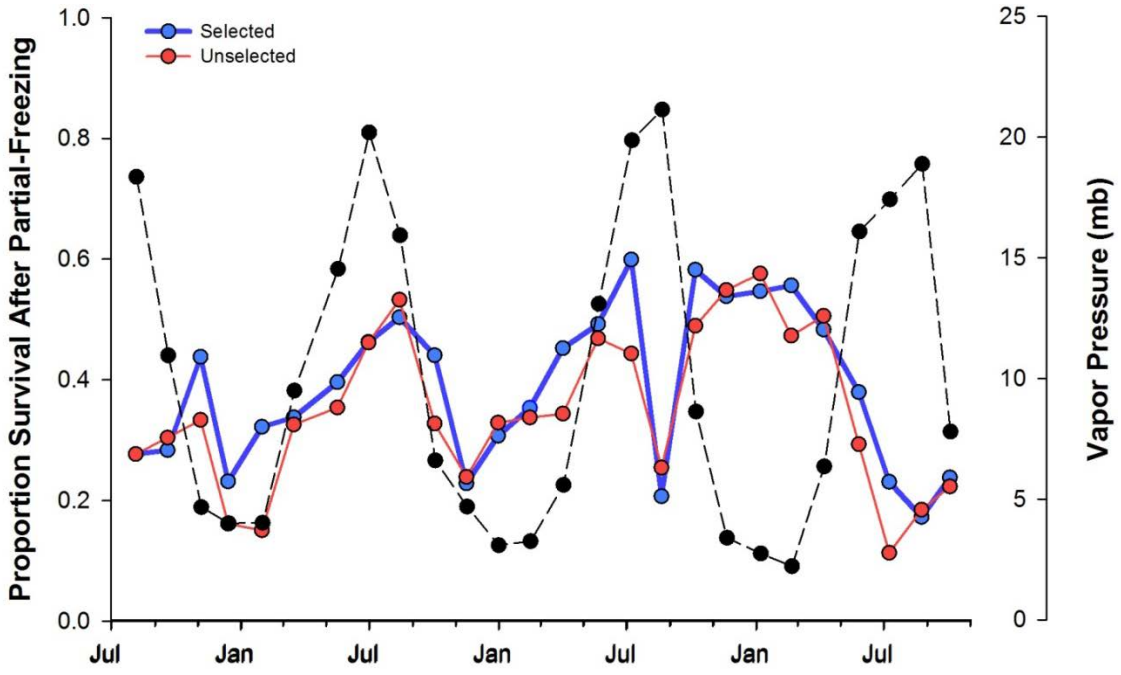


Figure 4.4. Comparison of *E. postvittana* survival after partial freezing with the atmospheric vapor pressure in St. Paul, MN, though time (July 2011–Nov 2014). Solids lines are experimental lines from the first population replicate (25 gens). *Blue* lines indicate the proportion survival of late instars following partial-freezing in selected line. *Red* lines indicate the proportion survival of late instars following partial-freezing in the unselected line. *Black* dotted lines indicate the average vapor pressure during the period each selection pressure was applied during a given generation. Vapor pressure measurements were converted from sub-hourly records of dewpoint temperatures (°C) from the Minneapolis/St. Paul International Airport weather station (mesonet.agron.iastate.edu/request/download.phtlml?network=MN_ASOS). Dew points were converted into vapor pressure (mb) using the formula $e = 6.11*10^{((7.5*T_d)/(237.3+ T_d))}$, where T_d is the dewpoint temperature (°C) (source: National Oceanic and Atmospheric Administration).

CHAPTER 5:

Could natural selection change the geographic range limits of light brown apple moth (Lepidotpera: Tortricidae) in North America?

[Reprinted with permission from: Morey et al. 2013. NeoBiota 18: 151–156. The article has been reformatted to meet the requirements of a University of Minnesota dissertation]

5.1 Summary

We artificially selected for increased freeze tolerance in the invasive light brown apple moth. Our results suggest that, by not accounting for adaptation to cold, current models of potential geographic distributions could underestimate the areas at risk of exposure to this species.

