PLASTICITY OF COLD-HARDINESS IN THE EASTERN SPRUCE BUDWORM, *CHORISTONEURA FUMIFERANA*

by

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Abstract

Of all abiotic factors that drive range boundaries, temperature is the best studied because of its pervasive influence on biological processes. For populations at high-latitudes, extreme cold and the populations’ cold-hardiness set the range boundary. Phenotypic plasticity, where a single genotype results in differentiated phenotypes under differential environmental conditions, can assist populations in managing changing temperatures. Local adaptation in phenotypic plasticity, which results in different responses in different populations, can assist with the variability in temperature a species can experience across its range, especially at range boundaries. I used the eastern spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae) as a model system for exploring local adaptation and phenotypic plasticity of insect cold-hardiness. The species is one of the most destructive forest pests in North America, therefore accurately predicting its range and population growth is essential for management. In this thesis, I show that there is no transgenerational plasticity in cold-hardiness. However, I found a fitness cost associated with repeated cold exposures. Additionally, across the species’ range, I found both local adaptation of seasonal cold-hardiness and short-term plasticity of this trait. Therefore, the findings of this thesis provide evidence for including phenotypic plasticity and local adaptation when modelling species distributions under climate change.
Lay Summary

For terrestrial insects enduring extreme temperatures is essential to survival. If insects encounter extreme temperatures, they can either migrate to more suitable areas, evolve increased tolerance to those temperatures, or exhibit phenotypic plasticity, which is the ability to change your response to environmental conditions without genetic change. The eastern spruce budworm is one of Canada’s most destructive forest pest species. It has a wide geographic range therefore populations are exposed to a variety of temperatures. In this thesis, I have tested for transgenerational plasticity (when the parental environment influences offspring responses) and local adaptation of phenotypic plasticity (different responses to environmental change in different populations) of cold-hardiness in the species. I found no evidence for transgenerational plasticity. However, I found evidence that northerly populations exhibit increased capacity for cold-hardiness when repeatedly cold-exposed compared to southerly populations. These results are important for accurately modelling population growth and species distributions.
Preface

The research presented in this thesis is my original work. Under the guidance of my supervisor, Dr. Katie E. Marshall, I designed the experiments, collected the data, performed relevant analyses and summarized the findings here. Additional suggestions and feedback were given by Drs Amy L. Angert, Amanda D. Roe, Patricia M. Schulte and Michelle Tseng.
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As we let our own light shine, we unconsciously give other people the permission to do the same.
Chapter 1: General Introduction

1.1 The limits to species ranges

All species have a distinct geographical range. Range boundaries can be determined by a broad array of factors (Spicer & Gaston, 1999; Price & Kirkpatrick, 2009), including abiotic factors like temperature (Tomanek, 2008) and biotic factors like predation (Holt & Barfield, 2009). The North-South hypothesis (reviewed in Cahill *et al*., 2014) is that equatorward range limits are more often set by biotic factors, whereas abiotic factors (particularly climate) play a bigger role at poleward limits. However, it is more likely that a combination of factors limits ranges (Harley, 2000; Merrill, 2007). In addition, historical and spatial aspects like past glaciation and mountain ranges may also play a role in the boundaries of current ranges (Gaston, 2000). Range boundaries have been extensively studied but the mechanisms that set those limits are still poorly understood.

Of all abiotic factors that drive range boundaries, temperature is the best studied because of its pervasive influence on biological structures and functions. Temperature drives biochemical reaction rates due to direct Arrhenius effects (Somero *et al*., 2017). It can influence protein stability, therefore changing both binding affinity and catalytic rate (Somero *et al*., 2017), further influencing reaction rates. In addition, changing temperatures alter membrane fluidity, where decreasing temperature decreases membrane fluidity, in turn, decreasing
the reaction rate for membrane-bound proteins (Somero et al., 2017). Extreme temperatures can result in macromolecule denaturation, apoptosis and consequently, mortality of the organism. For example, mussel hemocytes experience DNA damage after acute temperature exposure (Yao & Somero, 2012), which decreased genome integrity and initiated apoptosis. This stress can also activate downstream molecular responses important for dealing with stress, such as heat shock or antifreeze proteins, which can further contribute to whole-organism thermal tolerance (Somero et al., 2017). For ectotherms, whose body temperature closely matches their environmental temperature, temperature effects integrate from biochemical reactions up to the whole organism.

Some species have broad geographic ranges such that different populations experience substantially different thermal regimes. For populations at high latitudes, extreme cold temperature is an integral temperature stress limiting their distribution. Small ectotherms in these environments are especially at risk of freezing their body fluids due to their relatively lower thermal inertia (Rubalcaba et al., 2019). Freezing is deadly for most species because of the mechanical damage in cells caused by ice crystal formation and cellular dehydration, which can result in an accumulation of solutes and altered biochemical gradients (Lee, 2010). By comparison, populations at lower latitudes experience on average warmer temperatures and less variability in thermal conditions (Sunday et al., 2011a). Therefore, high latitude populations potentially
experience much higher selection pressure on cold tolerance than low latitude populations.

1.2 Evolved responses to temperature

The ability to survive stressful temperatures can come from two evolved responses: phenotypic plasticity and local adaptation. Plasticity is the altered phenotypic expression of a single genotype induced by different environmental conditions and can also integrate from molecular to whole-organism scale (Pigliucci, 2001). For example, fathead minnows can increase their critical thermal maxima (CT$_{\text{max}}$) when acclimated to warmer water temperatures (Salinas et al., 2019), and similarly, they are able to seasonally increase heat shock protein (Hsp70) expression as water temperatures increase (Feder et al., 1994). By contrast, local adaptation occurs because of spatial differences in forces associated with natural selection. This results in a population evolving traits that increase fitness in its local habitat, regardless of its impact in other habitats (Kawecki & Ebert, 2004). For example, damselflies (Ischnura elegans) show strong allelic turnover along their range associated with climatic variables such as maximum summer temperature (Dudaniec et al., 2018). Consequently, we might expect that high latitude populations exhibit either local adaptation in cold tolerance or higher phenotypic plasticity of cold tolerance.

In addition, phenotypic plasticity can undergo local adaptation (Kawecki & Ebert, 2004; Calosi et al., 2008), resulting in differentiated responses in different
populations. For example, Yampolsky et al. (2014) tested for both local adaptation and adaptive plasticity in high temperature tolerance in the widely distributed zooplankton, *Daphnia magna*. Individuals from sites with higher average temperature had higher heat tolerance suggesting local adaptation. Acclimation to warmer temperatures also increased heat tolerance in all populations (but to different degrees) suggesting adaptive plasticity. Therefore, local adaptation in phenotypic plasticity can assist in driving range edges by providing locally-suited capacities for tolerance shifts.

Phenotypic plasticity can occur on multiple timescales: hardening, acclimation and over development. Stress hardening occurs rapidly after short exposures to sublethal conditions and is usually reversible (Mitchell et al., 2011). Rapid cold-hardening (RCH) occurs after cold exposures on the scale of minutes to hours (Lee et al., 1987; Lee & Denlinger, 2010). Marshall & Sinclair (2012) differentiate between a change in response after a single compared to repeated cold shocks, which are more common in nature. In contrast, acclimation such as seasonal cold-hardening occurs over longer timescales of days to months (Lee, 2010). Since both hardening and acclimation result in a similar response of increased cold tolerance, they might share biochemical underpinnings such cryoprotectants and membrane modifications. In addition, there is also evidence that they can layer on top of each other (Kawarasaki et al., 2013), which suggests there might also be non-shared mechanisms, although the degree of reliance on these mechanisms are unknown (Teets & Denlinger, 2013). Across developmental
stages, individuals can be plastic in their responses. As an example, Jensen et al. (2007) showed large variation in cold tolerance of *Drosophila melanogaster* depending on the lifestage tested. Additionally, *Drosophila* showed the capacity to undergo RCH across all lifestages, however, some lifestages had negative or neutral effects of RCH. This provides evidence for the interaction between the timescales of plasticity and the costs associated with them.

