Epigenetic underpinnings of freeze tolerance in the goldenrod gall fly *Eurosta solidaginis* and the goldenrod gall moth *Epiblema scudderiana*

By

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Chair, Department of Biology

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Abstract

The goldenrod gall fly *Eurosta solidaginis* and gall moth *Epiblema scudderiana* are cold hardy insects that inhabit stem galls on goldenrod plants and survive subzero temperatures during the winter. *Eurosta* uses a freeze tolerance strategy where it restricts the formation of ice to extracellular spaces and preserves vital intracellular functions. *Epiblema* supercools its bodily fluids to remain unfrozen below 0°C. This thesis examined the role of epigenetic enzymes in insect cold hardiness strategies. Cold and subzero temperature exposure in *Eurosta* resulted in upregulation of several DNA Methyltransferase (DNMT) enzymes, increases in DNMT and Histone Acetyltransferase (HAT) activities, and decreases in Histone Deacetylase (HDAC) and Ten-Eleven Translocation (TET) enzyme activities. *Epiblema* showed upregulation of several DNMT enzymes with concurrent decreases in DNMT, HAT, and HDAC activities and no change in TET activity. These findings suggest that epigenetic regulation of genes and histones underpins the winter survival strategies of these insects.

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List of Abbreviations

5-mC	5-methylcytosine
5-hmC	5-hydroxymethylcytosine
Acetyl-CoA	acetyl coenzyme A
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride
AMPK	AMP-activated protein kinase
ANOVA	Analysis of Variance
C5	five prime carbon on cytosine
CAT	Catalase
CpG	cytosine and guanine separated by a phosphodiester bond
CREB	cAMP response element-binding protein
CS	citrate synthase
DNA	Deoxyribonucleic acid
DNMT	DNA Methyltransferase
DHAP	di-hydroxyacetone phosphate
EDTA	Ethylenediaminetetraacetic acid
G3P	glycerol-3-phosphate
G3PDH	glycerol-3-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GNAT	Gcn5-related N-acetyltransferases

GST	Glutathione S-transferase
H2A	Histone 2A
H2B	Histone 2B
H3	Histone 3
H4	Histone 4
H2A.K5.Ac	Histone H2A acetylated at lysine residue 5
H2B.K5.Ac	Histone H2B acetylated at lysine residue 5
H3.K9.Ac	Histone H3 acetylated at lysine residue 9
H3.K14.Ac	Histone H3 acetylated at lysine residue 14
H3.K18.Ac	Histone H3 acetylated at lysine residue 18
H3.K23.Ac	Histone H3 acetylated at lysine residue 23
H3.K27.Ac	Histone H3 acetylated at lysine residue 27
H3.K56.Ac	Histone H3 acetylated at lysine residue 56
H4.K8.Ac	Histone H4 acetylated at lysine residue 8
H3K9me3	Histone H3 trimethylated on lysine residue 9
H3K27me3	Histone H3 trimethylated on lysine residue 27
H3K36me3	Histone H3 trimethylated on lysine residue 36
HAT	Histone Acetyltransferase
HDAC	Histone Deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1	Hypoxia-inducible factor 1
HMT	histone methyltransferase
HRP	Horse radish peroxidase

HSP	Heat Shock Protein
MBD	methyl-CpG-binding domain protein
$MeCP_2$	methyl CpG binding protein 2
MEF2	myocyte enhancer factor-2
mTOR	mammalian target of rapamycin
MW	molecular weight
NAD^+	Nicotinamide adenine dinucleotide
NAD-IDH	NAD-isocitrate dehydrogenase
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaF	Sodium fluoride
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
Nrf2	Nuclear factor (erythroid-derived 2)-like 2
p300/CBP	CREB-binding protein
PCAF	P300/CBP-associated factor
PDH	polyol dehydrogenase
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PMSF	phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene fluoride
qRT-PCR	quantitative real time polymerase chain reaction
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase

sRNA	small RNA
siRNA	short interfering RNA
SIRT	sirtuin
TBST	Tris-buffered saline with Tween 20
TET	ten-eleven translocation enzymes
TGF-β1	Transforming Growth Factor β1
t-RNA	transfer ribonucleic acid

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Chapter 1

INTRODUCTION

1.1 Epigenetics

Epigenetics refers to heritable changes in gene expression that are not the result of a primary change in DNA sequence. Epigenetics encompasses a variety of mechanisms that alter the structure and packaging of DNA in such a way as to influence the transcriptional machinery of genes (Howlett and McGee, 2016). Although epigenetic mechanisms can produce stable transgenerational changes in physiology, they can also acutely respond to dynamic environmental input and alter organismal phenotype in an adaptive response to environmental demand. The classic epigenetic mechanisms at the level of DNA are DNA methylation and histone modifications (Wolffe and Matzke, 1999; Burggren, 2014; Zhao *et al.,* 2014; Storey, 2015; Howlett and McGee, 2016).

1.2 DNA Methylation and Methyltransferases

DNA methylation was first identified in the 1960s and has since become recognized as a key epigenetic regulatory mechanism. DNA methylation is characterized by the covalent addition of a methyl group ($-CH_3$) to the C5 residues of cytosine nucleotide bases in DNA. The vast majority (~60-70%) of DNA methylation occurs at CpG sites; these are regions of DNA where cytosine and guanine nucleotides are adjacent to each other separated by a phosphodiester bond. However certain regions containing an atypically high density of CpG sites, called CpG islands, are comparably unmethylated and become highly differentially methylated in different tissues in response to environmental stimuli (Bird, 2002; Wijenayake and Storey, 2015; Alvarado *et al*., 2014; Howlett and McGee, 2016). DNA methylation is necessary for normal patterns of gene expression and can result in permanent phenotypic modification as indicated by the critical role of this modification in tissue differentiation early in embryonic development

and by the fact that aberrant patterns of DNA methylation are common biomarkers of cancer. However, DNA methylation has also recently been acknowledged as a dynamic mediator of transcriptional regulation in response to environmental signals. Recent literature has suggested that DNA methylation is also more than merely a random response to environmental signals modulating DNA methylation but instead represents an organized programmed response to predictable and specific environmental signals (Weaver *et al*., 2004; Bollati *et al*., 2007; Alvarado *et al.*, 2015). The methylation of DNA produces steric hindrance due to the bulky methyl group and interferes with access by transcriptional regulators to the promoter region of genes. Consequently, DNA methylation usually results in transcriptional repression and the silencing of gene expressions (Alvarado *et al*., 2014; Zhao *et al*., 2014; Howlett and McGee, 2016).

DNA methylation is catalyzed by a family of enzymes known as DNA methyltransferases (DNMTs). DNMTs function by removing a $-CH_3$ group from a donor molecule, usually S-adenosylmethionine (SAM), and covalently attaching it to the C5 residue of cytosine nucleotides. Currently, five DNMTs have been characterized: DNMT1, DNMT2, DNMT3A, DNMT3B and DNMT3L (Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016). DNMT1 functions as the maintenance methyltransferase and is responsible for propagating patterns of methylation from parent to daughter cells by specifically targeting hemi-methylated strands of DNA in daughter cells. DNMT2 does not actually possess any DNA methyltransferase activity and instead is responsible for methylating aspartic acid t-RNA in the cytosol of cells. DNMT3A and 3B are responsible for establishing de novo patterns of methylation in new cells. DNMT3L also does not possess any inherent methyltransferase activity but instead

interacts with DNMT3A to assist in the stimulation of de novo methylation (Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016). DNA methylation can also facilitate transcriptional repression through the recruitment of methyl-CpG-binding domain proteins (MBDs): MBD1, MBD2, and $MeCP_2$ which bind specifically to methylated strands of DNA and create steric hindrance that enhances transcriptional repression (Howlett and McGee, 2016). MBDs are also believed to associate with chromatin modifiers such as histone deacetylases to promote condensed chromatin structure and transcriptional silencing (Bogdanovic and Veenstra, 2009).

The downstream biological consequences of DNMT1 activity have not been clearly demonstrated, however, in mammals, DNMT1 appears to be involved in the regulation of the cell cycle and the maintenance of normal tissue differentiation and prevention of pathogenesis (Klein *et al*., 2011). Similarly, the exact biological function of DNMT2 remains vague but it seems to be involved in the proper formation of blood cells as well as ensuring proper polypeptide synthesis (Durdevic *et al*., 2013; Tuorto *et al.,* 2015). DNMT3A and 3B are heavily involved in regulating cellular growth and proliferation as well as tissue differentiation, especially during early embryonic development (Kaneda *et al*., 2004; Linhart *et al*., 2007; Gao *et al*., 2011; Yuanhui *et al*., 2016). DNMT3L has lost its methyltransferase motif and by itself does not have any identified biological function. However, it acts as a methylation regulator and binds with DNMT3a and 3b to stimulate de novo methylation (Aapola *et al*., 2002; Chedin *et al.*, 2002; Deplus *et al*., 2002; Hata *et al*., 2002). DNMT3L is also thought to interact with HDAC1 to assist in transcriptional repression (Aapola *et al*., 2002; Deplus *et al.,* 2002). Similarly, the biological functions of MBD1 and MBD2 are not yet identified but they

appear to mediate the biological consequences of DNA methylation (Hendrich and Bird 1998; Fujita *et al*., 1999). $MeCP_2$ is similar in function to MBD1 and MBD2 in that it can bind specifically to methylated DNA and mediate the biological consequences of methylation. However, it is essential for the proper development and functioning of nerve cells. Mutations in the $MeCP_2$ gene are the cause of Rett syndrome (Amir *et al*., 1999; Chahrour *et al*., 2008). It is worth noting at this point that the biological functions of these enzymes have been investigated primarily in mammalian species. It is not known if they serve the same role in distantly related non-mammalian species as well.

1.3 DNA Demethylation and Ten-Eleven Translocation Enzymes

It's worth reiterating at this point that DNA methylation is not necessarily a static permanent process but rather is often an active and reversible process that coordinates physiological processes with dynamic feedback from the environment (Howlett and McGee, 2016). The flip side of DNA methylation is DNA demethylation. Patterns of DNA methylation are removed through the actions of several demethylase enzymes, however for the purposes of this thesis the focus will remain on the demethylase enzymes known as ten-eleven translocation enzymes (TETs).

TETs function by converting methylated 5Cs on cytosine residues to 5-hydroxy-meythlated cytosine residues (5-hmC) through a hydroxylation followed by an oxidation reaction (Coulter *et al*., 2013). It is believed that the formation of 5-hmC serves to remove patterns of methylation by either impairing remethylation or further methylation by DNMTs. As a consequence, this "erases" methylation patterns through gradual dilution as cells divide or through the active replacement of modified cytosines as a stand-alone mechanism (Bhutani *et al*., 2011). Hence, the action of TET enzymes is

believed to be a key mechanism involved in regulating the dynamic reprogramming of the epigenome in response to environmental and developmental signals (Pera 2013). It is suspected that 5-hmC serves as a key intermediate in the remodification of cytosine bases. It is worth clarifying that TETs are not considered to be true demethylases as they do not fully restore 5-mC back to normal cytosine. Instead they produce the 5-hmC intermediate that can be further modified back to normal cytosine by the action of other enzymes not studied in this thesis. As of this date there are three distinct enzymes in the TET family: TET1, TET2 and TET3 (Bhutani *et al*., 2011).

TET1 catalyzes the hydroxylation and then oxidation of 5-mC to 5-hmC in an Fe^{2+} and α-ketoglutarate dependent reaction (Tahiliani *et al*., 2009; Ito *et al*., 2011). TET2 is the close cousin of TET1 and behaves in much the same way (Ko *et al*., 2010). Mechanistically, TET3 also functions the same way as TET1/2 although it appears to be more important at different stages of development. TET1 is believed to be involved in embryonic stem cell renewal capacity as well as the regulation of pluripotency and lineage specific genes (Bhutani *et al*., 2011; Ficz *et al*., 2011; Costa *et al*., 2013; Gao *et al*., 2013). The function of TET2 is not very well understood but it appears to be involved in the normal generation of bone marrow and impairments of TET2 activity correlate with the presence of myeloid cancers (Ko *et al*., 2010; Wang *et al*., 2015). Mutations of the TET2 gene are associated with dysregulations of the cell cycle control as well as cytokine and growth factor signaling (Metzeler *et al*., 2011). TET3 seems to be most important post-fertilization and is involved in regulating methylation patterns very early in development, particularly during the zygote and oocyte stages. It seems to play a stronger role in oxidizing 5-mC in paternal DNA compared to maternal DNA in zygotes

and is important in regulating zygotic transcriptional repression (Wossidlo *et al*., 2011; Shen *et al*., 2014; Tsukada *et al*., 2015). It is believed that TET3 plays a role in erasure/remodeling of sperm genome 5-mC methylation patterns in order to produce a state of pluripotency in zygotes to prepare them for differentiation in response to developmental signals (Gu *et al*., 2014; Peat *et al*., 2014). However, much like DNMTs, the biological roles of TETs have been studied much more extensively in mammalian species and it is unknown at the present time how similar their functions are in non-mammalian species.

1.4 Histone Modifications

As previously mentioned, the other classic epigenetic mechanism for controlling gene expression is the post-translational modification of histone proteins. Histones are amongst the oldest and most evolutionarily conserved proteins known as are the posttranslational modifications that control them, appearing in both prokaryotes and eukaryotes (Thiagalingam *et al*., 2003). Genomic DNA is localized to the nucleus of eukaryotic cells, where it exists in a highly folded, constrained, and compacted state by histone proteins organized in units called nucleosomes. Nucleosomes consist of strands of DNA wrapped around eight histone core proteins: H2A, H2B, H3 and H4. Histone proteins are comprised of a globular domain and a more flexible, charged, protruding NH2-terminus (tail) (Luger *et al*., 1998; Jenuwein and Allis 2001; Roth *et al*., 2001; De Ruijter *et al*., 2003; Thiagalingam *et al.,* 2003). Each nucleosome is defined by two approximately two helical turns (~146 base pairs) of DNA wrapped around an octamer of core histone proteins, which form several pairings: an H3-H4 tetramer and two H2A-H2B

dimers (Luger *et al.*, 1997; Jenuwein and Allis 2001; Roth *et al*., 2001; Thiagalingam *et al*., 2003).

Repeating nucleosomes form a dynamic polymer called chromatin (Jenuwein and Allis 2001; Howlett and McGee, 2016). Chromatin organization is contingent on the patterning of nucleosomes and largely determines the transcriptional state of genes. Gene transcriptional states are represented according to differences in histone modifications that are defined as either euchromatic (on) or heterochromatic (off) (Jenuwein and Allis 2001; Thiagalingam *et al*., 2003). Whether or not a gene is expressed at any given point is largely determined by how accessible its promoter region is to transcription factors. When chromatin is tightly wrapped, condensed and in a closed conformation, the DNA associated with that chromatin is largely sheltered from transcription factors and will thus not be expressed. Conversely, when chromatin is in a more unfolded and open, its corresponding genes and their promoters are more exposed and so are more likely to be expressed. Open chromatin is largely associated with transcriptionally active states whereas closed chromatin is associated with transcriptionally inactive states (Li *et al*., 2007; Howlett and McGee, 2016). Posttranslational modifications of histones alter the chemical structure of histones and consequently affect whether the histone is in a transcriptionally active or inactive state. Some of these posttranslational modifications include but are not limited to: methylation of lysine and arginine residues; methylation, acetylation, ubiquitination, ADP-ribosylation, or sumoylation of lysine residues; and phosphorylation of serine and threonine residues (Li *et al*., 2007). Much like MBDs in DNA methylation, the presence of many of these posttranslational modifications can also recruit transcription factors to the modified histones which in turn can also have effects

on gene transcription (Kouzarides 2007; Howlett and McGee, 2016). For the purposes of this thesis, only histone lysine acetylation and deacetylation will be discussed and investigated.