5.2 Introduction

Forecasting future distributions of invasive insects is important for many management and regulatory decisions. However, numerous challenges exist in creating accurate, biologically relevant models and maps that are meaningful over time (Venette et al. 2010). In particular, no models currently account for the potential of an invasive species to adapt to a new environment. In fact, demographic models in invasion biology commonly treat species as "homogenous immutable entities" (Lee 2002). For invasions by alien species in North America, adaptation to cold temperature may be especially important at northern latitudes or high elevations; cold often prevents species from surviving year round (Huey 2010, Venette 2013). For example, cold is likely to constrain the future distribution of the light brown apple moth, *Epiphyas postvittana* (Walker), a recent insect invader to North America. No model for *E. postvittana* currently accounts for the possibility of evolution of increased cold tolerance. The objective of this study was to determine if it was possible to artificially select for increased cold tolerance in this species, and if so, to begin exploring the subsequent geographical repercussions. *Epiphyas postvittana* is considered to be predominately freeze intolerant during its purported overwintering stage, the late instar larva (Bürgi and Mills 2010), but

preliminary data suggests that a small proportion of the population may also be freeze tolerant (Venette, unpublished data). This phenomenon could be considered at least "partial freeze tolerance" (Sinclair 1999), and enabled freezing to act as a strong selection pressure for enhanced cold tolerance in our study.

5.3 Materials and Methods

Epiphyas postvittana eggs were obtained from USDA-APHIS (permit P526P-11-03713). All subsequent rearing and experimentation was conducted in a Biosafety Level 2 Containment Facility in St. Paul, MN. Eggs were held at 23°C, 60% RH, and resulting neonates were reared on artificial bean diet until late instars. We cooled 4-6th instars (verified through head capsule measurement; Danthanarayana 1975) individually inside gelatin capsules at ~1°C/min to their supercooling point inside a -80°C freezer (modified from Carrillo et al. 2004). Once freezing occurred, larvae were immediately returned to 23°C and given fresh diet. Mortality was measured as failure to eclose. Surviving moths were randomly mated in 0.47L (16 fluid oz) containers with 1-3 individuals of each sex. Randomly selected offspring were subsequently reared and supercooled as previously described. This procedure was repeated for nine generations. A minimum of 102 larvae were supercooled in each generation. A control population was maintained simultaneously and identically, save exposure to freezing.

Survival following freezing after nine generations was compared between the selected and control populations using non-parametric cumulative incidence functions (CIF) in SAS 9.3 (SAS Institute, Cary, N.C.) to address competing risks (Satagopan et al. 2004). Competing risks were those individuals that froze and survived. These estimates were then used to calculate the temperature required to kill 50% (LT_{50}) of each population.

We used NAPPFAST (Magarey et al. 2009) to map where temperatures might fall below the LT_{50} of the selected or control populations. For each 10 x 10km grid cell, NAPPFAST calculated the proportion of the last 10 years in which the lowest

temperature of the year was colder than the LT_{50} for each population. We ran the model with 3-D interpolated climate data.

5.3 Results and Discussion

After only nine generations of selection, the probability of survival following freezing was significantly (α=0.1) greater for the selected population than the unselected control (Figure 5.1; P= 0.078, df=1, χ^2 =3.11). The LT_{50} for the unselected and selected populations were estimated to be –16.5°C and –19.0°C, respectively.