Apart from intragenerational plasticity, there is also transgenerational plasticity. This occurs when parental environmental conditions influence offspring phenotype. Transgenerational plasticity (TGP) is most commonly studied as maternal effects (reviewed in Fox & Mousseau, 1998), but also include paternal, grandparental, and potentially earlier generations’ effects. For example, in the cabbage beetle, *Colaphellus bowringi*, the incidence of diapause in offspring is influenced by the temperature and photoperiod conditions parents are exposed to. He et al. (2018) showed that parental and offspring diapause incidence are negatively related, where if parents are exposed to conditions that increase entering diapause, when offspring are exposed to similar conditions, they decrease entering diapause. Therefore, the conditions prior generations are exposed to can have large effects on subsequent generations.

Transgenerational plasticity acts through two known mechanisms: epigenetic and non-epigenetic. Epigenetic mechanisms do not directly influence base pair sequences, instead they include chemical modifications that increase or decrease transcription and translation rates. They include chromatin remoulding
(DNA methylation or acetylation and histone modifications) and RNA-mediated modifications (non-coding RNA and microRNA; Ho & Burggren, 2010; Gladstad et al., 2019). Non-epigenetic mechanisms of transgenerational plasticity include shifts in egg size or provisioning (Gibbs et al., 2010). Although maternal effects like changes in egg size can influence offspring fitness (Benton et al., 2005), it is unclear whether it is a direct response to different environmental conditions or a consequence of reproduction (e.g. egg size is temperature-dependent, influencing total yolk in each egg; Ernsting & Isaaks, 2000). Therefore, TGP has multiple mechanisms and can provide a rapid response to rapidly changing thermal conditions.

Local adaptation does not occur in every population because there are factors that can inhibit the accumulation of differentiated characters. Dispersal can result in the sharing and mixing of characters through mating (Bohonak, 1999). Migration between geographically isolated populations could either hinder or enhance local adaption (Hoffman & Sgrò, 2011) through the homogenization of alleles or through the introduction of new alleles. At the individual level, dispersal to new environments is risky but at the population level, it is necessary to track changing environments. Smaller population sizes could result in less genetic variation within a population which can lead to less differentiated ways of dealing with local conditions (Kirkpatrick & Barton, 1997). All these drivers limit evolution broadly as well.
Local adaptation at range edges should encourage range expansion. There is evidence that these locally adapted range-edge populations might not be the best suited for expansion via an accumulation of detrimental traits from high incidence of inbreeding and genetic drift (Pironon et al., 2017). However, along a continuous environmental gradient, locally adapted populations at the edge could be primed for areas beyond the range, which might facilitate range shifts. Hargreaves & Eckert (2019) provide some evidence for both ideas, although high edge populations had higher lifetime fitness in comparison to lower elevation plants, this benefit disappeared under climate warming. Therefore, under projected climatic changes, there is no evidence that edge populations are better suited for range expansion.

Climate change is expected to alter the already variable climatic landscape. Climatic conditions have already changed faster than ever before (IPCC, 2014). In Canada, winter and higher latitudes have experienced the most change in temperature (Zhang et al., 2019). Therefore, populations are experiencing novel thermal environments across their ranges and understanding the responses of populations across a species’ range is necessary for predicting the changes in population growth and range that may occur in the future.

1.3 **Insect cold tolerance**

Climate extremes or thermal limits for insects are important to determining range boundaries because they set the absolute absence or presence
of a species with regards to the temperature in that area. Insects survive cold temperatures with cold-hardiness adaptations usually associated with diapause. Diapause is an endocrine-mediated state of metabolic arrest that usually occurs during a certain developmental stage (reviewed in Tauber et al., 1986). Diapausing insects are either obligate (independent of environmental factors) or facultative (programmed by environmental conditions like temperature and diurnal cycles) diapausers (Koštál, 2006). Diapause is a very common example of seasonal phenotypic plasticity in insects.

During the diapausing lifestage cold-hardiness enhances survival at lower temperatures through physical or metabolic changes. Cold-hardiness can occur without undergoing diapause, however cold-hardiness is a component of diapause (Denlinger, 1991). Among cold-hardy insects, cold-hardiness strategies can be divided into freeze avoidance and freeze tolerance (Bale, 1993). Freeze-avoidance is the tolerance of a larger range of low temperatures through the depression of the supercooling point (SCP; the temperature at which their internal fluid freeze; Lee, 2010). The SCP is depressed through the dehydration of body water content (Holmstrup et al., 2002), expressing cryoprotectants like glycerol (Lee & Baust, 1987) or sorbitol (Duman et al., 1985), and antifreeze proteins (AFPs; Block & Duman, 1989). By contrast, freeze-tolerant animals can survive internal ice formation by deploying a similar suite of macromolecules, but also initiating freezing using ice-nucleating proteins (INPs; Duman et al., 1985).
1.4 The eastern spruce budworm

Here I used the eastern spruce budworm, Choristoneura fumiferana (Lepidoptera: Tortricidae) as a model system for exploring local adaptation in plasticity and TGP of insect cold-hardiness. The species is considered one of the most destructive forest pests in North America and has been well-studied (Régnière & Duval, 1998; Royama et al., 2005; Gray, 2008; Pureswaran et al., 2016) primarily due to its large economic and ecological impacts (MacLean, 2004). It outbreaks in cycles of 30 – 40 years and in the past, three major outbreaks in eastern Canada (1910-20s, 1940-50s and 1970-80s) have caused defoliation on the scale of 11, 15 and 58 million hectares, respectively (Kettela, 1983). Additionally, there is spatial variation in the severity and duration of outbreaks (Candau et al., 1998). Therefore, apart from large-scale impacts on forest dynamics and carbon-sink potential, the economic cost associated with managing outbreaks compared to no management is significant (Chang et al., 2012a; Chang et al., 2012b). Using this species as a model therefore allows testing theoretical questions while producing tangible applied answers.

The eastern spruce budworm has a wide distribution across Canada and the northern United States (DuPuis et al., 2017). The ecology of spruce budworm is well-understood, including larval feeding strategies, extensive moth dispersion and outbreak patterns associated with climate (Greenbank, 1956; Royama, 1984; Thomson et al., 1984; Nealis, 2015). They are univoltine and overwinter as second-instar larvae. No larval feeding occurs from hatching until the end of
diapause, therefore larvae rely fully on yolk provisioning to survive overwintering. At the end of winter, they feed and rapidly grow, and moult to sixth-instar larvae before pupating and eclosing into an adult moth (Sanders, 1991). The overwintering mortality when in hibernacula and the two dispersal events associated with it (into and out of the hibernacula) are the dominant sources of mortality as larvae (Miller, 1958). There is spatial as well as temporal variation in the overwintering conditions across the species' large geographic range. The species is predicted to shift its range poleward as a response to warming winters (Gray 2008; Régnière et al., 2012). This suggests that the current northern range boundary is set by cold temperatures.

The eastern spruce budworm survives winter by undergoing obligate diapause for approximately six months, from August to February (Régnière, 1990, Han & Bauce, 1993). During this period, they become more cold-hardy. They are freeze-avoidant animals and are able to depress their SCP to ~-35°C (Han & Bauce, 1995a), by accumulating ~ 0.8 M glycerol (Han & Bauce, 1995b), expressing AFPs (Tyshenko et al., 1997; Qin et al., 2007) and decreasing their water content (Han & Bauce, 1998; Bauce & Han, 2001). While capable of surviving very brief exposures to just about their SCP, exposure to -15 °C for more than ten days can result in mortality (Han & Bauce, 1995a). Their basic mechanisms of cold-hardiness are well-understood, making them an excellent model organism for studying plasticity in cold-hardiness.
The role of plasticity and local adaptation in how this species survives different environmental conditions across its range is still poorly understood. Offspring fitness is influenced by parents’ nutritional status within the sixth-instar (Carisey & Bauce, 2002). They showed that parents that are fed on low quality diets result in reduced egg hatching success and early instar survival, but that surviving offspring have an increased tolerance to starvation compared to offspring with parents fed on high quality diets. Harvey (1983a, 1983b & 1985) showed that there is a geographical cline in egg weight, where northwestern populations have fewer, large eggs and southwest populations produce many, small eggs. Additionally, the work in this thesis stems from a Natural Resources Canada project (see interim report Candau et al., 2018) intended to validate predicted species distribution models. This project has showed that development times differ across the populations tested, where northern populations (Inuvik and Alberta) have faster larval development compared to more southern populations. Therefore, this thesis aims to further increase our current understanding of plasticity and local adaptation, specifically with regards to cold-hardiness measures, in this species.