The acetylation of histones on the ε-carbons of the side chains of lysine residues is arguably the most well-studied histone posttranslational modification. The effects of histone lysine acetylation are largely dependent on which lysine residues are specifically acetylated. However, histone acetylation generally causes a relaxation of chromatin structure, exposing gene promoter regions to transcription factors and thereby promotes transcriptional activation. Acetylation of lysine residues occurs in the histone tail of H3 and H4 histones and disrupts the association between the positively charged histone tail and the negatively charged DNA backbone resulting in the relaxation of chromatin structure and a more open conformation (Hong *et al*., 1993). Lysine residues can be monoacetylated, di-acetylated, or tri-acetylated (Roth *et al*., 2001; Abshiru *et al*., 2015). Histone lysine acetylation is regulated by the actions of two classes of opposing enzymes referred to as Histone Acetyltransferases (HATs) and Histone Deacetylases (HDACs). The action of HATs usually results in a more relaxed chromatin structure and transcriptional activation whereas HDACs remove acetyl groups from lysine residues on histones tails and promote a more condensed chromatin structure and transcriptional repression (McKinsey *et al*., 2001; Roth *et al.,* 2001; De Ruijter *et al*., 2003).

1.5 Histone Acetyltransferases (HATs)

Much like DNA methylation, histone lysine acetylation is a dynamic mediator of transcriptional regulation in response to environmental signals and also likely represents a programmed response to predictable and specific environmental signals. The

acetylation of lysine residues can have different effects on transcription depending on which residues are acetylated and the extent of the acetylation (mono, di or tri-acetylation). Mono acetylation usually results in transcriptional activation whereas di and tri-acetylation often result in transcriptional repression (Howlett and McGee, 2016).

There are currently four classes of HAT enzymes: Gcn5-related N-acetyltransferases (GNAT), MYST, p300/CBP (CREB-binding protein) and other undistinguished HATs (Roth *et al*., 2001; Vetting *et al*., 2005; McCullough and Marmorstein, 2015; Howlett and McGee, 2016). GNAT can be further divided into the enzymes GCN5 and PCAF (P300/CBP-associated factor). The MYST class contains the enzymes: Tip60, MYST1 (MOF), MYST2 (MOZ), MORF, and HBO1. The p300/CBP family includes p300. Two other enzymes from a class of HATs which haven't yet been delineated including TFIIC and CLOCK (McKinsey *et al*., 2001; Roth *et al.,* 2001; Vetting *et al.,* 2005; Doi *et al.,* 2006; Howlett and McGee, 2016). Each of these HATs share a conserved acetyl-CoA binding core and catalytic mechanism, which transfers the acetyl moiety of acetyl-CoA to its target (Lahm *et al*., 2007). For this thesis specifically, only four HATs were analyzed: PCAF, Tip60, MYST1, and MYST2.

PCAF preferentially acetylates lysine 9, 14, and 18 on histone H3 as well as lysine 8 and 16 on H4 although to a much lesser degree on H4 than on H3 (Poux and Marmorstein 2003; Kouzarides 2007). Tip60 has been identified as acetylating lysines 5, 8, 12, and 16 on H4, lysine 14 on H3, and lysine 5 on H2A (Kimura and Horikoshi 1998; Kouzarides 2007; Gao *et al*., 2014). MYST1 acetylates lysine 5, 8 and 16 on H4 (Dou *et al*., 2005; Thomas *et al*., 2008; Cai *et al.,* 2010). MYST2 acetylates lysine 5, 8, 12, 16 on H4 (Doyon *et al*., 2006; Kouzarides 2007).

PCAF is believed to be involved in regulating cell growth and differentiation (Yang *et al*., 1996; Watts *et al*., 2004; Okumura *et al*., 2006; Modak *et al*., 2013). Tip60 similarly plays roles in transcriptional regulation as well as has involvement in the regulation of DNA repair and apoptosis (Legube *et al*., 2004; Sun *et al*., 2005; Murr *et al*., 2006; Van Den Broeck *et al*., 2011). The biological roles of MYST1 remain unclear however there is evidence for its involvement in the regulation of stem cell pluripotency, autophagy and cell growth (Fullgrabe *et al*., 2013; Jaganathan *et al*., 2014; Mu *et al*., 2015). Similarly, the biological roles of MYST2 have not been well characterized but it is believed to be involved in the regulation of cell growth and DNA replication (Doyon *et al*., 2006; Lizuka *et al*., 2016). Once again it should be noted that the biological roles of these enzymes have been elucidated in mammalian species but it is not yet known how similar their functions are in non-mammalian species.

1.6 Histone Deacetylases (HDACs)

A key theme of this thesis is that epigenetic modifications are not necessarily permanent, irreversible, stable changes. Instead, they are active and dynamic processes that can coordinate physiological adaptations in organisms with the demands placed on them by the environment. The same holds true for histone acetylation. The actions of HATs are antagonistically regulated by the actions of HDACs. There are three classes of HDACs: HDACs I, II, and III (De Ruijter *et al*., 2003; Howlett and McGee, 2016). Class I HDACs include HDACs 1,2, 3 and 8 and are distinguished by the need for a Zn^{2+} cofactor in order to have catalytic activity (De Ruijter *et al*., 2003; Thiagalingam *et al*., 2003; Howlett and McGee, 2016). Class 1 HDACs are usually ubiquitously expressed (McKinsey *et al*., 2001). Class II HDACs are further categorized as Class IIa and Class

IIb HDACs. Although Class II HDACs also require Zn^{2+} cofactors for catalytic activity, they are distinct from the class I enzymes in important ways. For example, class II HDACs are usually expressed in the heart, brain, and muscles. Additionally, class II HDACs typically have molecular masses approximately twice as high as class I HDACs (McKinsey *et al*., 2001; De Ruijter *et al*., 2003; Thiagalingam *et al*., 2003; Howlett and McGee, 2016). Class IIa HDACs include HDACs 4, 5, 7, and 9 and are responsible for the recruitment of corepressors. Class IIa HDACs do not possess any lysine deacetylase activity (due to an amino acid substitution at the catalytic site) but serve as platforms which connect transcription factors with other HDAC-containing complexes in order to assist regulation of transcriptional repression (De Ruijter *et al*., 2003; Thiagalingam *et al*., 2003; Lahm *et al*., 2007; Howlett and McGee, 2016). Class IIb HDACs includes HDACs 6 and 10 and although they possess HDAC activity they are primarily responsible for deacetylating non-histone proteins (De Ruijter *et al*., 2003; Howlett and McGee, 2016). Class III HDACs are comprised by a group of enzymes known as sirtuins (SIRTs), specifically SIRTs 1-7. SIRTs are distinct from other classes of HDACs in that they require NAD^{+} to have catalytic activity. Much like Class IIb HDACs, they are also responsible for deacetylating non-histone proteins although their exact functions haven't been clearly delineated yet (Thiagalingam *et al*., 2003; Howlett and McGee, 2016). For this thesis, HDACs 1-7 were analyzed.

In general, HDACs are responsible for deacetylating the lysine residues of the N-terminal region of H2A, H2B, H3 and H4 core proteins, however in contrast to HATs, they appear to show little substrate specificity regarding which lysine residues they deacetylate. HDAC1 appears to be involved in the regulation of cell growth and

proliferation (Magnaghi-Jaulin *et al*., 1998; Kawai *et al*., 2003; Noonan *et al*., 2009; Oh *et al*., 2009; Suzuki *et al*., 2009). HDAC2 has shown involvement in regulating cellular responses to oxidative stress and inflammation (Sun *et al*., 2007; Kong *et al*., 2009; LaBeouf *et al*., 2010). Similarly, HDAC3 has shown involvement in the regulating cell growth as well as responses to oxidative stress (Juan *et al*., 2000; Zhang *et al*., 2002; Martin *et al*., 2014). HDAC4 has a critical role in skeletogenesis and tissue differentiation (Lu *et al*., 2000; Vega *et al*., 2004; Glenisson *et al*., 2007). The biological role of HDAC5 is not very well studied however evidence has suggested an involvement in regulation of the cell cycle, apoptosis, and energy metabolism (Haberland *et al*., 2009; McGee *et al*., 2009; Fan *et al*., 2014). HDAC6 can influence Growth Factor signaling, as well as modulate cellular redox status and the removal of misfolded proteins from the cytosol (Kawaguchi *et al.,* 2003; Gao *et al*., 2007; Kwon *et al*., 2007; Parmigiani *et al*., 2008; Kekatpure *et al*., 2009). Finally, HDAC7 is known to be involved regulating cell growth and proliferation, especially during early embryonic development (Jensen *et al*., 2007; Margariti *et al*., 2010; Zhu *et al.,* 2011; Bradley *et al.,* 2015). It is worth reiterating one final time that the roles of HDACs have mainly been studied in mammalian models and it is not known if they serve the same functions in non-mammals.

1.7 Metabolic Rate Depression

Most organisms regularly experience environmental challenges that can threaten their survival. These challenges can include conditions such as extreme heat, extreme cold, lack of oxygen (anoxia/hypoxia), food (starvation) and/or water (dehydration), excessive radiation exposure, and disease, to name a few. Organisms can cope with environmental stresses in numerous ways. Some develop behavioural adaptations to

combat or elude the stress, whereas others have developed physiological mechanisms to help them survive, especially when stresses are chronic, severe and persistent (Storey *et al*., 2015). One of the most commonly observed methods in nature for surviving environmental stresses is a coordinated global reduction of metabolic rate and entry into prolonged periods of hypometabolism. A large and diverse range of species utilize metabolic rate depression to minimize energy expenditure under unfavourable conditions before returning to an active state once the environment is more beneficial to their survival (Storey *et al*., 2015). Examples of global metabolic rate depression are seen in phenomena such as hibernation, aestivation, and survival of freezing and anoxia.

Hypometabolic states are characterized by suppression of energy expenditure, reallocation of metabolic resources to favour vital cellular functions that are necessary for survival, and the preservation of cellular macromolecules via a variety of protective mechanisms (Storey *et al.,* 2015). During periods of hypometabolism, organisms redirect metabolic resources away from nonessential biological functions such as growth, membrane transport, reproduction, and degradation/turnover of proteins, lipids, carbohydrates and nucleic acids. Energy-expensive functions such as transcription and translation are also suppressed to conserve energy and extend the time that viability can be maintained. They must also prevent/minimize physical damage to their cells and organs resulting from prolonged exposure to stressors. In order to achieve a state of hypometabolism and survive, organisms must enact a global coordinated suppression of cellular and physiological activities (Storey 2015). Epigenetic modulations are crucial physiological mechanisms underpinning the coordinated entry into hypometabolic states. Transcription on its own can account for anywhere from 1-10% of total metabolic energy

expenditure. Therefore, it follows that epigenetic mechanisms could underpin the reordering and suppression of transcription and other cellular processes during hypometabolic states. Indeed, this has been supported by a wide body of empirical evidence in numerous and diverse organisms (Padilla *et al*., 2002; Morin and Storey, 2006; Krivoruchko and Storey, 2013; Alvarado *et al*., 2014; Storey, 2015; Wijenayake and Storey, 2015; Zhao *et al*., 2015).

1.8 Cold Hardiness and Metabolic Rate Depression

Earth can be a very unforgiving place. In Canada alone, winter surface temperatures on can commonly range anywhere from -10°C to -70°C depending on the latitude (Storey and Storey, 2012). Cold hardiness refers to a collection of adaptive physiological and biochemical processes that act in synchrony to protect organisms against damage due to low temperature exposure (Lyons *et al*., 2013). Subzero temperatures routinely threaten the survival of most complex organisms on planet Earth. Ice nucleation begins in extracellular fluids, often propagating through organisms and sometimes even penetrating membranes to freeze intracellular fluids. The larger volume of extracellular fluid compared to intracellular fluid means that single ice nucleation events are more probable and once a single ice nucleation event has taken place, a large volume means that there's more opportunities for the ice nucleation to propagate compared to a smaller volume. Substantial volumes of extracellular ice withdraw fluids from cells through osmosis and in doing so damages the cells through compression stresses. The structural damage that results from this is often lethal to the cells. Additionally most of the organism's body water can be withdrawn and frozen in the form of ice, which in turn prevents blood from delivering and recycling nutrients and wastes

throughout the organism. Furthermore, enough ice buildup also physically impede muscle contraction thereby restricting and/or halting the movement and respiration of organisms. In light of this, many organisms have evolved physiological and biochemical mechanisms to cope with the freezing temperatures commonly experienced in Earth's climate (Storey and Storey, 2013).

Seasonal changes in photoperiod and temperature can provide a source of environmental stimuli to trigger the epigenetic reprioritization of cellular resources and metabolic expenditure in organisms to prepare them for the subzero temperatures accompanying winter (Alvarado *et al*., 2014). Winter presents a number of survival challenges to organisms such as freezing temperatures, dehydration, as well as reduced availability of food and water. The environment provides a number of consistent cues that have allowed organisms to evolve epigenetic programs that can alter the organism's physiology in response to environmental signals indicating the change in seasons and facilitating metabolic rate depression. Many organisms enter into a state of hypometabolism during the winter in order to conserve energy during times of reduced food and water availability before resuming normal activity levels when temperatures have rewarmed again (Alvarado *et al*., 2014). Changes in the proteome during hypometabolism are necessary for survival of cold temperatures. In order to endure the long periods of hypometabolism during the winter organisms must suppress cellular and physiological functions that are too ATP-expensive to maintain during a frozen and/or dormant state (Storey and Storey, 2013). Furthermore, any physiological changes that an organism endures while entering into a hypometabolic state must be reversible for when the temperature warms up and the organism resumes normal levels of physical and

metabolic activity (Storey and Storey, 2013). Epigenetic modifications of genes and chromatin provide such a dynamic and reversible mechanism for the coordinated suppression of non-essential cellular and physiological activities and a reorganization of the proteome in response to seasonal cues indicating the impending freezing temperatures.

The class of multicellular organisms that are prominently capable of survival at temperatures below 0°C is the insects (Storey and Storey 2012). Understanding the biochemical/epigenetic underpinnings of insect cold hardiness has significant practical importance. Multiple species of agricultural and forests pests such as the rice stem borer (*Chilo suppressalis*), onion maggot (*Delia antiqua*), and spruce budworm (*Choristoneura fumiferana*) are cold hardy and have served as valuable model organisms for understanding adaptations that allow for cold and/or freezing survival (Storey and Storey 2012). Moreover, the capacity for cold hardiness will have impacts on species distributions resulting from climate change. Understanding cold hardiness can also be important for preserving economically beneficial species such as the silkworm *Bombyx mori* which is hugely important to the silk industry (Storey and Storey, 2012).

Insects commonly depress their overall metabolic rate to <10% of their normal values during the winter (Hoback and Stanley, 2001; Storey and Storey, 2012). Indeed, metabolic rate depression underlies a phenomena observed in insects known as diapause. Diapause is a physiological state of dormancy and a delay in development in response to adverse environmental conditions (Denlinger, 2002). Diapause is a common physiological adaptation for surviving winter subzero temperatures in many insects and often works in concert with other adaptations to enable cold hardiness. Diapause may

occur at numerous developmental time points and may be obligate or facultative depending on the species (Storey and Storey, 2012).