Figure 2 illustrates the geographic significance of a putative increase in cold tolerance for *E. postvittana*. Dark grey areas indicate the most dramatic effect of cold, where the LT_{50} was reached in 9-10 of the 10 years modeled. Cold was sufficient to exclude *E. postvittana* in many northern areas (e.g., Minnesota, the Dakotas, Wyoming, and much of Canada) using either the unselected (Figure 5.2a) or selected (Figure 5.2b) model. However, for other midwestern, eastern, and southern states, there was an overall reduction in the number of years where the LT_{50} was reached when using the selected population; the red to white colors shifted north. For example, the unselected model projected that nearly all of Michigan would reach the LT_{50} during 90-100% (dark grey) of the years modeled. In contrast, the selected model projected that most of the state would only reach the threshold between 50 to 80% of the modeled years (red and orange). Similarly, western states (e.g., Nevada and Idaho) showed an eastward shift in the number of years that temperatures did not reach the LT_{50}

Current risk maps that exist for *E. postvittana* (e.g., Fowler et al. 2009, Gutierrez et al. 2010, Lozier and Mills 2011), acknowledge the importance of cold in shaping this species' potential U.S. range. However, the parameter(s) used to describe cold tolerance is/are assumed to be static. If natural selection follows a pattern similar to what our research suggests, within a relatively short period, current models may underestimate the risk of *E. postvittana* exposure in some areas in the future.

Uncertainty is inherent in pest risk models and contending with it is an ongoing area of research (Venette et al. 2010). Our study attempted to address the uncertainty

related to the potential of a species to adapt to a cold environment, and highlighted the geographic consequences if adaptation to cold is not considered. However, other sources of uncertainty still remain and are important future directions of this work. For example, the time of year may influence the effectiveness of selection on *E. postvittana*, assuming multi-voltinism and a randomly mating population. Selection is only likely to increase cold tolerance when there is a strong pressure (winter). But if there is any trade-off between increased cold tolerance and fitness (see Watson and Hoffmann 1996, Huey 2010), cold adapted individuals may be selected against during months when selection pressure is reduced (summer). Similarly, particularly for a highly polyphagous insect like *E. postvittana*, host plant variability could also affect cold tolerance measures (e.g., Liu et al. 2009). Understanding the relationship between cold tolerance and these additional factors will undoubtedly continue to further enhance the accuracy and ultimate utility of pest risk mapping tools.