1.5 Thesis objectives

This thesis takes a macrophysiological approach to testing the influence of plasticity on setting range boundaries. I’ve used the eastern spruce budworm because understanding plasticity of cold-hardiness during overwintering is useful
for better predicting population dynamics and consequently, mapping potential range shifts and future outbreaks. Since we know mortality associated with overwintering is an important driver of *C. fumiferana* population size and distribution, and that winters in Canada are changing, improving our understanding of the current and predicted range of this species on all fronts is necessary.

1.5.1 **Chapter 2 objectives**

The objective of this chapter was to determine the extent of TGP of cold-hardiness in the F$_1$ after parental cold exposure.

**Chapter 2 hypotheses**

1. Repeated cold exposure drives physiological and fitness costs

2. F$_1$ second-instar larvae’s cold-hardiness is influenced by the parental thermal environment.

**Chapter 2 predictions**

1. F$_1$ whose parents have been repeatedly cold exposed will have higher cold-hardiness,

2. There is a fitness cost associated with increasing offspring cold-hardiness, such parents would have smaller clutch sizes to increase offspring cold-hardiness.
3. Repeated cold exposures in parents will result in increased cold-hardiness measures in offspring

1.5.2 Chapter 3 objectives

The objective of this chapter was to determine if there is local adaptation in *C. fumiferana* 1) basal cold-hardiness and 2) inducible cold-hardiness in populations across the species’ range.

*Chapter 3 hypotheses*

1. There is local adaptation in basal cold-hardiness,

2. Populations will exhibit local adaptation in phenotypic plasticity of cold-hardiness.

*Chapter 3 predictions*

1. Northern populations will exhibit higher levels of measures of cold-hardiness,

2. Northern populations will exhibit higher phenotypic plasticity.
Chapter 2: Transgenerational Plasticity of Cold-hardiness

2.1 Introduction

Climate change is exerting strong selective pressure on species. There are three potential adaptive responses to this pressure: migrate to suitable areas, adaptively evolve in place or use phenotypic plasticity (Hoffmann & Sgrò, 2011). Phenotypic plasticity, because it results in shifts in phenotype without changing genotype after exposure to different environmental conditions, can result in rapid responses to climate change (Pigliucci, 2001). Therefore, it is often the first response to changing environmental conditions both for species who have the geographical and dispersal capacity to migrate and those who do not.

While phenotypic plasticity can act within generations, it can also act across generations. Across generation plasticity, or transgenerational plasticity (TGP), include the most commonly studied maternal effects (Mousseau & Fox, 1998). It is when parents alter their offspring’s responses to changing conditions (often associated with stress; Allen et al., 2008). These effects can be adaptive if prior environment conditions are good predictors for future generations’ environments (Uller, 2008; Uller et al., 2013). This alteration in offspring response can be induced through epigenetic mechanisms such as changing DNA methylation patterns (Ho & Burggren, 2010; Gladstad et al., 2019) or non-epigenetic mechanisms like altering egg sizes and therefore, offspring energetic reserves (Gibbs et al., 2010). Therefore, the occurrence of TGP is usually
associated with predictable stress events between parental and offspring environments, and at lifestages that occur before offspring have a chance to mediate the stress (e.g. before feeding lifestages).

There is significant potential for TGP in overwintering eastern spruce budworm (*Choristoneura fumiferana*). The larvae overwinter as second-instar that do not feed after hatching, therefore the reserves that keep caterpillars alive through a seven-month obligate diapause is entirely dependent on parental investment (Sanders, 1991). During diapause, offspring cease development and increase cold-hardiness through the production of a suite of antifreeze proteins and the cryoprotectant glycerol synthesized from glycogen reserves (Han & Bauce, 1995b; Tyshenko *et al*., 1997; Qin *et al*., 2007). These large energetic costs are funded completely by parentally-invested reserves.

Cold-hardiness can also be plastic on short timescales, responding to brief temperature fluctuations (Marshall & Sinclair, 2012). However, fluctuating temperatures also often induce additional stress in insects, including spruce budworm. For example, repeated exposures to sub-zero temperatures cause short-term increases in glycerol content in overwintering insects, but also long-term decreases in survival or reproductive output (Marshall & Sinclair, 2015). Therefore, responding to variable thermal environments with phenotypic plasticity could induce a cost to long-term survival and fitness.

With climate change, *C. fumiferana* is predicted to increase population growth and shift its range poleward (Gray, 2008; Régnierè *et al*., 2012), however
phenotypic plasticity has not been incorporated into these models. There is some
evidence in plants that not including measures of plasticity in species distribution
models underestimates predicted range shifts (Benito-Garzón et al., 2011;
Valladares et al., 2014). In terrestrial insects broadly, currently poleward
distributions are underpredicted by the most commonly used measures of cold-
hardiness, and plasticity might explain this gap (Sunday et al., 2012). Current
spruce budworm species distribution models use basal thermal tolerance and do
not account for plasticity (Régnière et al., 2012). By including TGP, if it exists, in
species distribution models for spruce budworm, we may produce more accurate
models of population growth and range.

Therefore, in this chapter I hypothesized that C. fumiferana would display
TGP of cold-hardiness. Using the IPQL lab strain, I exposed second-instar larvae
to repeated cold exposures to test if short-term cold stress cause altered
investment in cold-hardiness or energetic reserves in the offspring.
2.2 Materials and methods

2.2.1 Experimental animals

Animals used in all experiments and measures were sourced from a colony established in 1961 at the Insect Production and Quarantine Laboratories ("IPQL", Great Lakes Forestry Centre, Sault Ste. Marie, Canada; Roe et al., 2018).

I ordered pre-diapausing caterpillars from Insect Production and Quarantine Laboratories (Sault St. Marie, Ontario, Canada) and they were shipped to the University of British Columbia with ice packs to maintain cool conditions. Once received, I left caterpillars in an incubator (MIR-154, Sanyo, Bensenville, USA) held at 2 °C in constant darkness for six weeks to induce diapause.

2.2.2 Treatments

After six weeks, I separated caterpillars into “control”, “single T1” (a single cold exposure to -10 °C for 12 hours including a ramping rate of 0.05 °C/minute), “single T2” (a single exposure cold exposure to -10 °C for 12 hours the same ramping rate, but keeping them at 2°C for 152 hours (~ 6 days and 8 hours) to account for differences in time since first cold exposure between “single T1” and “repeated”) and “repeated” (five exposures to -10 °C and 2 °C for 12 hours with the same ramping rate) (Figure 2.1). When separating experimental groups, I removed larvae from their hibernacula and put 20 caterpillars in individual 0.2
mL microcentrifuge tubes (for SCP measures), five sets of 10 caterpillars (for biochemical assays), five sets of 20 caterpillars (for LLT measures) or 10 sets of 10 caterpillars in 0.2 mL microcentrifuge tubes (mating group), the two latter groups being held in the gauze in the tubes (Table A1).

The single and repeated animal tubes were then put into a milled aluminium block (as in Sinclair et al., 2015) connected to a programmable refrigerated circulating bath (Lauda Proline RP 3530, Wurzburg, Germany) containing 50:50 ethylene glycol:water. Ten 36 AWG Type T (copper-constantan) thermocouples (Omega Engineering Inc., Laval, Canada) were placed into the block to monitor temperature. These thermocouples were interfaced with PicoTech TC-08 thermocouple interfaces connected to a computer running PicoLog software (Pico Technology, Cambridge, U.K.) taking temperature measurements in the block every 0.5 seconds. The refrigerated circulating bath was set to cycle between 2 and -10 °C for 12 hours each including ramping rates between these temperatures of 0.05 °C/min once (single) or for five full cycles (repeated). I put caterpillars back into the diapausing conditions in the incubator for 24 h recovery after this exposure. I exposed LLT caterpillars a week later due to capacity constraints on the circulating baths.
2.2.3 Measures of cold-hardiness

2.2.3.1 Supercooling point (SCP)

SCPs were measured as in Strachan et al. (2010). I attached individual caterpillars to 36 AWG Type T copper-constantan thermocouples with a thin layer of vacuum grease. These thermocouples were threaded through the pierced top of the 0.2 mL microcentrifuge tubes and held in place with adhesive putty.