Multiple factors contribute to the metabolic rate depression and diapause observed in insects during the winter. Insects begin by reducing and eventually stopping feeding in order to lower metabolic expenditures on digestion, the biosynthesis of proteins, glycogen, triglycerides, etc. (Storey and Storey, 2012). Decreasing temperatures and shortened photoperiod also trigger the commencement of diapause which shuts down energetically expensive processes such as cell division, growth, tissue differentiation, reproduction and movement. Diapause can also act in concert with other physiological processes activated during winter specifically to promote cold hardiness. These processes utilize and redirect the metabolic fuel reserves towards vital functions and can include the specific activation of genes encoding, for example, antifreeze proteins, ice nucleating agents, or enzymes of polyhydric alcohol synthesis (e.g. glycerol, sorbitol) that act as colligative cryoprotectants to protect the intracellular environment. Diapause may also include the increased expression and activity of chaperone proteins and antioxidant defenses to prevent and minimize damage to structural proteins, lipids and carbohydrates during periods where metabolic energy to degrade/resynthesize damaged macromolecules is unavailable (Courteau *et al*., 2012; Storey and Storey, 2012).

Many of the characteristics of hypometabolism have been conserved across the animal kingdom. As previously mentioned, transitioning into and out of metabolic rate depression does not depend on a complete overhaul of metabolic organization but instead is dependent on acute, dynamic and reversible controls. This suggests that, amongst other things, these controls may be partially governed by epigenetic mechanisms. The present

thesis will investigate whether epigenetic mechanisms underlie subzero survival in two species of overwintering cold hardy insects that use different cold hardiness strategies for survival: *Eurosta solidaginis* (freeze tolerant) and *Epiblema scudderiana* (freeze avoiding). Specifically, these studies will examine changes in the relative expression and total activity of several enzymes responsible for epigenetic modifications of DNA (DNMTs/MBDs, TETs) as well as changes in the total activity of chromatin modifying enzymes (HATs and HDACs) across a range of temperatures set to simulate the low temperature experience that these insects are exposed to in their natural environment.

Other studies in our lab conducted by a former student (Shostak 2015, data unpublished), examined changes in the expression of specific HDACs, HATs, and acetylated lysine residues on histone targets in both *Eurosta* and *Epiblema* across the same time courses used in this thesis (Appendix A; Figures 15-24). These data were included in this thesis to provide a more comprehensive and complete interpretation of the data presented in this thesis and will also be integrated into joint publications at a later date.

1.9 Objectives and Hypotheses

1.9.1 ***Objective 1:*** *Profile the behavior of DNMTs, TETs, HATs, and HDACs across a range of experimental temperatures in Eurosta solidaginis*

Studies in other models of metabolic rate depression have demonstrated changes in the expression and activity of epigenetic enzymes during periods of hypometabolism and stress exposure. Moreover, these changes seem to be widely conserved across different species of organisms. It therefore follows that similar patterns of behaviour in

epigenetic enzymes should be observed during freeze tolerance in *Eurosta solidaginis* as well.

Hypothesis 1: *Eurosta solidaginis* will show differential expression and activity of DNMTs, TETs, HATs, and HDACs corresponding to decreases in temperature.

Chapter 3 tests this hypothesis by measuring changes in the expression of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2, $MeCP_2$, TET1, TET2, and TET3 in *Eurosta solidaginis* during cold and subzero temperatures. It will also measure changes in: total DNMT activity, total TET activity, total HAT activity, and total HDAC activity across the same range of temperatures. The goal of this chapter is to examine how these enzymes respond to changes in temperature and profile the epigenetic underpinnings of freeze tolerance.

1.9.2 ***Objective 2:*** *Profile the behavior of DNMTs, TETs, HATs, and HDACs across a range of experimental temperatures in Epiblema scudderiana*

The same line of reasoning cited for *Eurosta* also serves as the imputes for studying *Epiblema*. Since other models of metabolic rate depression have demonstrated changes in the expression and activity of epigenetic enzymes during periods of hypometabolism and stress exposure, it's believed that similar changes should exist in *Epiblema* as well. It again follows that similar patterns of behaviour in epigenetic enzymes should be observed during freeze avoidance in *Epiblema scudderiana*.

Hypothesis 2: *Epiblema scudderiana* will show differential expression and activity of DNMTs, TETs, HATs, and HDACs corresponding to decreases in temperature.

Chapter 4 tests this hypothesis by measuring changes in the expression of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2, $MeCP_2$, TET1, TET2, and TET3 in *Epiblema scudderiana* during cold and subzero temperatures. It will also measure changes in: total DNMT activity, total TET activity, total HAT activity, and total HDAC activity across the same range of temperatures. The goal of this chapter is to examine how these enzymes respond to changes in temperature and profile the epigenetic underpinnings of freeze avoidance.

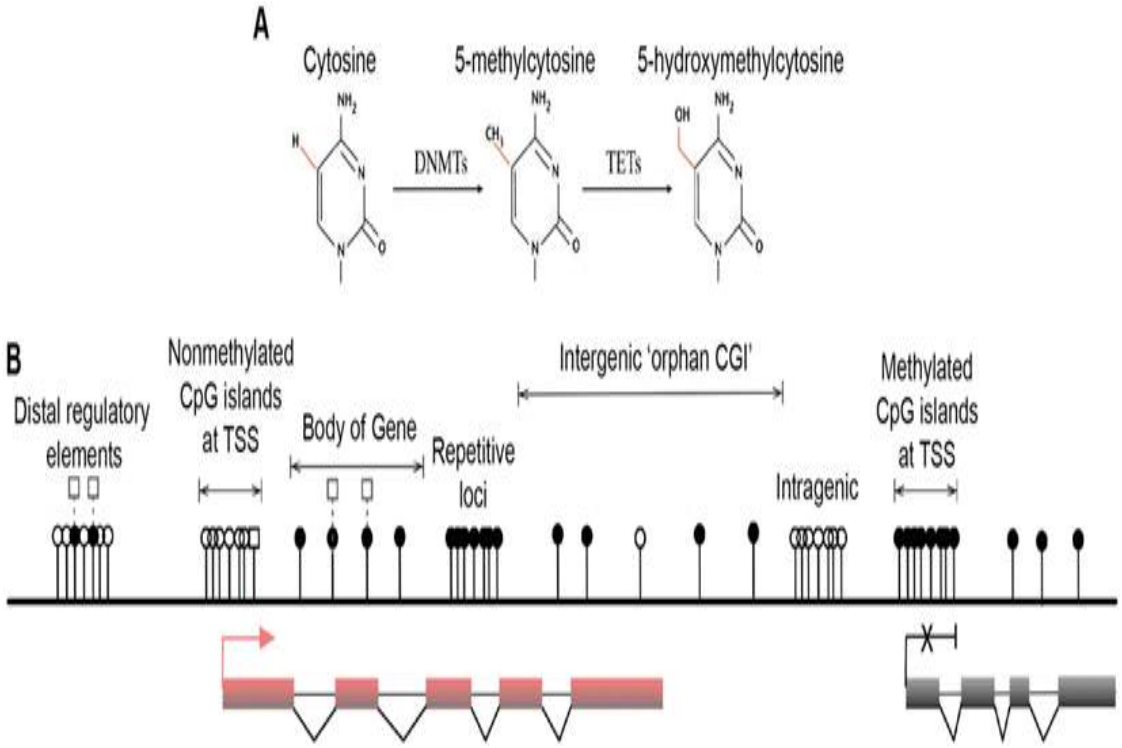

Figure I. A) illustrates the chemical modifications on cytosine bases of DNA catalyzed by DNMTs and TETs. B) schematic showing the effects of methylation on gene transcription (Figure taken from Meng *et al*., 2015)

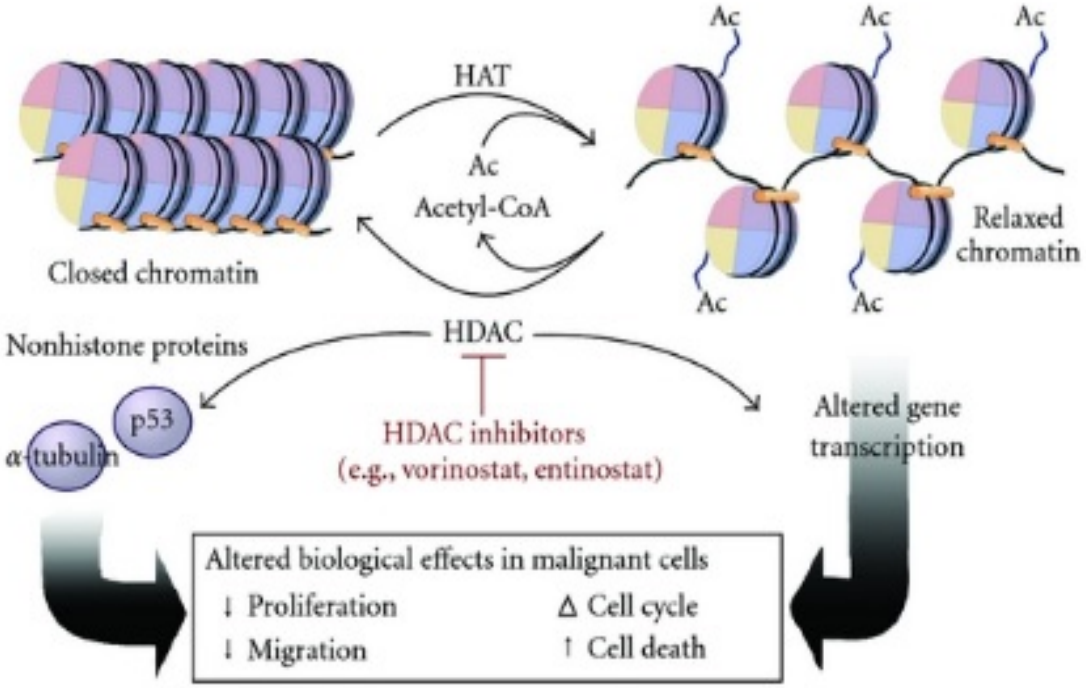

Figure II illustrates the chemical modifications of histones catalyzed by HATs and HDACs as well as the corresponding effect on chromatin structure following acetylation or deacetylation. (Figure taken from wikipedia.org)

Chapter 2
General Materials and Methods

2.1 Insect Collection

Goldenrod galls containing *E. solidaginis* or *E. scudderiana* larvae were collected from fields in the Ottawa, Canada region in 2006, 2007, and 2008. For the 2006/2007 samples, sample collection was performed on October 18-20 and October 24-27. The collected galls containing insects were first acclimated in an incubator in the lab at 15°C for 3 weeks. A group of galls were then randomly removed from the incubator, insects were quickly extracted and rapidly flash frozen in liquid nitrogen; these served as controls. The temperature of the incubator was then turned down to 5°C and remaining galls were exposed to this lower temperature for 4 hours, 1 week or 3 weeks, with sampling after each time. Subsequently, the temperature was lowered from 5°C to -15°C and galls were sampled after 4 hours and 1 week. For the 2008 samples (*E. solidaginis* only), sample collection was performed on October 15-17. Collected galls were acclimated in the lab at 15°C for 3 weeks and then transferred to 5°C for 3 weeks, and then to -15°C for 3 weeks. All insect acclimation experiments were conducted with the insects inside their galls *in situ*. After each time period, groups of galls were randomly removed and insects were rapidly extracted and flash frozen in liquid nitrogen. All samples were stored at -80°C until use.

2.2 Total Protein Isolation

To prepare soluble protein extracts, samples of frozen larvae of *Eurosta* or *Epiblema* (each n=a pool of 8-13 larvae/~0.5 grams of larvae) were quickly weighed, then ground into a powder under liquid nitrogen in with a mortar and pestle. Samples were then quickly added to homogenizing buffer at a 1:2 w:v ratio and homogenized using a Polytron homogenizer at a high speed for 15 seconds. Homogenizing buffer contained: 20 mM Hepes pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM

Na_3VO_4, 10 mM β-glycerophosphate with the addition immediately before homogenization of several crystals of phenylmethylsulfonyl fluoride (PMSF) and 10.0 µl/ml of Sigma Protease Inhibitor (components: 104 mM AEBSF, 80 µM aprotinin, 4 mM bestatin, 1.4 mM E-64, 2 mM leupeptin, 1.5 mM pepstatin A). Homogenates were then centrifuged at 4°C for 15 minutes at a speed of 10,000 rpm. Supernatants were transferred to clean tubes and stored on ice while the pellet was discarded. Soluble protein concentrations were then determined with triplicate readings on a microplate reader using the BioRad Protein Assay (Catalogue #: 500-0006) with Bovine Serum Albumin as the standard. Absorbance readings were measured at 595 nm on a BIO-TEK Power Wave HT Spectrophotometer using Gen5 software. Calculated volumes of homogenizing buffer were then added to each extract in order to standardize each sample to a highest common concentration. Aliquots of the standardized samples were then mixed at a 1:1 v:v ratio with 2x SDS blue buffer (components: 100 mM Tris-base, 4 % w:v SDS, 20 % v:v glycerol, 0.2 % w:v bromophenol blue, 10 % v:v 2-mercaptoethanol) and boiled for 10 minutes to denature the proteins. Finally, samples were stored at -40°C until use.

2.3 Western Immunoblotting

Samples containing 20-40 µg protein were loaded onto 6-10% SDS-polyacrylamide gels and subsequently separated via electrophoresis for 65-130 minutes at 180V and 0.160A in 1× Tris–glycine running buffer (75.5 g of Tris-base, 460 g glycine, 25 g SDS, ddH2O up to 2.5 L) using a BioRad Mini-Protean 3 System. Volume of sample used, gel percentage and run time were constant for individual proteins but varied between proteins depending on protein subunit MW and on the results of initial

optimization runs. Aliquots of 2-3 µl of pre-stained protein molecular weight ladders (either Froggabio; Cat. # PM005- 0500, PM007-0500 K or BLUeye Prestained Protein Ladder; Cat. # PM007-0500 for each gel) were run on the gel concurrently with the samples to serve as a molecular weight reference.

Following electrophoresis, proteins embedded in the gel were transferred onto 0.45 µm PVDF membranes (Millipore, Cat. #: IPVH00010) submerged in ~800 ml of transfer buffer (buffer contained 60.6 g Tris-base, 288 g glycine, 4 L methanol, 16 L ddH_2O) at 160-320 mA for 65–165 min (depending on the molecular weight of the proteins) using BioRad Mini-Protean Transfer cells.

After transfer, the PVDF membranes were washed for 5 minutes in 0.5×TBST (10 mM Tris, 150 mM NaCl, 0.05 % v/v Tween-20, pH 7.5) before blocking with 0.5-2.5% milk for 10-30 minutes followed by washing again 3 times for 5 minutes each in 0.5xTBST. Each PVDF membrane was then incubated with primary antibody at a dilution of 1:1000 v:v antibody: TBST overnight (17.5-22.5 hours) at 4°C. One antibody used in this analysis was purchased from Santa Cruz Biotechnology (DNMT1-sc-10221) whereas seven others were purchased from GeneTex (DNMT2-GTX13892; DNMT3A-GTX129125; DNMT3B-GTX129127; DNMT3L-GTX32565, TET1-GTX627420, TET2-GTX12424205, TET3-GTX121453), and three were purchased from Active Motif (MBD1-39857; MBD2-39547; $MeCP_2$-39188).