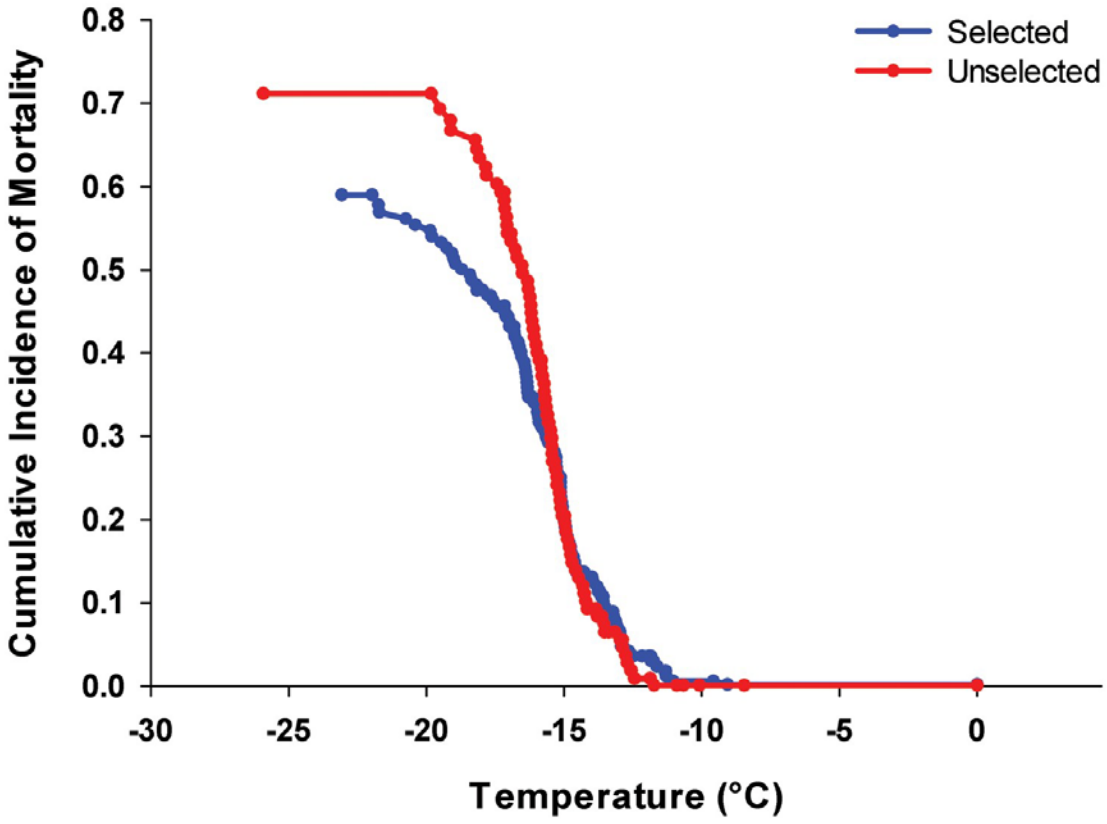


Figure 5.1: Cumulative probability distributions of the survival of *E. postvittana* late instars with (n=179) and without (n=109) nine generations of artificial selection for increased cold tolerance.