![Figure 2.1](image)

**Figure 2.1** Design of cold exposures conducted in a programmable refrigerated circulating bath. A refers to timepoint control was assessed, B is single T1, black dashed line refers to trajectory of single T2 and C is the timepoint both repeated and single T2 was assessed at.

Tubes were floated on 60:40 methanol: water in a programmable refrigerated circulating bath (Lauda ECO RE 1050, Wurzburg, Germany). The refrigerated circulating bath was cooled from 2 to -52 °C at a rate of 0.09
°C/minute. SCP was recorded as the point before the onset of the exotherm (Lee, 2010).

2.2.3.2 Biochemical assays

I homogenized groups of 10 caterpillars with approximately 90 0.5 mm Zirconium oxide beads (Next Advance Inc., Averill Park, USA) in a Bullet Blender (Storm 24, Next Advance Inc., Averill Park, USA) for two minutes at the highest speed. I added 50 µL of 0.05% Tween 20 and repeated blending. I added an additional 250 µL 0.05% Tween 20 and mixed the sample using a vortexer (Vortex-Genie 2, Scientific Industries Inc., Bohemia, USA). I then centrifuged (Allegra 64R, Beckman Coulter Canada Inc., Mississauga, Canada) the sample for 10 min at 15,000 × g. The supernatant was removed into two aliquots and stored at -80 °C for later assays.

I measured glycerol, glucose, glycogen and protein content using spectrophotometric assays following Gefen et al. (2006) using glycerol, glucose and Type II glycogen from oyster and bovine serum albumin as standards, respectively. Briefly, glycerol was measured using a Free Glycerol kit (MAK117, Sigma-Aldrich Canada Co., Oakville, Canada). Glucose was measured using a hexokinase-based Glucose assay kit (GAHK20, Sigma-Aldrich Canada Co.). Glycogen content was measured using the same kit following an 8-hour amyloglucosidase (A9228, Sigma-Aldrich Canada Co.) digestion in a dark drawer at room temperature. Soluble protein was measured using a Bicinchoninic acid
kit (BCA1, Sigma-Aldrich Canada Co.). Absorbance of each reaction was measured in a spectrophotometer (Spectra Max M2, Molecular Devices, San Jose, USA) and calculated concentrations are reported in μmol/individual.

2.2.3.3 Lower lethal temperature (LLT)

To estimate LLT, I exposed five groups of 20 caterpillars from each treatment group to either -15, -20, -25, -30, or -35 °C, as described in Sinclair et al. (2015).

Groups of larvae were exposed to their corresponding temperature treatment for 4 hours, by placing the pierced 0.2 mL microcentrifuge tubes into a milled aluminium block. This block was connected to a programmable refrigerated circulating bath (Lauda Proline RP 3530, Wurzburg, Germany) with 50:50 ethylene glycol:water. Ten 36 AWG Type T (copper-constantan) thermocouples (Omega Engineering Inc., Laval, Canada) were placed in the block to monitor temperature. These thermocouples were interfaced with PicoTech TC-08 thermocouple interfaces connected to a computer running PicoLog software (Pico Technology, Cambridge, UK) recording temperature in the block every 0.5 seconds. After exposure, I transferred them to Petri dishes to avoid stress when doing mortality assessment. Mortality was assessed under the microscope (MEB126, Leica, Wetzlar, Germany) by checking if caterpillars were dehydrated, out of their hibernaculum or immobile, one week after exposure, at the end of
diapause, at thinning (10 days after ending diapause) and at pupation. Dead caterpillars were removed at each time point.

2.2.4 F₁: Mating and rearing

After the exposures, I put the mating group back into an incubator at diapausing conditions (2 °C in constant darkness) for a total of 20 diapausing weeks. At this point, I removed them and put them into feeding/developing conditions (24 °C, with a 16:8 light: dark cycle). I followed the rearing methods as described in the standard operating procedure (Great Lakes Forest Centre Insect Production Services, 2015).

Briefly, groups of 10 caterpillars were put onto diet cups with artificial diet (McMorran, 1965; purchased through Insect Production and Quarantine Services). Diet cups were changed once a week to avoid the accumulation of microbes and mould. Ten days later (thinning), I put one caterpillar in each cup. At pupation, I sterilized caterpillars with a 10% bleach solution under a fume hood, rinsed them with deionized water and left them to dry on paper towels. They were then sexed under a microscope and the first 40 were weighed on a microbalance (CP 124 S, Sartorius, Göttingen, Germany). Males and females were moved to separate ventilated plastic containers to emerge. These emergence chambers were checked daily for emerged adult moths. I put the first 20 male and female moths (total of 40 moths) that emerged into 20 L clear plastic bags with 5 × 5 cm strips of waxed paper stapled together. I maintained a 50:50 female
and male ratio in each bag. I sprayed each treatment’s mating chamber with deionized water before and after take-down. The three mating chambers were then kept in environmental chambers (23 ± 3°C, 55 ± 10% RH, 16L:8D) in the Faculty of Forestry at the University of British Columbia. Mating chambers were set-up for a week, after which moths died and egg masses were collected.

I set up emergence pans using 30 × 25 × 5 cm baking trays lined with gauze and sealed with Parafilm and electrical tape to avoid larval escape. Once second-instar caterpillars began spinning their hibernacula, I opened the emergence pans, counted how many larvae had emerged and spun their hibernacula. I then removed the gauze, wrapped it with parafilm, and put it in an incubator held at 2 °C in constant darkness.

After six weeks into diapause, I repeated all measures of cold hardiness with F1 second-instar larvae.

2.2.5 Statistical analyses

All statistical tests and data plots were conducted in RStudio (version 1.1.463, 2018). I fitted a Type II ANOVA model using aov function in the car package (Fox & Weisberg, 2019) with generation and treatment as predictors, after testing the model assumptions. For biochemical assays, I set protein concentration as a covariate in the model. LT50s were calculated using a generalized linear model with binomial error distribution and the dose.p function with p set to 0.5 from the MASS package (Venables & Ripley, 2002). Alpha was
set to 0.05 and p-values less than 0.01 are reported as such. All significant interactions were further investigated using TukeyHSD posthoc tests.
2.3 Results

2.3.1 Life history

$F_0$ pupae had a mean weight of 0.098 g (± 0.034) and cold exposures had no effect on pupal weight (Treatment: $F_{(2,119)}=1.11$, $p=0.34$).

After rearing out $F_0$ exposed to no (control), a single, or five repeated cold treatments, I counted first-instar larvae in each group. Repeated cold exposures resulted in the smallest number of offspring number in comparison to control (51% more) or singly exposed $F_0$ parents (45% more; Figure 2.2). Since rearing was conducted as mass matings, I am unable to estimate the variability in offspring number per mating.

![Figure 2.2 Number of offspring. $F_1$ first-instar IPQL (Choristoneura fumiferana) larvae following parental ($F_0$) exposure to -10 °C once (“Single”) or five times (“Repeated”).]
2.3.2 Mortality and supercooling point

Cold exposure did not significantly change SCP of either generation, although SCP was higher in F₁ generation (Generation: F_{(1,139)}=63.77, p=p<0.01; Figure 2.3).