Following overnight incubation with the primary antibody, all PVDF membranes were washed 3 times for 5 minutes each in 0.5xTBST in order to remove any non-specific primary antibody. Subsequent to washing, all PVDF membranes were incubated in 1:8000 v:v diluted HRP-conjugated anti-rabbit IgG secondary antibody (Bioshop; Cat.

APA007P) in 0.5xTBST for 30 minutes at room temperature. Each membrane was then washed three-seven times for 5 minutes each time with 0.5×TBST. Target proteins were finally quantified using chemiluminescence; membranes were exposed to a mixture of 600 µl of Luminol and H_2O_2 (1:1 v:v) for 3 minutes and then imaged in a Chemi-Genius Bio-Imaging System (Syngene, Frederick, MD) and Gene Tools software. Membranes were then stained with Coomassie blue (0.25 % w:v Coomassie brilliant blue, 7.5 % v:v acetic acid, 50 % v:v methanol) and protein band densities were also quantified with the Chemi-Genius Bio-Imaging System using normal light settings to image the blue bands. For every target examined, the image analysis box covered the entire protein band and all image boxes used were the same size.

2.4 DNMT Activity

Total DNMT activity levels were assessed across the full experimental time course for the 2006 *E. scudderiana* samples (15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours, -15°C 1 Week). Total DNMT activities were also assessed across experimental time points for the 2006 *E. solidaginis* samples (15°C 3 Weeks, 5°C 3 Weeks, -15°C 1 Week); fewer time points were used due to limited availability of *E. solidaginis* samples. The activity assay kit purchased was an Epigentek EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit (Catalog # P-3009). The assay was carried out according to the user manual provided by the manufacturers.

Broadly, sample aliquots containing 30 µg of *E. scudderiana* or 20 µg of *E. solidaginis* larvae total soluble protein were used for each assay. These were mixed with ~45 µl of diluted assay buffer (Adomet 50X in DNMT Assay Buffer, 1:50 v:v) provided with the kit and incubated in a microplate for 120 minutes at 37°C. A well containing 50

µl of diluted Assay Buffer (Adomet 50X in DNMT Assay Buffer, 1:50 v:v) was used as a blank and a purified DNMT enzyme positive control (50 µg/ml; provided by Epigentek) was run with each plate. Following incubation, each well in the microplate was washed 3 times with 150 µl of 1X Wash Buffer prior to the addition of 50 µl of Capture Antibody (1000 µg/µl) per well and incubation at room temperature for 60 minutes. Following this incubation, the Capture Antibody was removed through 3 washes of 150 µl of 1X Wash Buffer and then 50µl of Detection Antibody (400 µg/µl) was added to each well before incubation at room temperature for 30 minutes.

Subsequently, 50 µl of Enhancer Solution was added to each well and incubated for 30 minutes at room temperature. Finally, 100µl of Developing Solution was added to each well and incubated for 7.5 minutes at room temperature away from direct light. Once a sufficient amount of methylated DNA was detected (as determined by the medium blue colouration of the positive control well), 100 µl of Stop Solution was added to each well to halt the reaction and the plate was read using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450nm and a reference wavelength of 655 nm.

Total DNMT activity was calculated using the formula:

$$\text{DNMT Activity (OD/h/mg)} = \frac{\text{(Sample OD – Blank OD) x 1000}}{\text{[protein amount (µg) x incubation time (h)]}}$$

Although the protein amounts used in this activity assay were in micrograms, the final total activity values were given in activity units (OD) per milligram amounts of protein according to the above calculation as per manufacturer's instructions.

2.5 HAT Activity

Total HAT activities were assessed across the same experimental time courses for *E. scudderiana* and *E. solidaginis* as described above for DNMT activity analysis. The activity assay kit purchased was an Epigentek EpiQuick HAT Activity/Inhibition Assay Kit (Catalog # P-4003). The assay was carried out according to the user manual provided by the manufacturers.

Broadly, 20 µg of *E. scudderiana* or 20 µg of *E. solidaginis* larvae total soluble protein were used for each assay. Diluted HAT substrates were first incubated in a microplate for 45 minutes at room temperature. Each well was then washed 3 times with diluted Wash Buffer before the addition of 26 µl of HAT Assay Buffer, 2 µl of acetyl-CoA diluted in HAT Assay Buffer (1:20 v:v), and 2 µl of total soluble protein samples to each well. A well containing 28µl of Assay Buffer and 2µl of diluted Acetyl-CoA in Assay Buffer (1:20 v:v) was used as a positive control and a well containing 30 µl of HAT Assay Buffer alone was used as a blank for each respective time course. Sample mixtures were incubated for 60 minutes at 37°C. Following incubation, each well in the microplate was washed 3 times with 150 µl of 1X Wash Buffer prior to the addition of 50 µl of Capture Antibody (1000 µg/µl) per well and incubation at room temperature for 60 minutes. Following this incubation, the Capture Antibody was removed through 4 washes of 150 µl of 1X Wash Buffer and 50 µl of Detection Antibody (0.2 µg/ml) was then added to each well before incubation at room temperature for 30 minutes.

Each well was then aspirated and washed with 150 µl of 1X Wash Buffer five times. This was then succeeded by the addition of 100 µl of Developing Solution to each well and an incubation for 7.5 minutes at room temperature away from direct light. Once

a sufficient amount of acetylated histones were detected (as determined by the medium blue colouration of the positive control well), 50 µl of Stop Solution was added to each well to halt the reaction and the plate was read using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450nm.

Total HAT Activity was calculated using the formula:

$$\text{HAT Activity (OD/h/mg)} = \frac{\text{(Sample OD – Blank OD) x 1000}}{\text{[protein amount (µg) x incubation time (h)]}}$$

Although the protein amounts used in this activity assay were in micrograms, the final total activity values were given in activity units (OD) per milligram amounts of protein according to the above calculation as per manufacturer's instructions.

2.6 HDAC Activity

Total HDAC activities were assessed across experimental time courses for *E. scudderiana* and *E. solidaginis* as described above for DNMT activity analysis. The activity assay kit purchased was an Active Motif HDAC Assay Kit (Colorimetric; Catalog # 56210). The assay was carried out according to the user manual provided by the manufacturers.

Broadly, aliquots of extracts containing 50 µg of *E. scudderiana* or 85 µg of *E. solidaginis* soluble protein were used for each point in their respective time courses. Total soluble protein samples for each time point were mixed with HDAC Assay Buffer (~35 µl) and 10 µl of diluted HDAC Substrate (1:10 v:v) in HDAC Assay Buffer. A well containing 40 µl of HDAC Assay Buffer and 10 µl of diluted HDAC Substrate (1:10 v:v) in HDAC Assay Buffer served as the blank whereas a well containing 10 µl of HDAC Assay Buffer, 10 µl of diluted HDAC Substrate (1:10 v:v) in HDAC Assay Buffer, and

30 µl of diluted HeLa nuclear extract (1:3 v:v) in HDAC Assay Buffer served as the positive control.

Samples were incubated in a microplate for 60 minutes at 37°C. Following incubation, 50 µl of diluted Trichostatin A (1:100 v:v) in HDAC Developer solution was added to each well before an incubation at room temperature for 15 minutes in order to stop the reactions. The plate was read using a microplate reader (Multiscan Spectrum, Thermo Labsystems) at 405nm.

The total HDAC Activity was calculated using the formula:

$$\text{HDAC Activity (OD/h/mg)} = \frac{\text{(Sample OD – Blank OD) x 1000}}{\text{[protein amount (µg) x incubation time (h)]}}$$

Although the protein amounts used in this activity assay were in micrograms, the final total activity values were given in activity units (OD) per milligram amounts of protein according to the above calculation as per manufacturer's instructions.

2.7 TET Activity

Total TET activity levels were assessed across the same experimental time course for *E. scudderiana* as described above for DNMT activity analysis. Due to limited availability of tissues, total TET activity levels were assessed across the 2008 experimental time course for *E. solidaginis*. The activity assay kit purchased was an Epigentek Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay Kit (Catalog # P-3086). The assay was carried out according to the user manual provided by the manufacturers.

Broadly, 1.25 µg of *E. scudderiana* and 1.00 µg of *E. solidaginis* larvae total soluble protein were used for each assay. First, wells containing 80 µl of Binding Solution and 2 µl of diluted TET Substrate in TET Assay Buffer (1:20 v:v) were incubated in a microplate for 90 minutes at 37°C. Each well was then washed 3 times with diluted Wash Buffer before the addition of ~49 µl of Final TET Assay Buffer (Co-Factor 1, Co-Factor 2, Co-Factor 2, TET Assay Buffer, 1:100 v:v) and ~1.00 µl of insect protein samples to each well. A well containing 50 µl of Final TET Assay Buffer was used as a blank for each respective time course. Sample mixtures were incubated for 90 minutes at 37°C. Following incubation, each well in the microplate was washed 3 times with 150 µl of 1X Wash Buffer prior to the addition of 50 µl of a Capture Antibody (1000 µg/µl) that binds specifically to 5-hmC per well and incubation at room temperature for 60 minutes. Following this incubation, the Capture Antibody was removed through 3 washes of 150 µl of 1X Wash Buffer and 50 µl of Detection Antibody (0.2 µg/ml) was then added to each well before incubation at room temperature for 30 minutes.

Each well was then aspirated and washed with 150 µl of 1X Wash Buffer four times. This was then succeeded by the addition of 50 µl of Enhancer Solution to each well and an incubation for 30 minutes at room temperature. The wells were then washed with 150 µl of 1X Wash Buffer five times before adding 100 µl of Developing Solution to each well. Once added the sample was incubated for 7.5 minutes at room temperature away from direct light. Once a sufficient amount of hydroxymethylated DNA was detected (as determined by the medium blue colouration of the wells), 50 µl of Stop Solution was added to each well to halt the reaction and the plate was read using a

microplate reader (Multiscan Spectrum, Thermo Labsystems) at 450nm with a reference wavelength of 655 nm.

The total TET Activity was calculated using the formula:

$$\text{TET Activity (OD/h/mg)} = \frac{\text{(Sample OD – Blank OD) x 1000}}{\text{[protein amount (µg) x incubation time (h)]}}$$

Although the protein amounts used in this activity assay were in micrograms, the final total activity values were given in activity units (OD) per milligram amounts of protein according to the above calculation as per manufacturer's instructions.

2.8 Statistical Analysis

In order to ensure constant protein loading across each time course and to control for confounds caused by variability in protein loading, band intensities on PVDF membranes were normalized against the total intensity of a group of Coomassie-stained protein bands in the same lane that showed constant expression across the experimental time course. Aliquots of 2-3 µl of pre-stained protein molecular weight ladders (Froggabio; Cat. # PM005- 0500 and PM007-0500 K and BLUeye Prestained Protein Ladder; Cat. # PM007-0500) as well as 5 µl of mammalian positive control samples (liver extract from ground squirrel, *Ictidomys tridecemlineatus*) were run on each gel along with the experimental samples. One-way ANOVAs and a Tukey post hoc test ($p < 0.05$) were conducted using Sigmaplot 12.0 software (Systat Software Inc., San Jose, CA) to test for significant differences among the different experimental conditions as well as construction of data plots for all expression and activity data.

Chapter 3

Epigenetics of Freeze Tolerance in *Eurosta solidaginis*

3.1 Introduction

The goldenrod gall fly *Eurosta solidaginis* (Diptera, Tephritidae) is an insect that inhabits the goldenrod plant (*Solidago canadensis*). The species distribution of this fly ranges from the Gulf of Mexico all the way up to northern and central Canada (Lee and Costanzo 1998; Morin *et al*., 2005). Adult flies emerge in April-May (in the Ottawa area) from stem galls on the previous year's goldenrod plants. After mating, females lay eggs in the tips of the newly-growing goldenrod shoots (goldenrod is a perennial) and when they hatch the larvae bore into the center of the new stem. There the larvae secrete compounds which have hormomimetic properties of goldenrod hormones and these trigger the plant to form tissue swellings (galls) around the larvae and maintain nutrient-rich cells within the central gall cavity (Courteau *et al*., 2012). The larvae feed on these nutrient-rich cells throughout the summer and early autumn, progressing through three instars. During the autumn the third instar larva eats out a tunnel to just under the "skin" of the gall before retreating into the central cavity and entering diapause for the winter. In April/May the larvae pupate and when the non-feeding adults hatch, they walk up the tunnel and push out of the gall to start the cycle again. The galls themselves offer only limited protection from the ambient air temperature and therefore *Eurosta* are often exposed not only to very low ambient temperatures (sometimes as low as -30°C in the Ottawa area) but also to rapidly fluctuating temperatures, especially in galls that project above the snowpack throughout the winter. It is not uncommon for *Eurosta* to be exposed to daily changes in temperature of as much as 25-35°C (Lee and Costanzo 1998; Morin *et al*., 2005).

During the autumn, *Eurosta* larvae detect changes in photoperiod and thermoperiod and this stimulates the development of a variety of physiological and biochemical changes that implement cold hardiness. *Eurosta* larvae use a freeze tolerance strategy of cold hardiness (Lee and Costanzo 1998; Morin *et al*., 2005; Rider *et al*., 2011). Freeze tolerant species can typically endure the freezing of about 65% of total body water in extracellular spaces while defending the liquid state of intracellular fluids (Duman 2001). *Eurosta* larvae are commonly used as a model of insect freeze tolerance in scientific research (Pfister and Storey 2006; Lyons *et al*., 2013; Lyons *et al*., 2015). Freezing presents a number of physiological challenges including: hypoxia/anoxia, dehydration, oxidative damage to cells, physical shearing of cells and tissues by surrounding ice crystals, etc. Consequently, understanding natural models of freeze tolerance can lend itself to understanding the principles and mechanisms underlying the biological adaptations to these stresses (Lee and Costanzo 1998; Morin *et al*., 2005).

Freeze tolerance in *Eurosta* consists of the coordinated implementation of metabolic rate depression (diapause) in concert with the production of very high concentrations of cryoprotectants (mainly glycerol and sorbitol), ice nucleators and general changes in patterns of gene expression (Duman 2001; Pfister and Storey 2006; Courteau *et al*., 2012). During the summer and early autumn, *Eurosta* accumulate large glycogen stores (Storey and Storey, 1986). Low temperatures in the autumn and early winter activate glycogen phosphorylase (via phosphorylation activation of glycogen phosphorylase kinase and inhibition of protein phosphatase 1) resulting in the breakdown of glycogen and the production of large amounts of glycerol and sorbitol. At the same time gluconeogenesis is inhibited by the phosphorylation mediated inhibition of glycogen

synthase (Storey 1997; Pfister and Storey 2006; Rider *et al*., 2011). *Eurosta* larvae also produce ice nucleating agents that act at relatively high freezing temperatures (-8°C to -10°C) to commence the freezing of the insect in a slow and highly regulated way that prevents the damage that can otherwise be caused by very rapid freezing from a more deeply supercooled state (e.g. the supercooling point of the larvae in summer is much lower, about -15°C) (Duman 2001; Courteau *et al*., 2012; Storey and Storey 2013). This is crucial to the survival of the larvae, as they often tolerate upwards of 65% of their total body water being frozen while still remaining viable (McMullen and Storey 2008; Lyons *et al*., 2013; Lyons *et al*., 2015). The exact ice nucleating agents employed by *Eurosta* are not yet known, however it is believed that they use potassium phosphate, calcium phosphate, sodium urate and/or potassium urate crystals (Duman 2001). Ice nucleating agents in effect restrict most of the ice formation to extracellular spaces where it is not lethal to cells. Ice in the extracellular spaces excludes solutes from its crystal matrix and thereby increases the osmolality of the remaining unfrozen extracellular water. This results in a net outflux of water from cells and reduces the freezing and nucleation point of intracellular fluids, preventing the accumulation of intracellular ice (Duman 2001; Storey and Storey 2013). Ice nucleating agents act in concert with glycerol and sorbitol, which help prevent the accumulation of too much ice in the extracellular space and the death of the cell by osmotically driven dehydration which can cause physical damage to the bilayer structure of cell membranes (Duman 2001; Storey and Storey 2013).