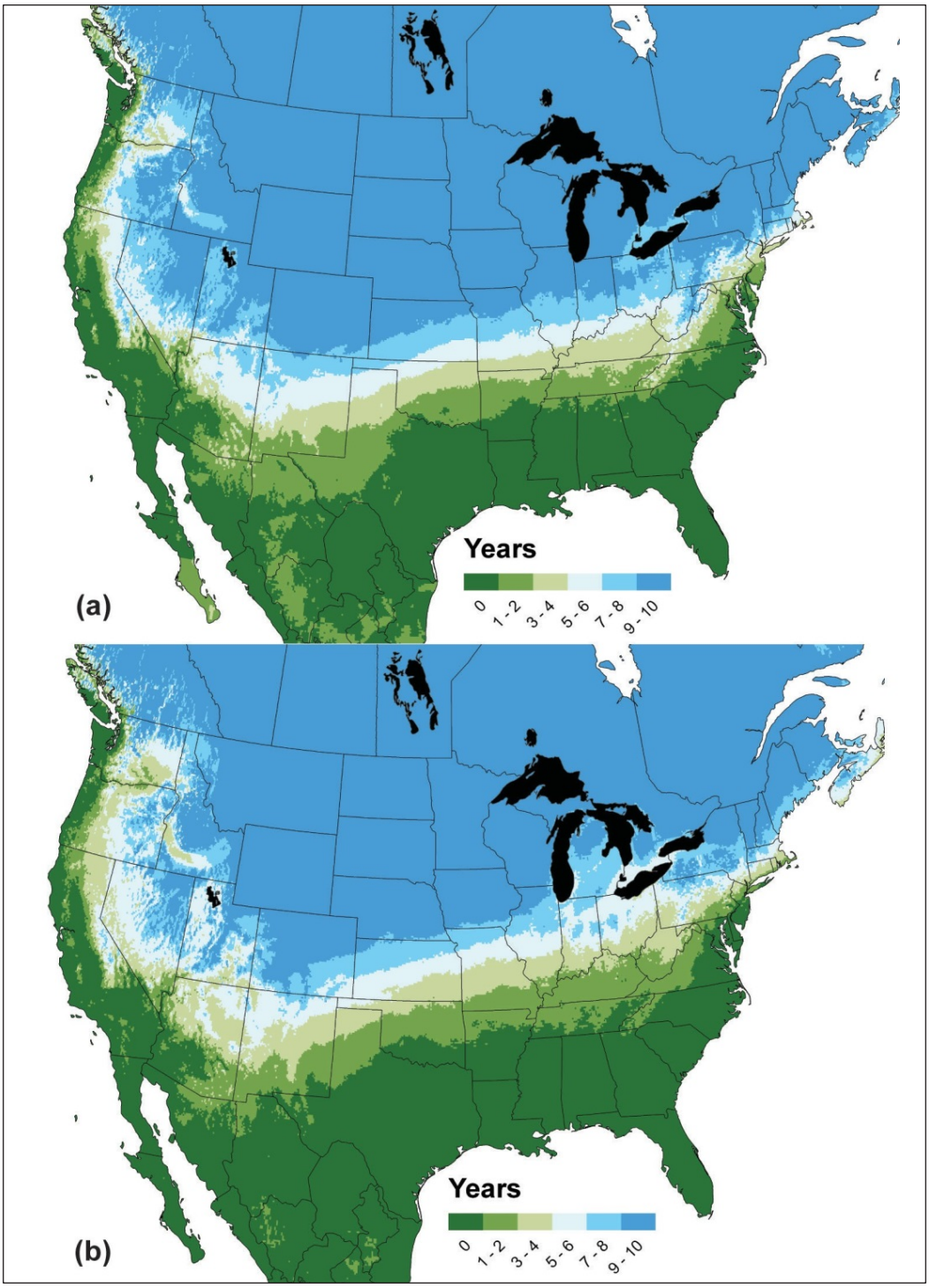


Figure 5.2: The frequency of years (2002-2012) in which temperatures fell below the threshold required to cause 50% mortality (LT_{50}) of *E. postvittana* in North America: **(a)** without selection, and **(b)** after nine generations of selection for increased freeze tolerance. Dark grey indicates where the LT_{50} was reached in 9-10 of the 10 years, white indicates where the LT_{50} was never reached during the 10-yr period.

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APPENDIX 1:
Report submitted to Better Border Biosecurity upon completion of training at Lincoln University, March 2014:
Preliminary analysis of phosphoglucose isomerase in the Light Brown Apple Moth (Epiphyas postvittana)

A1. Objectives

- To continue exploratory analysis of the potential genetic basis of environmental tolerance.
- To receive training in molecular methods relating to phosphoglucose isomerase (PGI) allozyme analysis in insects, which has been correlated with fitness and temperature tolerance in insects. Two former Lincoln University graduate students used and optimized these methods for portions of their entomological dissertations, including techniques based specifically on my study organism, *Epiphyas postvittana*. This invasive species has recently established in North America and many questions remain about its future geographic spread on the continent.
- To apply analytical techniques developed at Lincoln to *E. postvittana* specimens housed in quarantine at the University of Minnesota in the United States. Specimens in Minnesota are part of a selection experiment aimed at studying the evolutionary potential for a species to increase cold tolerance within a population. PGI analysis is likely to add a novel and informative molecular characterization of the selection results and further knowledge towards determination of the genetic basis of environmental tolerance.

A2. Background

For invasive insects, knowledge of thermal tolerance is an essential component to accurately predict their potential geographic spread and establishment. In temperate areas, cold temperature is often a major selective agent dictating when and where species

successfully occur. Research at the University of Minnesota (Dept. of Entomology) explores how an insect recently invasive to the U.S., the light brown apple moth (*Epiphyas postvittana*), responds to cold temperature. In particular, the research investigates the potential for *E. postvittana* to adapt to cold temperatures and how such adaptation could affect the prediction and management of this invasive species. One objective is to attempt to select for increased cold tolerance in a laboratory colony of *E. postvittana*. Currently, any response to selection is characterized using only physiological metrics of fitness and development. However, in collaboration with Lincoln University and the Better Border Biosecurity (B3) consortium, molecular and genetic analysis of the research colonies will be carried out to develop a better understanding of the mechanism underlying the results of cold temperature adaptation experiments. This will specifically involve an investigation of any variation in the allozymes of phosphoglucose isomerase and cold tolerance.