Mortality in F₀ generation was assessed after a cold exposure to five different temperatures at four different timepoints: one week after exposure, at the end of diapause (20 weeks from the onset of diapause), thinning (between instar 3 and 4) and pupation to understand the long-term effect of cold exposures (Figures A1-4). The effect of cold exposure on LT₅₀ depended on the timepoint mortality was assessed at and treatment (Timepoint: F_{(3,15)}=58.63, p<0.001; Treatment: F_{(3,15)}=4.18, p=0.047). Individuals exposed to cold once (but sampled at the same time as the repeated group) died at higher temperatures at every timepoint assessed, except at the end of diapause, compared to other cold exposures (Figure 2.4). Additionally, calculated LT₅₀ increased with time as mortality effects accrued through development. Larvae that received repeated cold exposures had the lowest calculated LT₅₀ at every timepoint which suggests increased cold-hardiness after repeated cold exposure.
Figure 2.3 Supercooling points (SCP) (°C) as a result of cold exposures and generation in second-instar IPQL Choristoneura fumiferana larvae. Bold line inside box shows median, lower and upper box boundaries show 25th and 75th percentile, respectively, lower and upper error lines show 10th and 90th percentile, respectively. Treatment groups are Single1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 12 hrs and assessed 24 hours after). Different letters indicate statistically significant comparisons (p≤α).
2.3.3 Metabolites

Cold exposure and generation had a significant effect on energetic reserves and metabolites. Total carbohydrate significantly differed among treatments (Treatment: $F_{(3,34)}=5.42$, $p=0.0047$). Post-hoc tests indicated single timepoint 2...
and repeatedly exposed larvae had significantly higher than total carbohydrate content the control and single timepoint 1 (Figure 2.5). There was no significant interaction between treatment and protein mass (Treatment × Protein mass: $F_{(3,34)}=2.03 \ p=0.13$). Similarly, glycogen content significantly differed among treatments (Treatment: $F_{(3,34)}=7.60, \ p<0.001$), following the same trend with control and single timepoint 1 being significantly lower than single timepoint 2 and repeatedly cold exposed larvae (Figure 2.6). Protein mass also differed with glycogen concentrations (Total protein: $F_{(1,34)}=43.81, \ p<0.001$).

Cold exposure caused no significant differences in glycerol content (Treatment: $F_{(3,34)}=1.039, \ p=0.39$). This can be attributed to the large variance (sd=0.08) compared to the other treatment groups in F0 (sd<0.01; Figure 2.7).
Figure 2.5 Total carbohydrate per larva (μmol) across the different treatments and two generations in second-instar IPQL *Choristoneura fumiferana* larvae. Refer to figure 2 caption for explanation of boxplot display. Treatment groups are Single1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 12 hrs and assessed 24 hours after). Different letters indicate statistically significant comparisons (p≤α).
Figure 2.6 Glycogen per larva (μmol) across the different treatments and two generations in second-instar IPQL Choristoneura fumiferana larvae. Refer to figure 2 caption for explanation of boxplot display. Treatment groups are Single1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 12 hrs and assessed 24 hours after). Different letters indicate statistically significant comparisons (p≤α).
Figure 2.7 Glycerol per larva (μmol) across the different treatments and two generations in second-instar IPQL Choristoneura fumiferana larvae. Refer to figure 2 caption for explanation of boxplot display. Treatment groups are Single1 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed 24 hours after), Single2 (larvae exposed to a single cold exposure of -10 °C for 12 hrs and assessed at the same time as the repeated group) and Repeated (larvae exposed to five cold exposures of -10 °C for 4 hrs and assessed 24 hours after). Different letters indicate statistically significant comparisons (p≤α).
2.4 Discussion

Here I show that, despite inducing a significant reduction in \( F_0 \) fitness, repeated cold exposures do not induce transgenerational plasticity in \( C. \) \textit{fumiferana}. I found that repeated cold exposure reduces the number of offspring a parent can produce, but there is no difference in realized or potential cold-hardiness of those offspring. Therefore, repeated cold exposures in \( C. \) \textit{fumiferana} might not result in TGP, however could decrease population size over time.

While this experiment was not designed to test differences in reproductive output, there was a large (>50%) decrease in the number of offspring produced by parents who had experienced repeated cold exposure. However, whether the decrease was due to some individuals being sterile or clutch sizes being small because of repeated cold exposure is untestable with the present data. The decrease in the number of first-instar offspring of parents who received repeated cold exposures could be attributed to trade-offs between the energetic costs of surviving repeated cold stress and reproductive output. For example, there are priority effects associated with trade-offs, such that some processes take energetic precedent over others (Zera & Harshman, 2001).

One explanation for lack of TGP after repeated cold exposure could be that post-diapause feeding could mask the effect stress-induced TGP (Tauber \textit{et al.}, 1986). However, I showed that the reproductive cost occurred after the sole feeding opportunity in this species. There are then two possibilities for the lowered reproductive output: either an inability to replenish depleted energetic
reserves or long-term damage. The absence of a difference in pupal weights suggests that individuals were likely able to replenish themselves, and instead it is more likely that long-term damage was incurred.

In general, cold exposures negatively influence reproductive behaviour and output (Coulson & Bale, 1992; Shreve et al., 2004; Basson et al., 2011). In addition, similar trade-offs between reproductive output and cold-hardiness have been found in flies (*Drosophila melanogaster*) and the goldenrod gall fly (*Eurosta solidaginis*). Both species demonstrate reduced population growth after being repeatedly exposed to cold events in a fluctuating thermal regime even when total duration and intensity of cold exposure was matched (Marshall & Sinclair, 2010; Marshall & Sinclair, 2018). Therefore, the trade-offs between life-history traits due to energetic limitations can be detrimental to population growth.

Contrary to my expectations, there was no significant increase in cryoprotectant glycerol concentration after being repeatedly exposed to cold. Marshall & Sinclair (2015) found that five exposures to -10 °C was enough to induce increased investment in glycerol content through increased glycerol synthesis from glycogen. By contrast, I found increased carbohydrate content in larvae that received repeated or a single cold exposure the matched timepoint. It could be that they are utilizing lipid reserves, however this is not well understood in *C. fumiferana* (Marshall & Sinclair, 2018).

While I found that SCP did not change among the F₀ treatment groups, there was a decrease in LT₅₀ in caterpillars that received repeated cold
exposures, which indicates increased cold-hardiness. Additionally, mortality increases at over time, and is different for different treatment groups. Although I did not observe a significant increase in glycerol content as a result of cold exposure, it is possible that other cold-hardiness mechanisms (e.g. heat shock proteins or antifreeze proteins) that I didn’t measure may be responsible for increased cold-hardiness. Previous work has shown that second-instar larvae in the middle of diapause had increased survival to sub-zero exposures and that acclimation can increase survival to cold exposures (Han & Bauce, 1995a). Therefore, although tolerance to acute temperatures is very high as measured by SCP, the resultant chilling injury resulting from temperatures above the SCP is still significant.

In general, I found no difference in cold-hardiness in F₁ offspring. Therefore, although I was able induce fitness costs in parents, there was no evidence of differential investment as a result of parental experience. Due to lower than expected F₁ offspring numbers, I was unable to test LT₅₀ as done in F₀, however SCP as an acute measure of cold-hardiness did not change. In addition, no difference in glycerol or glycogen (source of glycerol) was found.

There was a difference in cold-hardiness between the F₀ and F₁ generations. Although I followed the standard operating procedure for rearing spruce budworm as set by the Insect Production and Quarantine Laboratory, it is assumed that there are differences in the control of these practices between IPQL and myself. The differences between the two generations are attributed to this.
TGP can only be adaptive when environmental conditions are predictable between parent and offspring lifecycles. Larval *C. fumiferana* don’t disperse far, they are capable of dispersing between a few trees by “ballooning” on silken threads or walking to different parts of the tree crown (Johns & Eveleigh, 2013; Nealis, 2014). By comparison, adult moths disperse much larger distances, sometimes 100s of kms (Sturtevant *et al*., 2013). Therefore, it could be that the probability of shared environmental conditions between generations is low, and TGP of cold-hardiness in second-instar is not favoured by selection.

It is also possible that TGP occurs in other winter-related traits rather than in absolute cold-hardiness or supplied energetic reserves. For example, Harvey (1961) found that some fourth-instar *C. fumiferana* undergo a second diapause. It could be that the occurrence of second diapause could be a plastic trait. Therefore, since it is likely that the predictability between second- and fourth-instar larvae environmental conditions is higher than between generations, it is possible that TGP could occur in the occurrence of second diapause.

The lack of TGP in cold-hardiness could also be due to using a lab-selected strain. The IPQL strain has been in culture for over 70 years and has gone without the addition of wild alleles for the past 20 of those years (Roe *et al*., 2018). Therefore, the relaxed selection of cold-hardiness under lab conditions could have resulted in a very different response compared to wild populations (Hoffmann & Ross, 2018). So perhaps wild populations would demonstrate TGP.
The absence of evidence for TGP in IPQL strain diapaus ing cold-hardiness suggests that TGP does not need to be considered for population growth or species range models in *C. fumiferana*. However, it does provide further evidence that repeated cold exposure is more complicated and that fitness trade-offs in overwintering insects exist. Further work should focus on untangling the potential mechanisms of these trade-offs, and future modelling should include fitness effects of repeated cold exposure.
Chapter 3: Local Adaptation in Phenotypic Plasticity of Cold-hardiness

3.1 Introduction

Temperature has ubiquitous effects on all organisms. In small ectotherms, it both directly and indirectly drives the rates of physiological and biochemical processes (Somero et al., 2017). In addition, there is variability on multiple, interacting timescales in the thermal environments that animals are exposed to (Marshall & Sinclair, 2012). Therefore, it is likely that selective pressure exists on animals to adjust their thermal tolerance rapidly.