Patterns of gene expression also commonly change as a result of freezing, often in ways involving the upregulation of various categories of genes such as those involved in antioxidant responses and maintenance of cellular redox status, such as HIF-1, GST,

SOD, CAT and Se-GPX as well as chaperone proteins such as HSPs. A regulated suppression of the activities of enzymes involved in mitochondrial oxidation (citrate synthase, NAD-isocitrate dehydrogenase and glutamate dehydrogenase) is also known to occur (Storey 1997; Pfister and Storey 2006; Zhang *et al*., 2011; Courteau *et al*., 2012; Storey and Storey 2012). Additionally, the Na^+/K^+-ATPase ion pump is the single greatest consumer of cellular ATP, often accounting for anywhere between 5-40% of the total ATP consumption depending on the tissue type. Consequently, huge savings in metabolic energy expenditure can be accomplished by the coordinated suppression of the Na^+/K^+-ATPase pump as well as other ion pumps (e.g. Ca^{2+}-ATPase) (Storey and Storey 2012). In light of this it is not surprisingly that *Eurosta* shows reduced activity of Na^+/K^+-ATPase by as much as 80-85% during the winter, as well as suppression of Ca^{2+}-ATPase activity by 65-80% (Storey and Storey 2012; Storey and Storey 2013). The AMP-activated protein kinase (AMPK) is also a key regulator of catabolic/anabolic activity in cells. AMPK is a protein kinase that is allosterically activated by AMP buildup under low energy conditions. It is often referred to as the fuel gauge of cells and acts as an energy sensor (Storey and Storey 2012). AMPK phosphorylates and inactivates acetyl-CoA carboxylase, thereby inhibiting lipogenesis and favouring fatty acid oxidation. AMPK also inhibits other anabolic enzymes as well as carbohydrate storage by inhibiting glycogen synthase. *Eurosta* has shown lower levels of AMPK and lipolytic enzyme activity during frozen states in midwinter (Storey and Storey 2012).

It is clear from the literature cited above that *Eurosta* employs metabolic rate depression mechanisms in conjunction with freeze tolerance to survive subzero winter temperatures. The speed, acuteness, and reversibility of the aforementioned mechanisms

suggests that epigenetic mechanisms may control many of the processes required for freezing survival. Epigenetic responses to stress have been observed in a number of other model organisms (Howlett and McGee 2016; Wijenayake and Storey 2016; Alvarado *et al*., 2015; Lyons *et al.,* 2015a; Storey 2015; Zhao *et al*., 2015; Alvarado *et al*., 2014; Lang-Oullette and Morin 2014; Storey and Storey 2013; Lyons *et al*., 2013a) and selected types of epigenetic control (specifically microRNA control of gene expression) has also been illustrated in *Eurosta* itself (Lyons *et al*., 2013b; Lyons *et al*., 2015b, Courteau *et al*., 2012). Collectively, these studies serve as the rationale for the current thesis. Since epigenetic responses to stress have been observed in other organisms as well as in *Eurosta*, it is believed that they are also involved in hypometabolism and freeze tolerance in *Eurosta* as well. This chapter examines the changes in expression and activities of key epigenetic enzymes (DNMTs/MBDs, TETs, HATs and HDACs), in *Eurosta solidaginis* larvae exposed to a range of temperatures for different durations, including both unfrozen (15 and 5°C) and frozen (-15°C) states (see **Chapter 2**). The different temperature and duration conditions were designed to simulate the conditions *Eurosta* is naturally exposed to in its environment during the winter, as well as provide an indication of how responsive its epigenetic enzymes are to changes in temperature. It is hypothesized that *Eurosta* will show differential regulation of DNMT, MBD, TET, HDAC and HAT expression and activity that is reflective of its exposure to different temperatures.

3.2 Materials and Methods

3.2.1. Insect Collection

Insect collection was performed as described in **Chapter 2.1**

3.2.2. Total Protein Isolation

Total protein isolation was performed as described in **Chapter 2.2**

3.2.3 Western Blotting

Western Blotting was performed as described in **Chapter 2.3**. Expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2, $MeCP_2$, TET1, TET2, and TET3 were analyzed. See **Appendix A: Supplementary Expression Data for Histone Modifications** for expression data on the Histone Acetyltransferases (HATs): PCAF, Tip60, MYST1 and MYST2; the Histone Deacetylases (HDACs): HDAC 1-7; and the Histone Acetylation levels of: H2A.K5.Ac, H2B.K5.Ac, Total Histone H3, Total Acetylated Histone H3, H3.K9.Ac, H3.K14.Ac, H3.K18.Ac, H3.K23.Ac, H3.K27.Ac, H3.K56.Ac, H4.K8.Ac. See **Appendix B: Antibody Information** for a full list of antibodies used during western blotting. See **Appendix C: Western Blotting Conditions** for a list of western blotting parameters for each protein analyzed.

3.2.4. Epigenetic enzyme activities:

Total activities of DNMTs, HATs, HDACs and TETs were assayed as described in **Chapter 2.4, 2.5, 2.6 and 2.7, respectively.**

Statistical Analysis

Statistical analyses were performed as described in **Chapter 2.8**

3.3 Results

3.3.1 DNMT and MBD protein expression in response to low temperature exposure

Relative protein expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2 and $MeCP_2$ were measured in samples of larvae from freeze tolerant *E. solidaginis*. DNMT3L expression levels showed a significant 1.6 fold upregulation ($P<0.05$) during the 5°C 1 Week condition compared to controls (15°C 3 weeks) and the 5°C 4 Hours condition before returning to control levels once larvae were frozen at -15°C (Figure 1). MBD2 expression levels in *E. solidaginis* were significantly upregulated during the -15°C 1 Week condition compared to all other conditions: values were 2.39-fold higher compared to the control, 4.36 fold compared to the 5°C 4 Hours condition, 2.66 fold compared to the 5°C 1 Week condition, and 1.92 fold compared to the -15°C 4 Hours condition ($P<0.05$; Figure 1). Similarly, expression levels during the -15°C 4 Hours condition were significantly upregulated by 2.27 fold compared to the 5°C 4 Hours condition ($P<0.05$; Figure 1). $MeCP_2$ expression levels showed a significant upregulation in response to 5°C exposure, rising by 2.24-fold after 4 Hours and 2.62-fold after 1 Week, compared to control levels ($P<0.05$; Figure 1). However, exposure to -15°C lowered $MeCP_2$ back to values not different from controls.

No significant differences were observed in the expression levels of DNMT1, DNMT2, DNMT3B, and MBD1 at any of the experimental time points and temperatures as compared with their control values. DNMT3A was not detected in *E. solidaginis*; this might be due to a lack of cross reactivity with the antibody used or to the absence of this DNMT family member in this insect species (Figure 2).

3.3.2 DNMT activity in larvae in response to low temperature exposure

Total DNMT activity was measured as the change in optical density (OD) per hour per mg of total soluble protein in *E. solidaginis*, representing the cytosine methylation of a polynucleotide substrate. There was no significant difference observed in DNMT activity between the control and 5°C 3 Weeks condition. However, there was a dramatic 20.1-fold increase in activity during the -15°C 1 Week condition compared to controls ($P<0.05$; Figure 3).

3.3.3 TET protein expression in response to low temperature exposure

Relative protein expression levels of TET1, TET2, and TET3 were measured in samples of freeze tolerant *E. solidaginis* larvae (Figure 4). There was an overall decreasing trend in expression levels across the time course for TET2, however the only significant difference observed was between the -15°C 4 Hours and control conditions. TET2 protein levels showed a significant 65% reduction after 4 Hours at -15°C 4 compared to controls (Figure 4). No significant differences in expression levels were observed between any of the experimental conditions for TET3. TET1 did not show any cross reactivity with the antibody used.

3.3.4 TET activity in larvae in response to low temperature exposure

Total TET activity was measured as the change in optical density (OD) per hour per mg of total soluble protein in *E. solidaginis*, representing the hydroxymethylation of a polynucleotide substrate. There was a significant 55% decrease in total TET activity during the 5°C 3 Weeks condition compared to the controls (Figure 5). Similarly, suppression of TET activity was sustained during the -15°C 3 Weeks condition with a

mean value that was 50% lower compared to the controls (Figure 5). No significant differences were observed between the 5°C 3 Weeks condition and the -15°C 3 Weeks condition (Figure 5).

3.3.5 HAT activity in larvae in response to low temperature exposure

Total HAT activity was measured as the change in optical density (OD) per hour per mg of total soluble protein in *E. solidaginis*, representing acetylation of a histone substrate. HAT activity increased significantly compared to the control during both the 5°C 3 Weeks and -15°C 1 Week conditions ($P<0.05$; Figure 6). Total HAT activity increased by 1.40 fold during the 5°C 3 Weeks condition compared to the control, and was 1.47 fold higher during the -15°C 1 Week condition compared to the control. However, no significant differences were observed in HAT activities between the 5°C 3 Weeks and -15°C 1 Week conditions (Figure 6).

3.3.6 HDAC activity in larvae in response to low temperature exposure

Total HDAC activities were measured as the change in optical density (OD) per hour per mg of total soluble protein in *E. solidaginis*, representing the deacetylation of a lysine substrate. HDAC activity levels in *E. solidaginis* were significantly reduced compared to the control during both the 5°C 3 Weeks and -15°C 1 Week conditions ($P<0.05$; Figure 7), with 20% and 30% decreases in total activity, respectively, compared to the control. Furthermore, HDAC activity levels were also significantly lower during the -15°C 1 Week condition compared to the 5°C 3 Weeks condition (Figure 7), showing a 13% decrease in total HDAC activity.

3.4 Discussion

Relative protein expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2 and $MeCP_2$ were measured in samples of freeze tolerant *E. solidaginis* larvae. DNMT3L expression levels showed a significant 1.6 fold upregulation after 1 Week at 5°C as compared to controls and the 5°C 4 Hours condition before returning to control levels when exposed to -15°C (Figure 1). DNMT3L does not possess inherent methyltransferase activity but it does interact with DNMT3A to help stimulate de novo methylation and it also acts to recruit HDACs to methylated DNA in order to assist in transcriptional repression (Aapola *et al*., 2002; Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016; Deplus *et al.,* 2002). Hence, DNMT3L may contribute to an epigenetic suppression of gene transcription as the larvae become cold-acclimated.

Seasonal changes in temperature and photoperiod signal *Eurosta* to begin the transition into diapause and the initiation of physiological processes involved in freeze tolerance (Lee and Costanzo 1998; Morin *et al*., 2005; Rider *et al*., 2011). Being held at 5°C for one week in an incubator likely had the same effect on *Eurosta* as exposure to seasonal autumn temperatures in a gall in its natural environment. This could signal *Eurosta* to initiate the mechanisms required for freeze tolerance during the winter months. It is possible that the temporary upregulation of DNMT3L was engaged to assist DNMT3A in establishing new methylation patterns in order to selectively silence gene transcription and shut down nonessential biological processes. DNMT3L has also been observed to specifically bind to unmethylated H3K4 in order to stimulate de novo methylation of H3K4 through its interactions with DNMT3A (Ooi *et al*., 2007). Thus,

DNMT3L may be involved in triggering the methylation of histones in *Eurosta* to promote gene specific transcriptional silencing prior to freezing.

In a similar line of reasoning, it also might be the case that DNMT3L may have been temporarily upregulated in order to help recruit other transcriptional repressors such as HDACs, again for the purposes of silencing nonessential processes prior to freezing. Studies of skeletal muscle in hibernating 13-lined ground squirrels (*Ictidomys tridecemlineatus*) have shown that acetylated H3 levels are reduced by 25% with a concomitant 82% increase in HDAC expression and activity during torpor (Morin and Storey 2006; Storey and Storey 2015). Similar mechanisms have also been observed in turtles (*Trachemys scripta elegans*) during anoxia exposure. These turtles endure weeks of prolonged anoxia during the winter when they are submerged in cold water. During this period their metabolic rate can be depressed to ~10% of its normal value. This was associated with a 40-60% decrease in acetylated H3 content compared to aerobic values as well as upregulation of HDACs 1-5 ranging from 1.3-4.6 fold. Total HDAC activity was also increased by 1.5 fold (Krivoruchko and Storey, 2010).

Changes in the status of histone acetylation and HDAC activity have been observed as key processes in regulating transcriptional repression when animals enter torpor. Accordingly, changes in the methylation status of histones can also result in transcriptional silencing. Collectively this suggests that chromatin condensation could be a conserved mechanism underpinning hypometabolism which might also be conserved in insects for entry into diapause, facilitated in part by interactions with DNMT3L. Caution must be taken though since DNMT3A did not show any cross-reactivity with the antibodies used in this study, so it cannot be determined whether DNMT3L interactions

with DNMT3A occurred or if changes in the expression of DNMT3A were associated with entry into diapause or exposures to low temperatures.

MBD2 expression levels in *E. solidaginis* were significantly upregulated by at least 1.92 fold during the -15°C 1 Week condition compared to all other conditions ($P<0.05$; Figure 1). This strong upregulation suggests that increases in MBD2 expression are biologically significant. MBD2 binds specifically to methylated strands of DNA to assist in transcriptional repression and is also known to associate with HDACs to promote transcriptional silencing (Howlett and McGee, 2016; Bogdanovic and Veenstra, 2009). As previously mentioned the condensation of chromatin into heterochromatin may be a conserved mechanism for regulating hypometabolism. Since the most pronounced upregulation of MBD2 expression was seen during the -15°C 1 Week condition (Figure 1) it is possible that MBD2 may be actively interacting with chromatin modifiers at this point to assist in the transcriptional silencing of nonessential biological processes the frozen state.

Given that total DNMT activity showed a major increase during -15°C exposure (Figure 3), the increased MBD2 expression may also be linked with the presence of a greater amount of methylated DNA in the frozen state and therefore an enhanced production of MBD2 and an increased amount of MBD2 protein binding to methylated DNA as part of transcriptional suppression. MDB2 has also been shown to suppress gene transcription indirectly through its interaction with the microRNA hsa-mir-496 (Alvarado *et al*., 2013). MicroRNAs can rapidly and reversibly alter gene/protein expression under stress conditions and as such are key epigenetic regulators of hypometabolism. Differential microRNA expression has been shown to be a conserved epigenetic

mechanism for regulating hypometabolism in both vertebrate and invertebrate species (Biggar and Storey 2011; Storey 2015) and is involved in responses to environmental stress including hibernation and prevention of muscle atrophy in 13-lined ground squirrels (Morin *et al*., 2008) and bats (Kornfeld *et al*., 2012), anoxia tolerance and cell cycle suppression in turtles (Biggar and Storey 2012; Biggar and Storey 2009), and estivation in sea cucumbers (Chen and Storey 2014; Chen *et al*., 2013). Additional studies have shown upregulation of selected microRNAs during -15°C exposure in *Eurosta* (Lyons *et al*., 2015b; Lyons *et al*., 2013b; Courteau *et al*., 2012).