Phosphoglucose isomerase (PGI) is an enzyme involved in an early step of glycolysis and has been shown to exhibit a high degree of polymorphism in many taxa, including insects (reviewed in Wheat 2010). The variation seen among PGI "allozymes" in insects has often been correlated with individual fitness-traits, such as locomotion, dispersal, and flight metabolism (e.g., Watt 2003, Haag et al. 2005, Dick et al. 2014), geographic distribution (e.g., Rank and Dahlhoff 2002), and fecundity (e.g., Saastamoinen 2007). Of importance to the current research, PGI variation has also been shown to co-vary with cold-stress tolerance. For example, Neargarder et al. (2003) found that willow beetle adults and larvae survived in greater proportion following cold stress if they expressed a particular PGI genotype. Similarly, Karl et al. (2008) found one PGI genotype dominated in populations of Copper butterfly that were more cold-stress resistant. Luo et al. (2014) also found allelic variation in the *pgi* gene of Glanville fritillary butterfly adults correlated with a measure of cold tolerance. Relatively few studies, however, have fully investigated this link and its potential application to invasion biology is beginning to be recognized (Hanski and Saccheri 2006, Lefort et al. 2014).

With respect to *E. postvittana*, the first research question is whether any allozyme variation is apparent in PGI for *E. postvittana*. Secondly, if variation is observed, does this correlate with either a response to cold tolerance selection or, if no clear response to

selection is seen, to any phenotypic plasticity in cold tolerance. While currently it is not yet clear if the selection study will yield a consistent response to selection (data collection and analysis is still on-going), significant plasticity in cold tolerance has been observed over time, suggesting that PGI variation could be compared among extreme phenotypic groups of the selected populations.

A3. Materials and Methods

Allozyme variation among individual moths was explored in two ways, 1) by looking for differences in protein electrophoretic mobility, and 2) identifying differences in single nucleotide polymorphisms (SNPs) within the *pgi* gene. To trial the methods, the *E. postvittana* specimens analyzed were predominately laboratory-reared moths, although four wild moths were eventually collected near the Lincoln University campus and included in the analysis. Detailed methodologies are described below.

A3.1 Specimen source and preparation

Adult *E. postvittana* were collected within 24hrs of eclosion from lab-colony pupae provided by Lincoln University collaborators. Each adult was placed inside individual centrifuge tubes and immediately frozen until sample preparation. For protein electrophoresis, specimens were crushed with a disposable pestle in 250µl of refrigerated Tris HCl buffer and then centrifuged for 90 s. Initially, whole specimens were used, but due to excessive scales from parts of the body contributing little tissue, subsequent samples contained only the abdomen. The supernatant was used immediately for analysis. For DNA analysis, one to two legs were removed from each specimen using forceps sterilized in EtOH in between each sample. Legs were placed in separate centrifuge tubes and then frozen if DNA extraction did not immediately follow.

A3.2 Protein-level analysis

PGI genotypes were characterized by electrophoretic mobility using cellulose acetate electrophoresis (based on Herbert and Beaton 1993). Sample application followed

Lefort (2013), using a 10 µl aliquot of each sample supernatant (above) and a Super z-12 application kit (Helena Laboratories). Electrophoresis was initially carried out at 100V, 500 mA, for 30 min, but parameters were eventually extended to 150V for 45mins to improve band separation. Staining recipes and procedure followed Lefort (2013), although the staining period was longer (20-30 min) and images were taken for band scoring while plates were still wet (rather than after oven-drying).

In total, 20 lab-colony individuals (males and females) were analyzed, as well as four wild-caught individuals (all males).

A3.3 DNA-level analysis

DNA was extracted using a *prep*GEM® Insect kit (ZyGEM), adding 40µl of extraction master mix (buffer, prepGEM, DI-water) to each sample. Extraction conditions were: 75°C for 15 mins, 95°C for 5 mins, and 4°C as a final holding temperature.

The region of *pgi* encompassing exons 7-9 was amplified in a 25µl total reaction volume containing GoTaq® Green Master Mix (Promega) (12.5µl), water (9.5 µl), 0.5 µl of each primer (200µM stock solution), and 2µl DNA extract. Two EPIC primers, developed by He (2010) for *pgi* in *E. postvittana*, were used: PGI-33_F (forward: TACTCCCTCTGGTCGGC) and PGI-34_R (reverse: CCTGGTGTATGAGCTGGTAG).