Evolved responses to this selection include phenotypic plasticity and local adaptation. Phenotypic plasticity is altering phenotype without changing genotype as a response to environmental change (Pigliucci, 2001). In contrast, populations that are locally adapted have genetic differences that increase fitness in their location-specific environments (Kawecki & Ebert, 2004; Barrett et al., 2011). Although they are thought of two different responses, they can evolve together (Jensen et al., 2008) as there can also be local adaptation of phenotypic plasticity (Ghalambor et al., 2007).

The eastern spruce budworm, Choristoneura fumiferana is a boreal defoliating caterpillar native to North America. The species has a large geographical range from Newfoundland to British Columbia, with populations as
far north as Inuvik, Northwest Territories. Therefore, populations across its
range are exposed to a wide range of temperatures. In addition, *C. fumiferana* is
predicted to move poleward with climate change (Gray, 2008; Régnierè *et al.*, 2012) as winters warm, particularly in the north (Zhang *et al.*, 2019).

The eastern spruce budworm survives cold temperature during a seven-
month long diapause as a second-instar larvae. As part of the diapause
programme, it increases its cold-hardiness by accumulating low molecular weight
cryoprotectant glycerol (Han & Bauce, 1995b) and antifreeze proteins (Tyshenko *et al.*, 1997; Qin *et al.*, 2007), and by decreasing body water content (Han &
Bauce, 1998) to suppress its supercooling point (the temperature at which
internal fluids freeze). It therefore invests a substantial amount of energy to
survive this lifestage.

Cold-hardiness can be phenotypically plastic. Cold developmental
acclimation can decrease CT\textsubscript{min} (critical thermal minimum, lowest temperature
before losing locomotory function; Overgaard *et al.*, 2011; Schou *et al.*, 2016).
Crosthwaite *et al.* (2011) showed significant seasonal plasticity in cold-hardiness
in the emerald ash borer (*Agrilus planipennis*) with increased glycerol content
and supressed SCPs in winter. In spruce budworm, repeated cold exposure of
second-instar larvae resulted in rapidly increased glycerol content (Marshall &
Sinclair, 2015). Therefore, plasticity in cold-hardiness is common in insects.

Most of the work on local adaptation of cold-hardiness in insects does not
explicitly include the possibility of local adaptation in plasticity of cold-hardiness.
Sinclair et al. (2012) reviews examples of local adaptation in thermal performance measures, e.g. metabolic rate, feeding rates and locomotion, across insect populations. In *Drosophila melanogaster*, basal cold-hardiness varies across populations (Schmidt et al., 2005). Local adaptation in cold tolerance limits (CT\textsubscript{min}) of natural populations was found in *Eldana saccharina* (Kleynhans et al., 2014). Therefore, while local adaptation of cold-hardiness occurs frequently, local adaptation of plasticity is less well-understood.

Much of the work on cold-hardiness in *C. fumiferana* has been conducted on the IPQL strain, which has been in culture for more than 70 years. Recently, to validate the population growth/species distribution models, Candau et al. (2018) showed that the IPQL strain is not reflective of wild populations of *C. fumiferana*. They found that wild populations differ vastly in developmental rate, compared to the IPQL. Additionally, Harvey (1983a) showed that genetically based phenotypic differentiation exists in this species. Number and egg size vary across *C. fumiferana* range; with fewer, larger eggs in the northwest and more, smaller eggs in the southeast of its range when in common-garden. Therefore, testing for local adaptation in wild populations of *C. fumiferana* is a logical next step.

Therefore, in this chapter I tested two questions using representative populations of *C. fumiferana* from across its range: 1) is there local adaptation in seasonal plasticity of cold-hardiness, and 2) is there local adaptation in short-term plasticity of cold-hardiness?
3.2 Materials and methods

3.2.1 Experimental animals

I used second-instar diapausing *Choristoneura fumiferana* caterpillars in all experiments and measures. These cultures were originally sampled from wild populations as reported in Candau et al. (2018). Populations used in this study were originally sampled around Campbellton, New Brunswick (47°59'13.1"N 66°40'37.8"W), Fermont, Quebec (52°51'07.1"N 67°06'35.5"W), High Level, Alberta (58°50'71" N 117°14'03" W), Inuvik, Northwest Territories (68°21’56.2"N 133°42'04.9"W) and a laboratory strain that has been in culture for over 70 years (“IPQL”, see Roe et al., 2018; Figure 3.1). Wild populations were kept in culture for 1 (late diapause measures) or 2 generations (all other measures) before use.

![Map of population sampling localities.](image)
Diapausing larvae in newly-spun hibernacula in gauze were shipped from Insect Production and Quarantine Laboratories (Great Lakes Forestry Centre, Sault Ste. Marie, Canada) to the University of British Columbia with ice packs to maintain cool diapausing conditions. Once received, larvae were placed into an incubator (MIR-154, Sanyo, Bensenville, USA) held at 2 °C in constant darkness.

3.2.2 Treatments

I removed larvae from the incubator at either six or twelve weeks into diapause, constituting the “early” and “late” diapause groups, respectively. Early diapause groups were further separated into “basal” (individuals immediately tested) or “inducible” (first exposed to a cold exposure, described below, before being tested). No caterpillars from the New Brunswick population were exposed to the inducible treatment due to limited sample numbers.

I divided larvae in the inducible group into groups of approximately 10 individuals each by cutting pieces of gauze (to avoid disturbing or stressing caterpillars) and putting them into pierced 0.2 mL microcentrifuge tubes. These tubes were then placed into a milled aluminium block (as in Sinclair et al., 2015) connected to a programmable refrigerated circulating bath (Lauda Proline RP 3530, Wurzburg, Germany) containing 50:50 ethylene glycol:water. I placed ten 36 AWG Type T (copper-constantan) thermocouples (Omega Engineering Inc., Laval, Canada) in the block to monitor temperature. These thermocouples were interfaced with PicoTech TC-08 thermocouple interfaces connected to a computer
running PicoLog software (Pico Technology, Cambridge, U.K.) taking
temperature samples in the block every 0.5 seconds. The bath was set to cycle
between 2 and -15 °C for 12 hours each including ramping rates between these
temperatures of 0.051 °C/min for five full cycles. I put the caterpillars back into
an incubator in diapausing conditions for 24 hours to recover after the exposure.

3.2.3 Measures of cold-hardiness

I extracted caterpillars from their hibernacula in the gauze and placed into
0.2 mL microcentrifuge tubes before all measurements. I separated them into 20
replicates of one caterpillar per pierced tube for SCP measures or five
microcentrifuge tubes with 10 caterpillars for biochemical assays.

I measured SCPs as in Strachan et al. (2010) and as described in Chapter 2
SCP methods. Late diapausing Inuvik caterpillars could not be frozen by the
previous method. In this case, I estimated SCPs by placing thermocouples
attached to individual caterpillars in microcentrifuge tubes in Styrofoam freezer
boxes and then into a -80 °C freezer. The cooling rate for this exposure can only
be estimated in this case as 9 °C/minute.

I conducted biochemical assays as described in Chapter 2 biochemical
assay methods.
3.2.4 Statistical analyses

All statistical tests and data plots were conducted in RStudio (version 1.1.463, 2018). I fitted a Type II ANOVA model using `aov` function in the `car` package (Fox and Weisberg, 2019) with population, diapause stage and treatment as predictors, after testing the model assumptions. For biochemical assays, I set protein concentration as a covariate in the model. Alpha was set to 0.05. p-values less than 0.01 will be reported as such. Significant interactions were further investigated using TukeyHSD posthoc tests. Means and SE are reported in results.
3.3 Results

Population and time in diapause had a significant effect on SCP, where the Inuvik late diapause individuals had a significantly lower SCP compared to other populations and times tested (Population: $F_{(2,119)}=4.87$, $p<0.001$; Time: $F_{(1,119)}=16.06$, $p<0.001$; Population $\times$ Time: $F_{(2,119)}=5.43$, $p<0.01$; Figure 3.2).