Importantly, the microRNAs identified as being upregulated in these studies have roles in regulating HDACs, HSPs, the Krebs cycle, PI3K activity and cell growth. Although hsa-mir-496 was not examined in any of these studies, these results collectively suggest a significant involvement of microRNA activity in regulating hypometabolism. Since MBD2 is known to interact with hsa-mir-496, it may be the case that it also interacts with other microRNAs during -15°C exposure since each microRNA species can typically target the transcripts of several different genes. It could be that the upregulation of MBD2 observed here reflects an interaction with microRNAs to silence nonessential gene transcription

$MeCP_2$ expression levels showed a strong increase in response to 5°C exposure, levels rising by 2.29-fold after 4 Hours and 2.62-fold after 1 Week, compared to controls (Figure 1). However, freezing at -15°C quickly reversed this and $MeCP_2$ returned to control levels. The pattern of changes in $MeCP_2$ expression levels showed a pattern more similar to that seen for DNMT3L than for MBD2. This could imply that $MeCP_2$ has a stronger role to play in preparing *Eurosta* for freezing rather than the active

transcriptional repression of genes once frozen. $MeCP_2$ serves very similar functions to MBD2 in that it binds specifically to methylated strands of DNA and can recruit HDACs to assist in transcriptional silencing (Howlett and McGee, 2016: Bogdanovic and Veenstra, 2009). It may be that the 5°C upregulation of $MeCP_2$ seen here reflects interactions with HDACs to silence metabolically expensive processes in order to conserve energy and prepare *Eurosta* for freezing. It is worth noting that $MeCP_2$ has also been shown to bind and activate the CREB1 transcription factor in mice to activate gene transcription (Chahrour *et al*., 2008). It is unknown if $MeCP_2$ performs the same function in insects but it could be the case that one result of $MeCP_2$ upregulation was increased interactions with CREB to alter gene transcription during entry into diapause.

No significant differences were observed in the expression levels of DNMT1, DNMT2, DNMT3B, and MBD1 in *Eurosta* at any of the experimental time points and temperatures as compared with their control values (Figure 2). However, one interesting observation was that DNMT1 expression levels did not change significantly across the time course. This is not surprising since a main role of DNMT1 is in control of cell division whereas this metabolically expensive process would be shut down during the winter.

Figure 3 shows a very large 20.1-fold increase in total DNMT activity during the -15°C 1 Week condition compared to controls. Since DNMTs generally cause transcriptional silencing (Howlett and McGee, 2016), this major increase in their activity could be a major factor in strongly shutting down transcription (and, by extension, translation as well) during the winter months when *Eurosta* is frozen. Protein translation can account for anywhere between 30-40% of total metabolic expenditure and a hallmark

mechanism for hypometabolism observed across the animal kingdom is the suppression of protein synthesis at the gene level via transcriptional silencing (Biggar and Storey, 2011). *Eurosta* likely follows this pattern with freezing, freeze-induced anoxia, and diapause all signaling a need for transcriptional silencing. DNMTs are one of the main epigenetic regulatory mechanisms and are required for transcriptional silencing of energetically expensive processes during metabolic rate depression. Therefore, the most economical means of achieving widespread transcriptional silencing during periods of limited energy availability would be to alter the activity of epigenetic enzymes rather than to upregulate their expression and degrade them when they're no longer required. It is likely that the increase in total DNMT activity seen here results from posttranslational modifications of DNMT enzymes since this would be metabolically inexpensive to achieve, could occur quickly in response to environmental cues, and would be reversible following an increase in temperature and a transition out of diapause.

Relative protein expression levels of TET2 and TET3 were measured in samples of freeze tolerant *E. solidaginis* larvae (Figure 4). TET2 demonstrated an overall decreasing trend in expression with a decrease in temperature with a significant 65% decrease in protein levels during the -15°C 4 Hours condition compared to controls (Figure 4). TETs all function in very similar ways by converting methylated 5Cs on cytosine residues to 5-hydroxy-meythlated cytosine residues (5-hmC) through a hydroxylation followed by an oxidation reaction (Coulter *et al*., 2013). They are believed to be key players in erasing methylation patterns (Bhutani *et al*., 2011) and regulating the dynamic reprogramming of the epigenome in response to environmental and developmental signals (Pera 2013). The biological importance of TET2 is not yet well

understood in humans, let alone in model organisms of metabolic rate depression. TET2 appears to be involved in the normal generation of bone marrow (in humans) as well as in cell cycle control, cytokine and growth factor signaling (Ko *et al*., 2010; Metzeler *et al*., 2011; Wang *et al*., 2015). Other studies have linked TET2 dysfunction with impaired stem cell differentiation (Figueroa *et al.*, 2010). Whether TET2 could serve similar functions in insects is not known, but the enzyme could similarly be involved in cell cycle control and growth factor signaling. If this were the case the observed downregulation of TET2 over time at cold temperatures would make sense and contribute to a cessation of cell proliferation and growth processes that would be expected in the diapause and frozen states.

Figure 4 also shows no change in the expression levels of TET3 across any of the experimental time points. Together, the expression results for TETs should be considered within the context of earlier results for DNMTs. The decrease or lack of change in TET expression patterns seen in Figure 4 makes sense when considering the already established increase in DNMT activity shown in Figure 3. It is highly unlikely that *Eurosta* would implement such an increase in DNMT enzyme activity only to have it immediately cancelled out via the actions of TET enzymes converting 5-mC to 5-hmC. This is especially true during periods of hypometabolism that must last for many months and that must be fueled by a fixed reserve of metabolic fuel acquired by the larvae before entry into diapause. Furthermore, metabolic fuels acquired by *Eurosta* larvae during the summer/autumn must also be stretched to support the radical reorganization of the insect's body during pupation and metamorphosis as well as all the energy needs of the non-feeding adult fly.

This point is further supported by the results shown in Figure 5. There was a significant 50-55% decrease in total TET activity at cold temperatures, beginning at 5°C 3 Weeks and sustained through -15°C 3 Weeks as compared to control levels (Figure 5). This can again be interpreted in the context of the earlier results (Figures 1-3). It was already demonstrated that decreasing temperatures were associated with increases in DNMT3L, MBD2, and $MeCP_2$ expression (Figure 1). It was also shown that total DNMT activity greatly increased during -15°C exposure. It was also speculated that the increase in MBD2 and $MeCP_2$ expression likely reflected an increased need for HDAC recruitment and chromatin condensation to promote transcriptional silencing. In light of these results, it is reasonable to expect a decrease in total TET activity as temperature decreases in order to sustain a high DNA methylation state during freezing. It would be self-defeating to concurrently convert 5-mC to 5-hmC and counteract the suppressive effects of DNMTs on gene expression, thereby wasting the limited metabolic energy available and impeding the ability of the larvae to regulate essential and nonessential processes during freezing.

Total HAT activity increased significantly by 1.40 fold and 1.47 fold during the 5°C 3 Weeks and -15°C 1 Week conditions, respectively, when compared to controls (Figure 6). This result was somewhat unexpected given that HATs catalyze the acetylation of histones which results in a euchromatic state that promotes transcriptional activation. It was particularly surprising that HAT activity was elevated at -15°C when larvae are frozen. Overall metabolic activity of the larvae would be very substantially reduced in the frozen state as compared to spring and summer values. However, in its natural environment *Eurosta* must be able to deal with occasional and rapid freeze/thaw

cycles when the ambient temperature fluctuates (Lee and Costanzo 1998; Morin *et al*., 2005). It could be that the increase in total HAT activity here reflects a mechanism to keep transcriptional activity on "standby" during freezing so that *Eurosta* could quickly alter metabolic activity in the event of a rapid thawing. Furthermore, it should also be noted that the increase of 1.47-fold may not be biologically significant.

This also needs to be considered along with the difference between HAT activities during the 5°C 3 Weeks condition compared to the control. Compared to the control there was a 1.40 fold upregulation in HAT activity after 3 weeks at 5°C. Again, it is not clear if this increase is biologically meaningful, but it may be a preparatory mechanism reflecting an upregulation of genes required for freeze tolerance. For example, perhaps enhanced HAT activity is involved in the upregulation of genes/proteins for enzymes involved in the synthesis of glycerol and sorbitol as winter cryoprotectants. Furthermore, HATs are known to be involved in regulating DNA repair and apoptosis (Legube *et al*., 2004; Murr *et al*., 2006). Thus, another potential role for elevated HAT activity at -15°C may be to selectively allow the expression of some genes, such as those involved in cellular repair processes, to be transcribed and translated during intermittent periods of thawing during the winter.

Total HDAC activity levels in *E. solidaginis* showed a 20% and 30% decrease compared to controls during the 5°C 3 Weeks and -15°C 1 Week conditions, respectively (Figure 7). Much like the results for total HAT activity, these results are also unexpected. HDACs catalyze the deacetylation of histones promoting a more condensed, heterochromatin state that results in transcriptional silencing. Again, given that during -15°C exposure *Eurosta* is frozen, it is unlikely that there would be substantial

gene transcription occurring. Much of what was said for the observed increase in HAT activity levels could be said for the decrease in total HDAC activity: e.g. that this might reflect some preparatory mechanism for a rapid thaw, that it could reflect a specific need to enhance transcription of genes involved in freeze tolerance, or that a 20-30% decrease might not be biologically relevant, etc.. However, the 20% decrease in HDAC activity during the 5°C 3 Weeks condition compared to the controls makes more sense. During 5°C exposure *Eurosta* would presumably be preparing for freeze tolerance. Hence, the decrease in HDAC activity observed during the 5°C exposure may reflect a need to transcribe a set of specific genes involved in the preparation for freeze tolerance. Finally, it should be noted that the kit used to measure total HDAC activity here does not measure the activity of Class III HDAC enzymes (SIRTs 1-7) since they require a NAD^+ cofactor to work, nor does the kit detect HDAC 2/8 activities well. It remains a possibility that the overall activity of HDACs measured by the kit could go down but that activities of SIRTs and HDACs 2/8 could contribute to transcriptional silencing in important ways that were not measured here. Hence, it is possible that the results seen here give a biased view of the involvement of HDACs in diapause and freeze tolerance.

In summary, the aforementioned evidence affirms hypothesis #1-that *Eurosta* shows differential regulation in the expression and activity of DNMT, TET, HAT and HDAC enzymes corresponding to low temperature exposure. The insect appears to rely on an increase in DNMT activity with a concomitant decrease in TET activity as major controls on gene expression as well as an increase in both specific DNMT/MBD enzyme expression and HAT activity to help mediate the transition into and help cope with the stress of freezing. The insect also appears to utilize a decrease in HDAC activity and a

downregulation of TET2 specifically. Collectively, these results are similar to the epigenetic processes observed in other species that serve as models of metabolic rate depression and extreme stress tolerance, suggesting that epigenetic responses may be a conserved mechanism across the animal kingdom for implementing hypometabolism and dealing with extreme environmental stress.

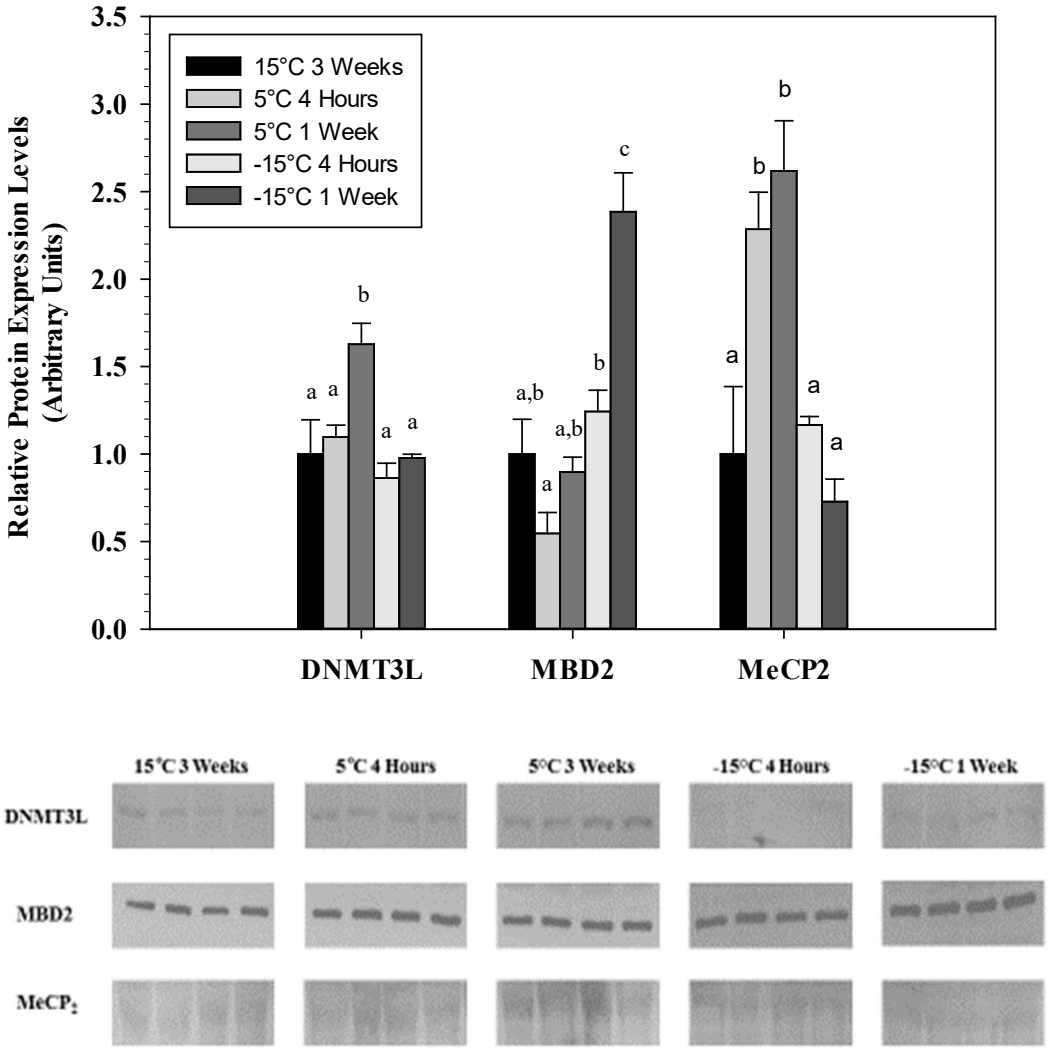

Figure 1: DNMT3L, MBD2 and $MeCP_2$ expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