PCR conditions were initially a 4-step "Touchdown program", which successfully produced bands. However, in attempt to reduce run time, subsequent programs had three total steps and extension time and annealing temperature were altered, with final conditions being: 95°C for 5 min, followed by 40 cycles of 95°C for 30 s, 50°C for 30 s, 72°C for 3 min, followed by a final extension of 72°C for 7 min (4 °C as final holding temperature). An annealing temperature of 54°C (for 30 s) was also tried, but no bands were produced. 51°C (for 30 s) was also used, and produced banding, though with less intensity than 50°C.

PCR products were visualized by loading 4 µl samples onto 0.08% agarose gel with 2µL RedSafe™ (Ecogen) stain added to TBE buffer, and submerged gel electrophoresis at 100V (500 mA) for 30 min. Bands were viewed and captured over UV light. Based on clarity of banding, some products were sent for in-house sequencing. To prepare products for sequencing, a reaction mixture for each primer using was made with

the following concentrations: 0.5 µl BigDye (v3, Applied Biosystems), 2 µl BigDye buffer, 3 µl water, 0.5 µl PCR product, and 4 µl of 5 µM primer. Sequences were subsequently edited and aligned using the program Geneious (Biomatters, Auckland).

A4. Results and Discussion

A4.1 Protein-level

As described in Herbert and Beaton (1993), PGI is a dimeric enzyme and its genotypes can be inferred based on characteristic migration patterns; homozygotes are indicated by a single band per individual, while heterozygotes have three bands where the two peripheral bands should align with the bands homozygous for those alleles. Based on this, all *E. postvittana* colony individuals analyzed appeared to be homozygous for the same allele (i.e., a single band with the same migration distance). A single wild individual, however, appeared to be heterogygotic (Figure A1, W4).

While little-to-no variation might be expected in a laboratory population with minimal out-crossing, conclusions are quite preliminary at this point. Many runs were subject to various user-errors while still learning the technique. Additionally, the staining reagents for some runs were old, and parameters such as electric voltage/time and buffer pH may not have been optimal for *E. postvittana.* As a consequence, visualization of bands on the acetate plates was generally quite poor (see Figure A1 for example of the most successful visualization). Importantly, the putative heterozygote seen in one wild sample indicates that *E. postvittana* may be polymorphic at the PGI locus; being polymorphic is vital for the hypothesis of correlation between cold tolerance variation and PGI to be possible.

A4.2 DNA-level

The majority of samples produced a product of the anticipated size of about 500 bp, including introns. However, primer-dimer was observed in most cases, as well as additional weak bands that could indicate nonspecific binding of the primers. This made sequencing difficult without additional clean-up. However, sequencing was desirable to

confirm that the correct gene region had been amplified. Therefore, specimen #2 (Figure A2) was sent for sequencing because it produced the clearest single banding pattern. However, an actual sequence could not be found and aligned with confidence; results were quite noisy. Additional products (wild #1 and colony #12) were prepared and submitted for sequencing on my final day at Lincoln, but those results have not yet been received.

While clear specific sequence-level variation was not obtained, PCR products showed some variation in migration patterns, indicating differences in their nucleotide sequence compositions (Figure A2). Future work should investigate the use of different primers. Of note, the wild individual (W4) that produced a unique electrophoretic pattern also produced a unique PCR pattern.

A5. Conclusions and Future Directions

Using both laboratory and wild individuals of *E. postvittana*, I received training in two techniques involving the analysis of the glycolytic enzyme, PGI. I also significantly strengthened my competency in multiple molecular concepts, which will allow my current research on insect cold tolerance to be viewed within a wider context. Based on the relative success of the preliminary investigation of PGI in *E. postvittana* in New Zealand, the intention to analyze Minnesota specimens for PGI variation will continue. Initial explorations will focus on protein-level analysis, but through the use of isoelectric focusing in a polyacrylamide matrix (BioRad) to detect genotypic differences. This change in methodology is due in part to the readily available equipment locally, but also because the preliminary work at Lincoln suggests cellulose acetate electrophoresis may not be the most suitable substrate for analyzing this enzyme system in this particular species. A different method may allow for improved migration patterns and visualization. Isoelectric focusing is known to achieve high resolution of enzyme banding patterns (Munderloh et al. 1994), and McIntosh and Ignoffo (1983) found greater separation of PGI variants among two related moth species using isoelectric focusing than with acetate electrophoresis.