Population and time in diapause both had a significant effect on basal total carbohydrate concentrations (Population: $F_{(2,29)}=3.196$, $p=0.020$; Time: $F_{(1,29)}=15.96$, $p<0.001$). This was driven by New Brunswick larvae in early diapause having lower total carbohydrate content than the Inuvik and Quebec early diapause larvae (Figure 3.3). Glycogen concentrations were significantly affected by time only (Time: $F_{(1,29)}=11.27$, $p<0.01$; Figure 3.4).

Population had a significant effect on basal glycerol content (Population: $F_{(2,29)}=13.85$, $p<0.001$; Figure 3.5). In this case, larvae from Inuvik in early diapause significantly increased their glycerol at late diapause, whereas larvae from New Brunswick significantly decreased glycerol throughout diapause.
Figure 3.2 Basal SCP (°C) of second-instar *Choristoneura fumiferana* across different populations tested at either early (6 weeks) or late (12 weeks) into diapause. Different letters indicate statistically significant comparisons (p≤α).
Figure 3.3 Basal total carbohydrate per larva (μmol) of second-instar *Choristoneura fumiferana* across different populations tested at either early (6 weeks) or late (12 weeks) into diapause. Different letters indicate statistically significant comparisons (p≤α).
Figure 3.4 Basal glycogen per larva (μmol) of second-instar *Choristoneura fumiferana* across different populations tested at either early (6 weeks) or late (12 weeks) into diapause. Different letters indicate statistically significant comparisons (p≤α).
When I examined the effects of repeated cold exposure on measures of cold-hardiness, I found that there was a significant interaction between population and cold exposure frequency on SCP (Population: $F_{(3,169)}=10.32$, $p<0.001$; Treatment: $F_{(1,169)}=15.80$, $p<0.001$; Population $\times$ Treatment: $F_{(3,169)}=3.654$, $p=0.014$; Figure 3.6). Alberta and Inuvik decreased their SCP after exposure,
whereas Quebec and the IPQL remained the same. For total carbohydrate, there were no significant effect of population or treatment (Population: $F_{(3,39)}=2.765$, p=0.058; Treatment: $F_{(1,39)}=0.88$, p=0.35; Figure 3.7). The same is true for glycogen; there was no effect of population or treatment (Population: $F_{(3,39)}=0.123$, p=0.12; Treatment: $F_{(1,39)}=0.534$, p=0.47; Figure 3.8). However, a significant effect of population and treatment was found on glycerol concentrations (Population: $F_{(3,39)}=36.47$, p<0.001; Treatment: $F_{(1,39)}=31.31$, p<0.001; Population × Treatment: $F_{(3,39)}=7.24$, p<0.001; Figure 3.9).
Figure 3.6 Supercooling point (°C) of second-instar *Choristoneura fumiferana* before (“basal”) and after (“induced”) five exposures to -15 °C. Different letters indicate statistically significant comparisons (p≤α).
Figure 3.7 Total carbohydrate (μmol) of second-instar *Choristoneura fumiferana* before ("basal") and after ("induced") five exposures to -15 °C. Different letters indicate statistically significant comparisons (p≤α).
Figure 3.8 Glycogen (μmol) of second-instar *Choristoneura fumiferana* before (“basal”) and after (“induced”) five exposures to -15 °C. Different letters indicate statistically significant comparisons (p≤α).
Figure 3.9 Glycerol (μmol) of second-instar Choristoneura fumiferana before (“basal”) and after (“induced”) five exposures to -15 °C. Different letters indicate statistically significant comparisons (p≤α).
3.4 Discussion

I found evidence for seasonal plasticity of cold-hardiness and local adaptation in the short-term plasticity of cold-hardiness in the *C. fumiferana* second-instar diapausing larvae. In particular, I found local adaptation in plasticity in SCP and glycerol content as individuals from the most northerly population (Inuvik) significantly decreased their SCP and increased their glycerol content at late-stage diapause, and a similar response was found in both Inuvik and Alberta (the next most northerly population tested) caterpillars after repeated cold exposure.

Populations did not differ in their basal SCP in early diapause; however, when tested at late diapause, Inuvik had a significantly lower SCP compared to other populations tested (Figure 3.2). This could be related to winters in this area, which are significantly longer and colder than the conditions from the collection localities of the other populations (Government of Canada, 2020). However, early in the experiment, the method of measuring SCP using a -80 °C freezer could have influenced this result. There is evidence that shows that cooling rates can influence absolute thermal limits (Salt, 1966; Terblanche *et al.*, 2007). Since the cooling rate used in the -80 °C freezer was very fast (9 °C/min), this might have resulted in a much lower SCP. However, the switch in methods was due to the inability to freeze any Inuvik larvae at temperatures that were sufficient for the other populations. In addition, following repeated cold exposures and using a refrigerated circulating bath method, the measured SCP of
the Inuvik population was also much lower than the other populations (Figure 3.2), suggesting that the lower SCP late in diapause is accurate.

The reductions in SCP were also matched by increases in glycerol content. By accumulating low-molecular weight solutes like glycerol, insects can increase their body fluid osmolality, thereby depressing their SCP (Zachararisssen, 1985). Late diapausing Inuvik individuals had significantly higher glycerol content compared to other populations tested. At early diapause, individuals from Alberta had significantly higher glycerol content than all other populations tested besides Inuvik. This could be also explained by comparatively colder and longer winters in Alberta and Inuvik. Williams et al. (2015) similarly showed evidence for local adaptation in seasonal plasticity in fall webworms (Hyphantria cunea). They showed that populations from more northerly compared to more southerly locations had differential cold-hardiness across seasons, driven by different rates of energy use, growth and development. Therefore, depending on local temperature conditions, the expression of cold-hardiness might differ over time.

In comparison, I found no differences in energy content across the population comparisons in early or late diapause. Most populations had lower carbohydrate contents later in diapause, since they are actively using these reserves throughout diapause. As mentioned in the previous chapter, it could be that spruce budworm is utilizing lipid reserves, however this is not well understood in the species (Marshall & Sinclair, 2018).
All the populations I tested showed some evidence for local adaptation in short-term phenotypic plasticity of cold-hardiness. This was clustered into two groups, Inuvik and Alberta, and Quebec and IPQL. Given the long culture duration of the IPQL strain, it may have adapted to laboratory conditions (Hoffmann & Ross, 2018) although it still maintained similar cold-hardiness to the Quebec population. Individuals from Alberta and Inuvik were able to significantly increase their cold-hardiness after being repeatedly exposed to -15 °C, compared to both Quebec and the IPQL which could not. This clustering is supported by Lumley et al., (2020) who showed that there are three C. fumiferana genetic subgroups: eastern (which would include the Quebec and IPQL populations), central (eastern Alberta and Manitoba) and western (which would include the northern Alberta and Inuvik populations). These subgroups are hypothesized to have been created and maintained through the Wisconsin continental ice sheet and proglacial lakes that formed on the southern border of the Laurentide ice sheet as it melted, and the western boundary is further maintained through prevailing westerly winds. This validates the separation and clustering of Alberta and Inuvik because it provides evidence for geographical isolation from the eastern and central subgroups.

Although the IPQL strain has been used extensively for understanding overwintering in C. fumiferana, further evidence is provided here on the importance of considering wild populations when determining range-wide responses. I do not have measures of cold-hardiness in the IPQL at late diapause,
however the evidence that I do have shows that short-term plasticity is comparatively lower in the IPQL compared to other wild populations compared here.

The climate variability hypothesis (CVH) states that populations in areas with high thermal variability (i.e. higher latitudes) should display stronger clines for phenotypic plasticity (Janzen, 1967; Stevens, 1989). I found evidence in support of this hypothesis with short-term plasticity of cold-hardiness in Alberta and Inuvik, which experience comparatively more thermal variability than the other populations tested (Government of Canada, 2020).