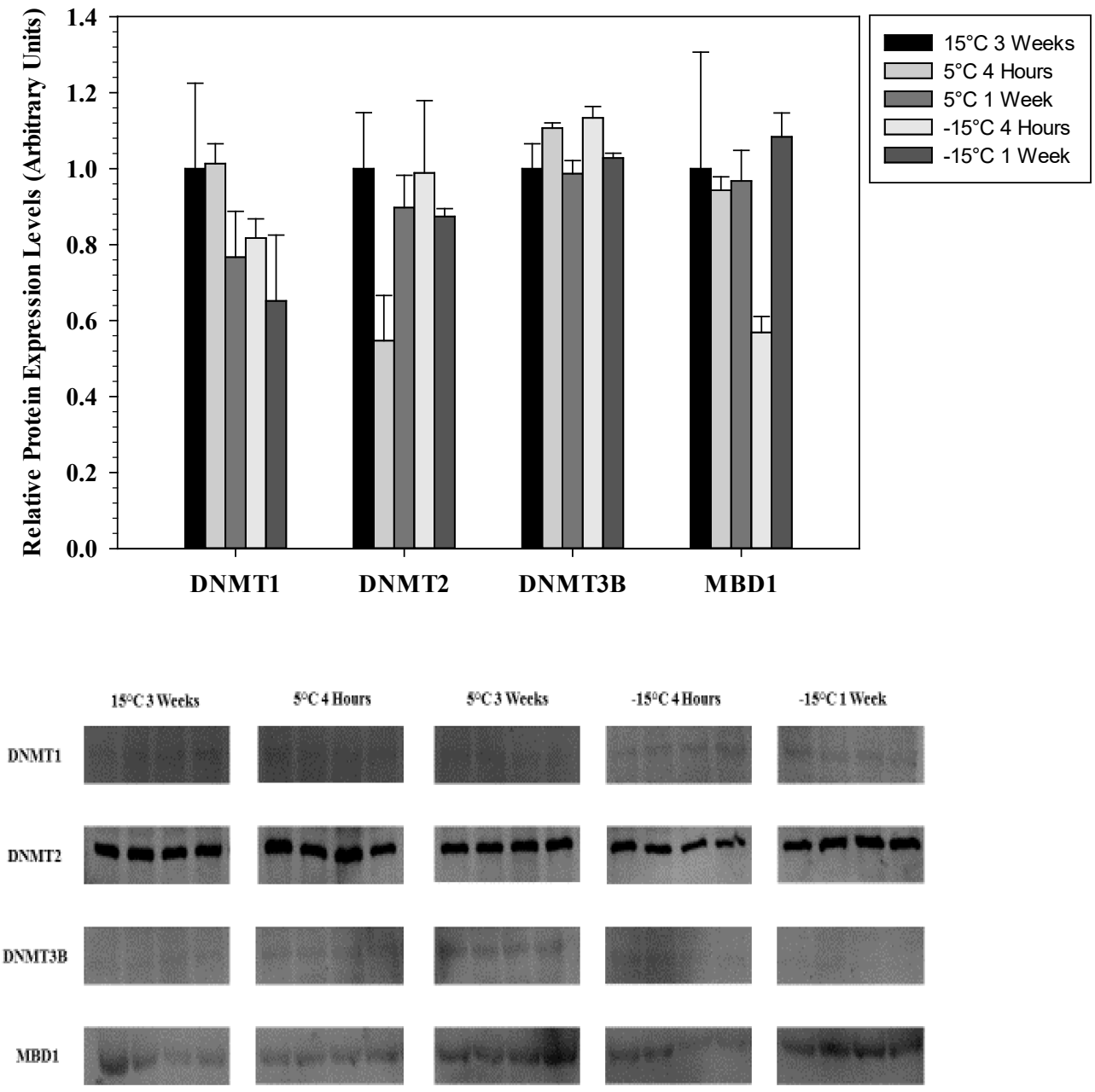

Figure 2: DNMT1, DNMT2, DNMT3B and MBD1 expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 3 Weeks, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were observed during any of the experimental conditions. DNMT3A showed no cross reactivity with the antibodies used for detection.

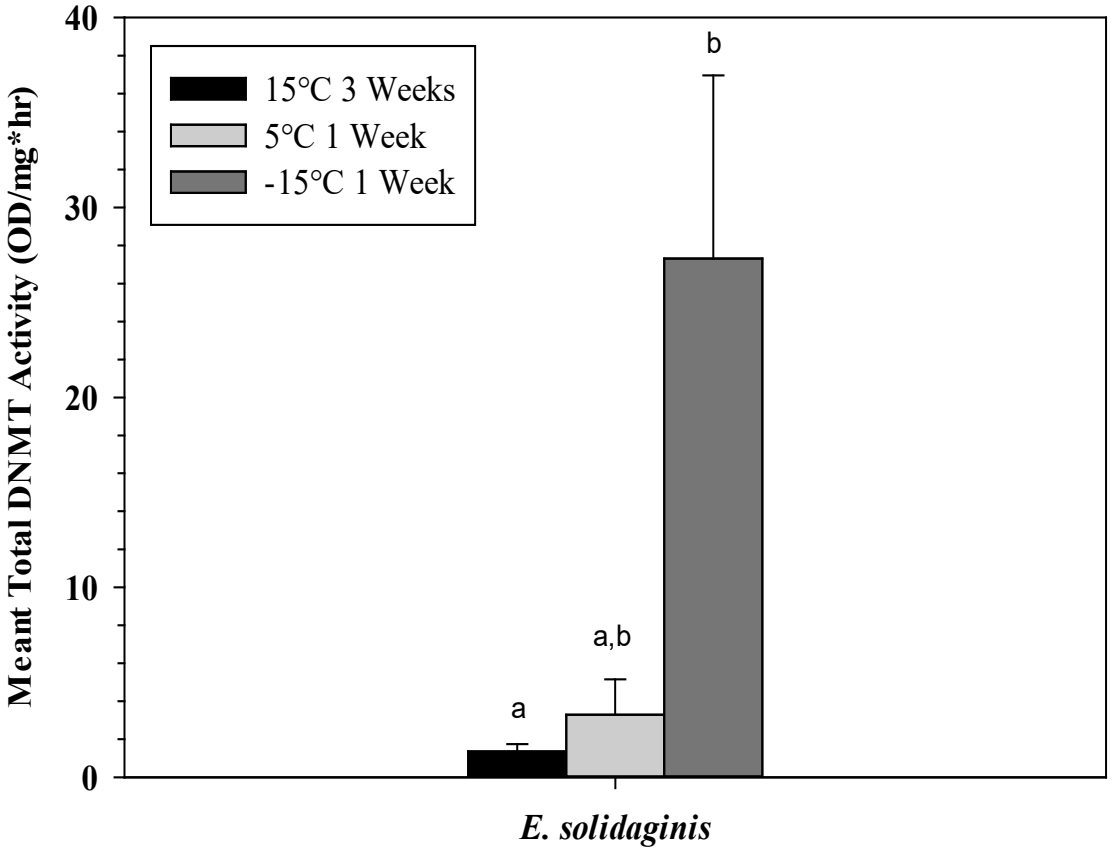

Figure 3: Total DNMT enzyme activity (measured in OD/mg*hr) in *E. solidaginis* larvae at 15°C 3 Weeks, 5°C 3 Weeks, and -15°C 1 Week. Activity was measured by an EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit from Epigentek. Histograms display mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

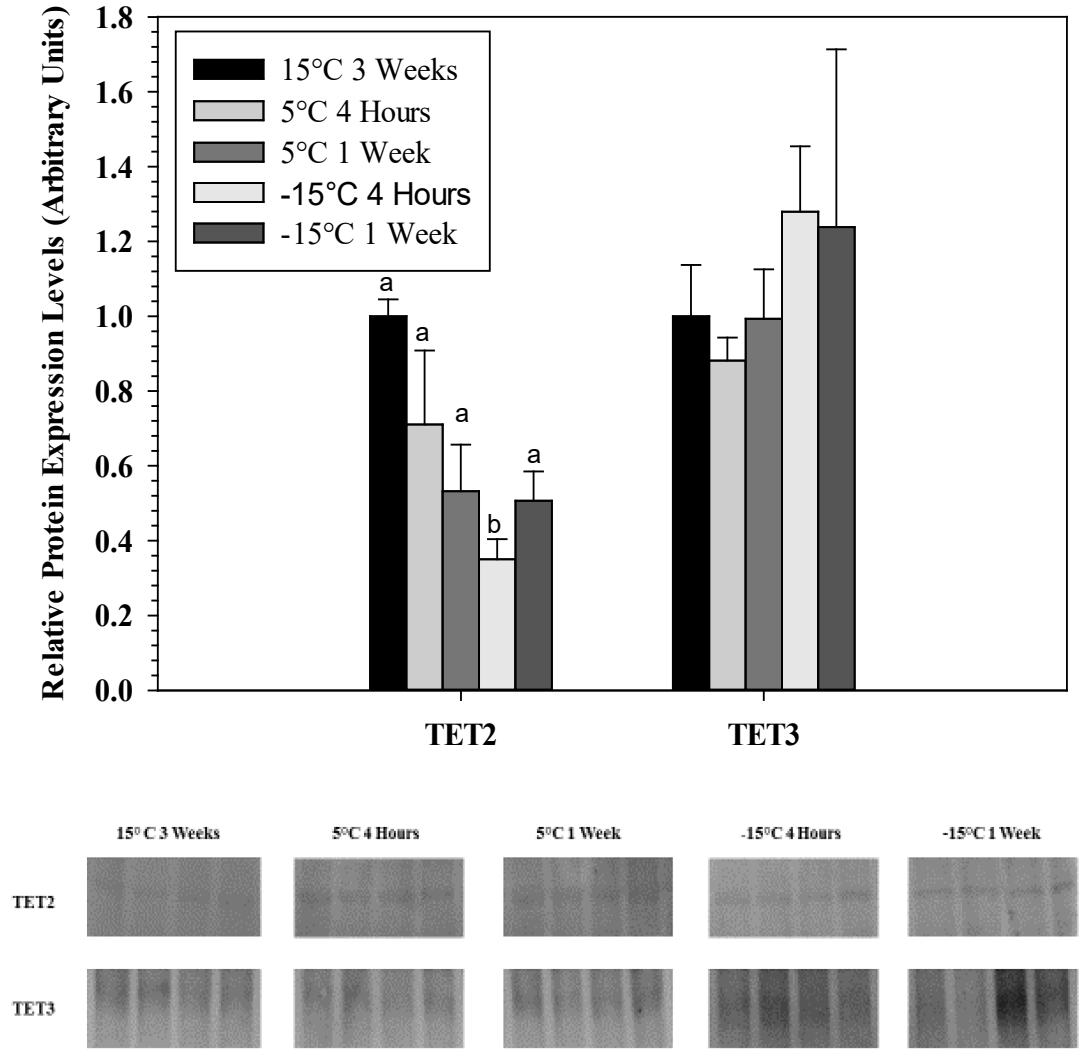

Figure 4: TET2 and TET3 expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.TET1 did not show any cross reactivity with the antibodies used. No significant differences in expression levels were observed between any of the conditions for TET3.

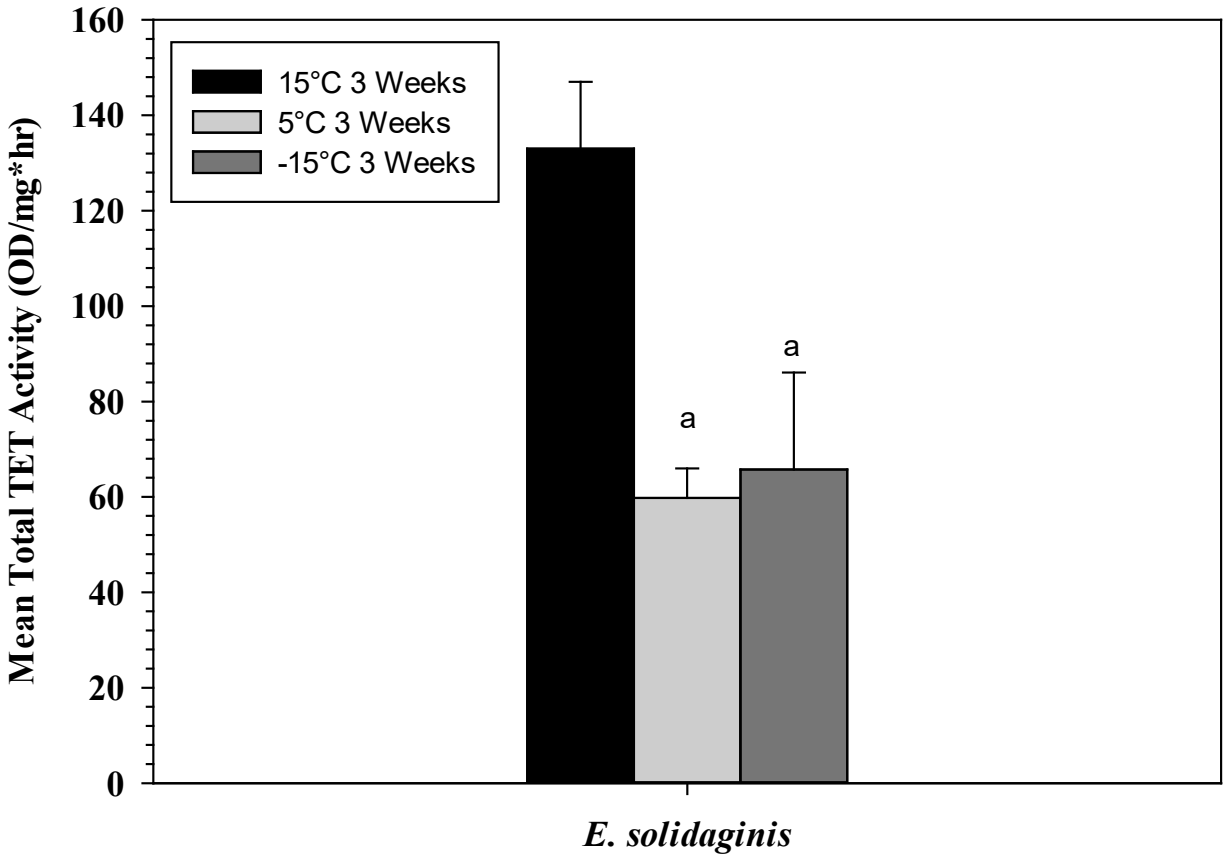

Figure 5: Total TET enzyme activity (measured in OD/mg*hr) in *E. solidaginis* larvae at 15°C 3 Weeks, 5°C 3 Weeks, and -15°C 3 Weeks. Activity was measured by an Epigentek Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay Kit from Epigentek. Histogram displays mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

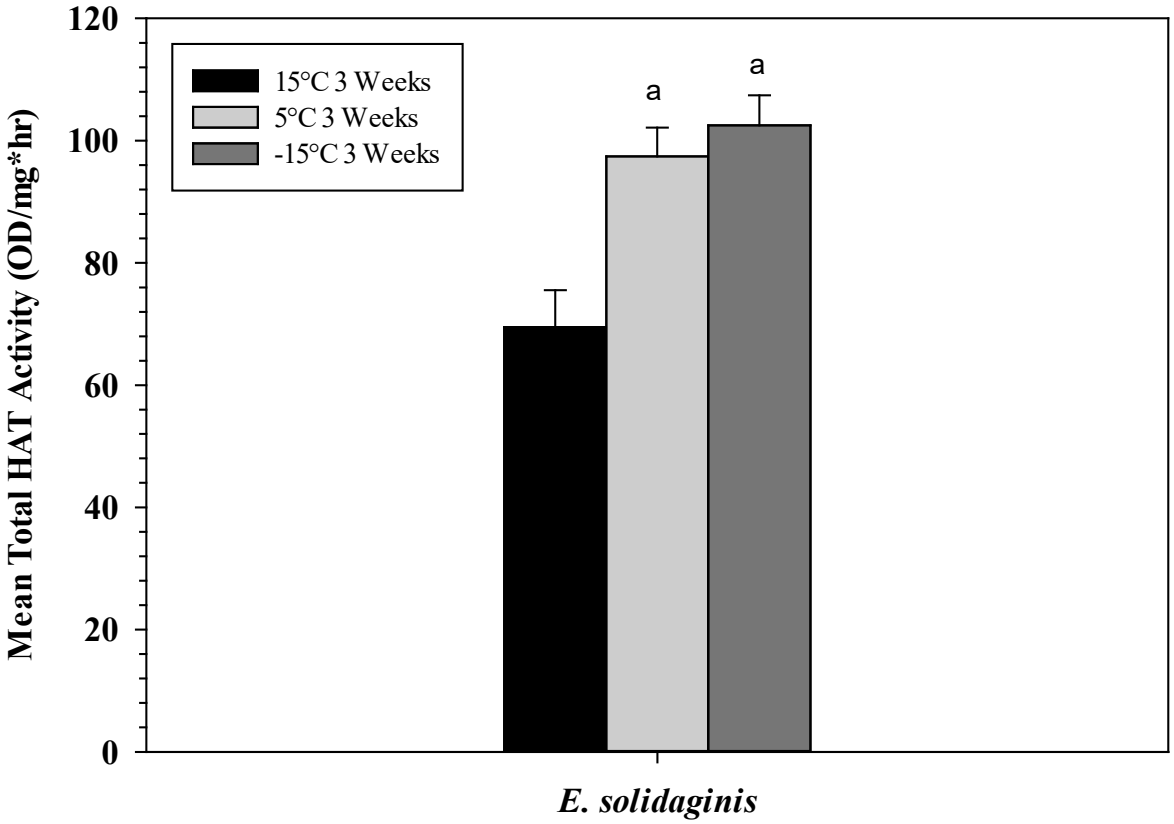

Figure 6: Total HAT enzyme activity (measured in OD/mg*hr) in *E. solidaginis* at 15°C 3 Weeks, 5°C 3 Weeks, and -15°C 1 Week. Activity was measured by an EpiQuick HAT Activity/Inhibition Assay Kit from Epigentek. Histogram displays mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