If initial protein analysis yields significant findings – e.g., genotypic variation that corresponds with variation in the thermal history of a population --, a more extensive specimen analysis will be conducted, and possibly further work will be completed to characterize the DNA-level polymorphisms of those samples. This work will comprise an additional component of my dissertation (e.g., chapter, appendix), and depending on the results, may produce further publication.

A6. Acknowledgements

I found my time at Lincoln University to be very insightful and am incredibly grateful for the unique opportunity. I was introduced to valuable skills and techniques outside of my expertise that I can now apply and expand upon with my own work, and that will undoubtedly serve me well in my future scientific career. Just as important are the collaborative relationships I strengthened and formed, including the generous guidance and support from Drs. Sue Worner and Karen Armstrong, as well as their students Sam Brown, Dr. Ana Chomic, and Marie-Caroline Lefort. My sincere thanks are extended to them all. Not least, I thank B3, Better Border Biosecurity Consortium, for funding my visit and subsequent training at Lincoln University.

Figures

Figure A1: Example of electrophoretic allozyme products. Plate from March 27th run. Electric current conditions were 150V, 4mA, for 45 minutes. From left to right: #5, #16, #17, #18 (lab colony individuals), W1, W2, W3, W4 (wild-caught individuals). Image enhanced in Photoshop. Genotypic distinction is difficult; however, W3 appears to be heterozygotic (three bands) with the rest possibly being homozygous for the same allele (one band).

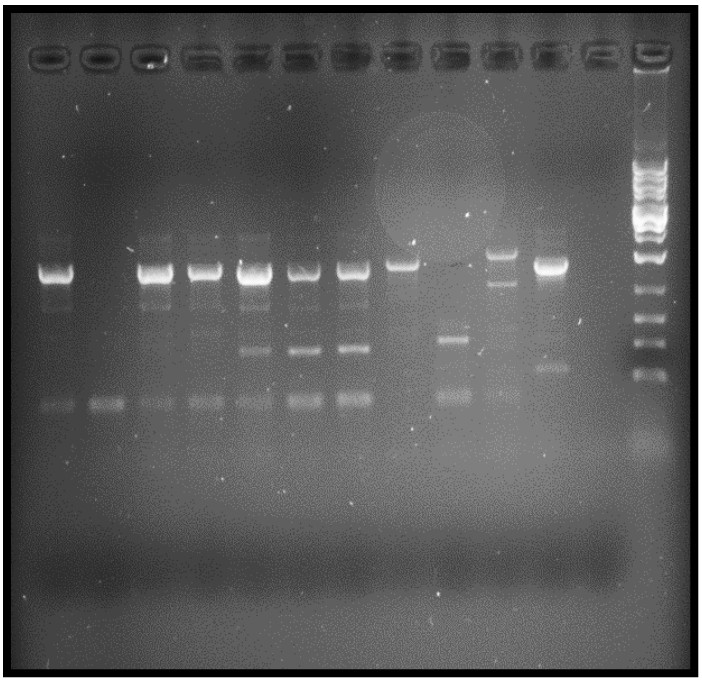

Figure A2: Example of PCR products. Gel from March 27th PCR. Samples, left to right: #2, #6, #12, #14, #16, #17, #18 (lab colony individuals), W1, W2, W3, W4 (wild-caught individuals), water, 1Kb DNA ladder (Axygen). Main banding occurred for most samples at ~500bp. Image enhanced in Adobe Photoshop.