Local adaptation and phenotypic plasticity can and should be included in species distribution models for *C. fumiferana*. Diamond (2018) highlights the use of hybrid species distribution models, which uses trait mean, variability, heritability and the plasticity of the trait to determine range shifts, therefore incorporating both plasticity and evolution. Bush et al., (2016) tested this *AdaptR* model and found that range loss for 17 species of *Drosophila* decreased by 33% in 2105 when incorporating these additional traits. For *C. fumiferana*, accurately modelling species distributions with these spatiotemporal adaptive models could prove useful for species management in future climates.

In summary, this chapter corroborates evidence found in Harvey (1983a) and Candau et al. (2018) of local adaptation in eastern spruce budworm. In addition, it provides novel evidence for local adaptation in seasonal and short-term phenotypic plasticity in cold-hardiness in *C. fumiferana*. Therefore, it gives
evidence for the use of these types of data in the building of species distribution and population growth models.
Chapter 4: Conclusion

The objective of this thesis was to assess the role of plasticity and local adaptation in overwintering survival of *Choristoneura fumiferana*. I specifically tested for transgenerational plasticity, local adaptation in seasonal plasticity and local adaptation in short-term phenotypic plasticity of cold-hardiness. These questions arose out of an attempt to understand the importance of these mechanisms to further improve the species distribution and population growth models for the species. The latter has implications for applied pest management under future climate change scenarios.

I found no evidence for TGP in overwintering *C. fumiferana*; however, I found that repeated cold exposures have significant reproductive consequences. In addition, I found significant evidence for local adaptation in phenotypic plasticity, both short-term and seasonally, of cold-hardiness across *C. fumiferana*’s range.

4.1 Chapter 2 summary

Transgenerational plasticity could evolve from selection on managing variability in temperature or could be a by-product from selection acting on a different suite of traits. Since *C. fumiferana*’s survival of extreme cold temperatures throughout overwintering is fully dependent on energetic provisioning of eggs, it was hypothesized that TGP was used to prime offspring to
manage harsh winters. The results from Chapter 2 do not support this hypothesis. I found evidence for a significant fitness cost associated with experiencing repeated cold exposures in $F_0$ parents, but no TGP in cold-hardiness in overwintering spruce budworm was found. This is attributed to two potential reasons: 1) long-term lab selection in the IPQL strain used relaxed selection for TGP, and 2) low predictability in environmental conditions between generations due to high dispersive capabilities of adult moths. It is also possible that TGP effects can persist across multiple generations (Shama & Wegner, 2014), however, this study did not have the resolution to pick up effects from prior generations excluding $F_0$. Martin *et al.* (2019) similarly found no TGP in thermal tolerance in acorn ants (*Temnothorax curvispinosus*). Evidence for TGP of cold-hardiness has been found in other invertebrates. For example, marine polychaete (*Ophryotocha labronica*) mothers increase cold tolerance corresponding to parental environments when temperatures were experienced in late oogenesis but not early oogenesis (Massamba-N’Siala *et al.*, 2014) and blow fly (*Calliphora vicina*) parents exposed to warmer conditions during diapause produced larvae with decreased cold-hardiness (Coleman *et al.*, 2014). This suggests that while TGP in temperature tolerance is certainly possible in insects, it is not always the case.
4.2 Chapter 3 summary

The eastern spruce budworm has a large range in North America, and consequently, populations are exposed to very different temperature regimes across this range. It was hypothesized that populations would exhibit local adaptation in seasonal as well as short-term plasticity. The results from Chapter 3 provide evidence in support of this hypothesis. Two populations tested, Alberta and Inuvik, showed similar levels of short-term plasticity, which is attributed to geographical connectivity between the populations. Generally, there were no differences in basal cold-hardiness among the populations tested, however seasonal and repeated cold exposures revealed evidence for local adaptation of plasticity in the populations. The chapter results have direct implications for the predicted population growth, range shifts, and current species distribution modelling for the species. The IPQL lab strain, which forms the basis of our current understanding of cold-hardiness in the species, shows comparatively low plasticity. Therefore, increased climate extremes that select for plasticity, and altered thermal regimes in local environments as a consequence of climate change, will mean that different populations across the species’ large range will respond very differently.

4.3 Limitations and future work

In my experiment testing for TGP in cold-hardiness, I used the IPQL lab strain. This strain has been used in most of the studies that form the basis of our
understanding of *C. fumiferana* cold-hardiness (Han & Bauce, 1993, 1995a, 1995b, 1998). However, since this strain has been in culture for over 70 years, it is likely that lab selection has removed much of the natural variation in this population (Hoffmann & Ross, 2018). Since we know now that local adaptation of phenotypic plasticity exists in wild populations, the existence of TGP of cold-hardiness should be tested in wild populations as well.

### 4.4 Implications

The results from this thesis have three broad implications.

The first implication is for modelling *C. fumiferana* population growth and ranges. The current models do not consider phenotypic plasticity or local adaptation of cold-hardiness (Régnière *et al*., 2012). Overwintering in this species is a significant lifestage taking up over half of their lifetime (Sanders, 1991). Therefore, differences in overwintering survival across the species’ range may account for substantial differences between projected models and realized population growth. However, I showed that TGP in cold-hardiness for second-instar is absent, therefore changes in these traits are not predictive across generations. However, evidence for local adaptation in phenotypic plasticity was found, and should be considered in future species distribution models (Diamond, 2018). This will strongly improve these models, with specific improvement in the northern range of the species.
The second major implication of my work is for understanding insect low temperature physiology. I show that repeated cold exposures have significant reproductive costs. Impacts of thermal stress is often only assessed after a single cold exposure (Nedvěd et al., 1998; Chown & Terblanche, 2007). Here I show that this might not be fully reflective of the trade-offs in insect cold tolerance. We know that in nature, insects are exposed to multiple bouts of cold temperatures at varying degrees (Gaines & Denny, 1993). Although a regime of five predictable cold exposures is also not truly reflective of natural thermal regimes, Marshall & Sinclair (2015) show that although simplified, this frequency results in similar impact to more intensive regimes.

The final implication of my work is for species range limits and species thermal tolerance. We are moving towards the synthesizing our understanding of thermal tolerances (Sunday et al., 2019). However, this provides evidence that although northern species might be suited for range expansion into areas of climate-suitability, depending on the type of climate, it won’t necessarily be so straightforward. Thermal limits are clearly only one part of the story: reproduction and population growth, biotic interactions (Harley, 2003), and other abiotic factors might play a role in range shifts.
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Appendix

Table A1. Sample sizes for all measures assessed. Measures assessed per treatment taken as: supercooling point, 20 caterpillars; metabolites, 5 biological replicates of 10 caterpillars; lower lethal temperature, 5 tubes with 20 caterpillars each per temperature treatment; mating, 100 caterpillars.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Sample size (n)</th>
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<tbody>
<tr>
<td>Supercooling point</td>
<td>20</td>
</tr>
<tr>
<td>Metabolites</td>
<td>5</td>
</tr>
<tr>
<td>Lower lethal temperature</td>
<td>100</td>
</tr>
<tr>
<td>Mating</td>
<td>100</td>
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</tbody>
</table>
Figure A1. Proportion of survival of Control F₀ second-instar IPQL (*Choristoneura fumiferana*) larvae assessed at different developmental times after 4-hour exposures to lower lethal temperature treatments (n=100 per treatment). Times assessed are A) 1 week, B) the end of diapause, C) thinning, and D) pupation.
Figure A2. Proportion of survival of Single T1 F₀ second-instar IPQL (*Choristoneura fumiferana*) larvae assessed at different developmental times after 4-hour exposures to lower lethal temperature treatments (n=100 per treatment). Times assessed are A) 1 week, B) the end of diapause, C) thinning, and D) pupation.
Figure A3. Proportion of survival of Single T2 F₀ second-instar IPQL (*Choristoneura fumiferana*) larvae assessed at different developmental times after 4-hour exposures to lower lethal temperature treatments (n=100 per treatment). Times assessed are A) 1 week, B) the end of diapause, C) thinning, and D) pupation.
Figure A4. Proportion of survival of Repeated F₀ second-instar IPQL (*Choristoneura fumiferana*) larvae assessed at different developmental times after 4-hour exposures to lower lethal temperature treatments (n=100 per treatment). Times assessed are A) 1 week, B) the end of diapause, C) thinning, and D) pupation.