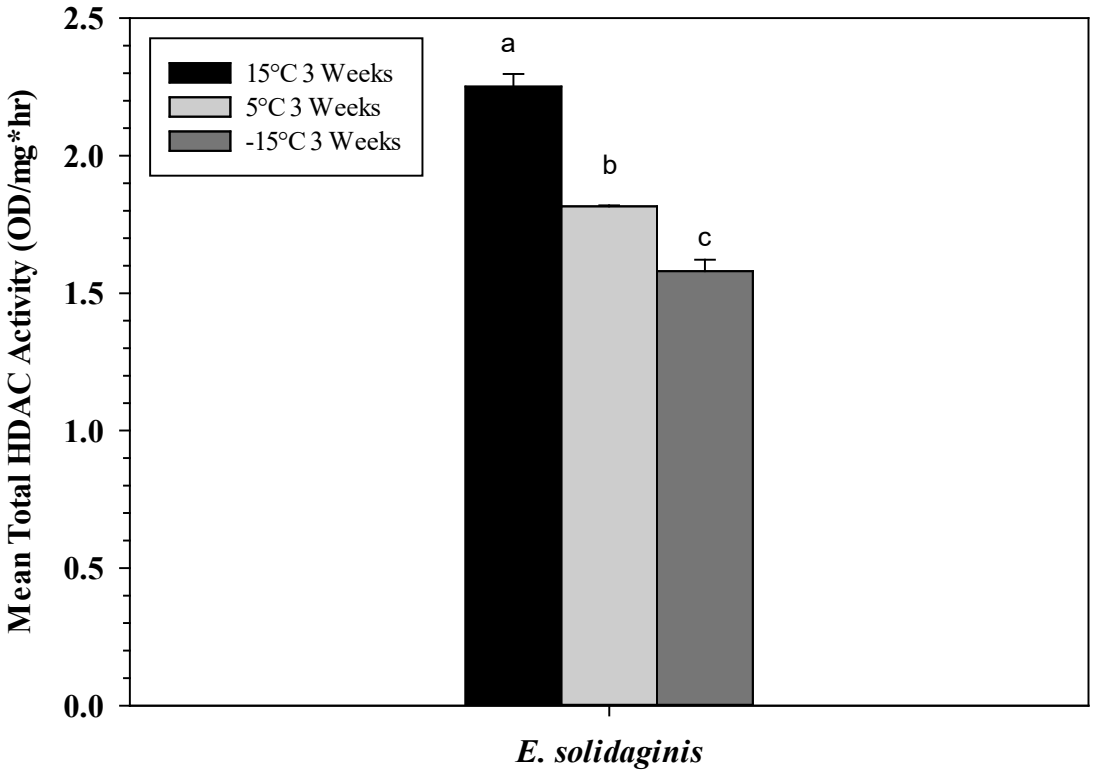

Figure 7: Total HDAC enzyme activity (measured in OD/mg*hr) in *E. solidaginis* at 15°C 3 Weeks, 5°C 3 Weeks, and -15°C 1 Week. Activity was measured by an Active Motif HDAC Assay Kit. Histogram displays mean activity levels (±S.E.M. n= 3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

Chapter 4

Epigenetics of Freeze Avoidance in *Epiblema scudderiana*

4.1 Introduction

There are many parallels between the lifestyle of *Eurosta solidaginis* and the goldenrod gall moth *Epiblema scudderiana* (Lepidoptera, Olethreutidae). The species distribution of this moth also ranges from the Gulf of Mexico all the way up to northern and central Canada much like *Eurosta* (Plakidas 1978). *Epiblema* also overwinter as larvae, pupate in the spring and adults emerge to lay eggs in the newly-growing tips of goldenrod plants. When eggs hatch, the larvae move into the center of the stem where they begin to feed. The combination of feeding on plant tissues and the secretion of compounds which have hormomimetic properties of goldenrod hormones trigger the plant to form tissue swellings (galls) around the caterpillars. However, unlike *Eurosta*, as the *Epiblema* caterpillars prepare to overwinter, they line the inside of their galls with silk to help prevent the penetration by ice crystals during the winter (Rickards *et al*., 1987).

Epiblema also detects changes in photoperiod and thermoperiod during the autumn and enters a state of diapause for the duration of the winter (Rider *et al.,* 2011). They also implement physiological mechanisms in order to survive cold exposure. However, unlike *Eurosta* which is freeze tolerant, *Epiblema* uses a strategy called freeze avoidance. Freeze avoidance is the deep supercooling of bodily fluids without the fluids themselves freezing (Storey and Storey, 2012; Lyons *et al*., 2013b; Lyons *et al*., 2015a; Rider *et al*., 2011; Bilgen *et al*., 2001). Freeze avoidance is made possible through the exploitation of two properties of water. Firstly, freeze avoiding animals take advantage of the fact that the higher the concentration of dissolved solutes in a solution, the lower the freezing point of the solution will be. Secondly, although we are most familiar with the freezing of water at about 0°C in nature, freezing at this temperature is actually triggered

by the presence of ice nucleators including both internal nucleators within a body (e.g. certain plasma proteins, food particles or bacteria in the gut, etc.) or nucleators on the skin (e.g. bacteria), or other nucleators in the environment that trigger external ice formation that in turn can nucleate the internal body fluids across the integument. However, in the absence of nucleators, water can actually be supercooled to -40°C which is the temperature at which water molecules themselves can form aggregates for long enough to trigger crystallization without the involvement of a nucleator. Hence, if organisms can clear their bodies of nucleators or mask the action of nucleators, then their body fluids can supercool to low values. Freeze avoiding organisms have taken advantage of this principle and evolved specific antifreeze proteins (AFPs) that can adhere to microscopic nascent ice crystals and prevent their further growth. With the use of AFPs as well as the accumulation of high concentrations of polyhydric alcohol cryoprotectants (glycerol in *E. scudderiana*) many insects and other arthropods and can stabilize a supercooled state down to near -40°C (Duman 2001; Storey and Storey, 2012). Indeed, the mean supercooling point of *E. scudderiana* larvae in midwinter is -38°C.

One of the key molecular differences between freeze tolerance and freeze avoidance pertains to the accumulation of polyhydric cryoprotectants. Much higher total levels of polyhydric cryoprotectants are accumulated for freeze avoidance than freeze tolerance. *Epiblema* also almost exclusively relies on glycerol as a cryoprotectant in comparison to *Eurosta* which uses a combination of glycerol and sorbitol (Lyons *et al*., 2013; Rider *et al*., 2011). Glycerol is an ideal cryoprotectant for several reasons. It is highly soluble in water and thus can achieve high concentrations in aqueous solutions. It can be made quickly from glycogen with minimal ATP expenditure. It can be produced

by shunting intermediates from other metabolic pathways and it helps stabilize protein conformation without effecting enzyme kinetic parameters. Finally, glycerol can also be used as an energy source in both aerobic and anaerobic pathways so that it can be catabolized as a fuel source during the spring without the need to first reconvert it to glycogen. Glycerol synthesis begins with the cold activation of glycogen phosphorylase. However, because glycerol synthesis is highly dependent on NADPH, carbon skeletons for glycerol synthesis are first shunted through the Pentose Phosphate Pathway to produce both NADPH and triose phosphate precursor molecules (Storey and Storey 2012). Glycerol can be synthesized from dihydroxyacetone phosphate (DHAP that is then converted to glycerol-3-phosphate (G3P) by glycerol-3-phosphate dehydrogenase (G3PDH), before being converted to glycerol by phosphatase enzymes (Joanisse and Storey 1994). However, the main route of glycerol biosynthesis in insects appears to be from glyceraldehyde-3-P produced from the pentose phosphate pathway that is then dephosphorylated to glyceraldehyde and then converted to glycerol via the NADPH dependent polyol dehydrogenase (PDH) enzyme. PDH's maximum activity is the same at 5°C to -15°C, however its affinity for its substrate (glyceraldehyde) was over twice as high at 5°C compared to -15°C, providing a mechanism for *Epiblema* to begin glycerol synthesis prior to subzero temperature exposure and allow for supercooling (Joanisse and Storey 1994; Holden and Storey 2011; Storey and Storey 2012). Moreover, PDH was observed to be highly phosphorylated and comparably inactive at -15°C. Glycerol synthesis is facilitated by the summer and early autumn accumulation of large storages of glycogen in its fat body. Amazingly, at its peak storage over 20% of *Epiblema* total body mass is in the form of glycerol (>2M concentrations) (Storey and Storey, 2012; Lyons *et*

al. 2015a). Furthermore, at the end of winter any leftover glycerol is catabolized for fuel when transitioning out of diapause and the commencement of pupation.

Another important physiological difference between the freeze avoiding *Eurosta solidaginis* and the freeze avoiding *Epiblema scudderiana* is the greater reliance on mitochondrial activity during the winter months in *Epiblema*. Mitochondrial activity is generally suppressed in both species of insects since lower temperatures depress enzymatic activity directly and nonessential biological processes are mostly shut off in order to conserve energy. Mitochondria activity in citrate synthase (CS), glutamate dehydrogenase (GDH), and NAD-isocitrate dehydrogenase (NAD-IDH) were reduced by 40-70% in both species during the winter, however mitochondrial activity still remained important in *Epiblema* in other ways (Joanisse and Storey 1994b). This is supported by the fact that anaerobic metabolic end products do not accumulate during the winter and certain mitochondrial enzymes involved in lipid oxidation (hydroxyacyl-CoA dehydrogenase, carnitine-palmitoyl transferase, and acetoacetyl-CoA thiolase) actually increase in activity during the autumn and winter despite a substantial suppression in cytochrome oxidase c activity, CS, GDH, and NAD-IDH (Churchill and Storey 1989; Joanisse and Storey 1996; McMullen and Storey 2008). Elsewhere, it has also been seen that *Epiblema* experiences a 2-fold increase in AMPK mediated phosphorylation of acetyl-CoA carboxylase (ACC) and a 70% increase in triglyceride lipase activity during the winter (Rider *et al*., 2011). Furthermore, and much like *Eurosta*, *Epiblema* also experiences a ~75% reduction in the activity of the Na^{+}-K^{+}-ATPase and Ca^{2+}-ATPase (McMullen 2004; Lyons *et al*., 2015a). Suppression of the activities of these ion channels is brought about via phosphorylation by protein kinases A, G, or C. *Epiblema* also

utilizes elevations in HSPs during the winter to prevent stabilize proteins (Zhang 2006). Also important to note, the activities of numerous important antioxidant enzymes: GPX, CAT, GST, and have all been observed to increase during the winter months whereas SOD activity decreased (Joanisse and Storey 1996b).

It has been clearly established that *Epiblema* employs metabolic rate depression and freeze avoidance to survive cold winter temperatures. Much like *Eurosta, Epiblema* uses fast, acute, and reversible molecular mechanisms to trigger physiological changes to survive the cold, suggesting that epigenetic regulations may control many of these processes. Epigenetic responses to stress have been observed in a number of other model organisms (Howlett and McGee 2016; Wijenayake and Storey 2016; Alvarado *et al*., 2015; Lyons *et al.,* 2015a; Storey 2015; Zhao *et al*., 2015; Alvarado *et al*., 2014; Lang-Oullette and Morin 2014; Storey and Storey 2013; Lyons *et al*., 2013a) and in *Eurosta* as well (Lyons *et al*., 2013b; Lyons *et al*., 2015b, Courteau *et al*., 2012). Since epigenetic responses to stress have been observed in other organisms, it is believed that they are involved in hypometabolism and freeze avoidance as well. The present chapter examines changes in the expression and activity of key epigenetic enzymes (DNMTs/MBDs, TETs, HATs and HDACs) in *Epiblema scudderiana* larvae exposed to a range of temperatures for different durations (see **Chapter 2**). The different temperature and duration conditions were designed to simulate conditions that *Epiblema* is naturally exposed to in its environment during the winter, as well as provide an indication of how responsive its epigenetic enzymes are to changes in temperature. It is hypothesized that *Epiblema* will show differential regulation of DNMT, MBD, TET, HDAC and HAT expression and activity that is reflective of its exposure to different temperatures.

4.2 Materials and Methods

4.2.1. Insect Collection

Insect collection was performed as described in **Chapter 2.1**

4.2.2. Total Protein Isolation

Total protein isolation was performed as described in **Chapter 2.2**

4.2.3 Western Blotting

Western Blotting was performed as described in **Chapter 2.3**. Expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2, $MeCP_2$, TET1, TET2, and TET3 were analyzed. See **Appendix A: Supplementary Expression Data for Histone Modifications** for expression data on the Histone Acetyltransferases (HATs): PCAF, Tip60, MYST1 and MYST2; the Histone Deacetylases (HDACs): HDAC 1-7; and the Histone Acetylation levels of: H2A.K5.Ac, H2B.K5.Ac, Total Histone H3, Total Acetylated Histone H3, H3.K9.Ac, H3.K14.Ac, H3.K18.Ac, H3.K23.Ac, H3.K27.Ac, H3.K56.Ac, H4.K8.Ac. See **Appendix B: Antibody Information** for a full list of antibodies used during western blotting. See **Appendix C: Western Blotting Conditions** for a list of western blotting parameters for each protein analyzed.

4.2.4. Activities of DNMT, HAT, HDAC and TET

All activities were assayed as described in **Chapter 2.4, 2.5, 2.6 and 2.7, respectively.**

4.2.5 Statistical Analysis

Statistical analyses were performed as described in **Chapter 2.8**

4.3 Results

4.3.1 DNMT and MBD protein expression in response to low temperature exposure

Relative protein expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2 and $MeCP_2$ were measured in samples of larvae from freeze avoiding *E. scudderiana*. DNMT3A showed a significant 1.57 fold upregulation compared to the control, and levels falling thereafter with values at -15°C being 29-31% of control values($P<0.05$; Figure 8). By contrast, DNMT3B levels showed a significant 5.52-fold upregulation during the 5°C 1 Week condition compared to the control levels, before returning to expression levels during the -15°C conditions that were not significantly different from controls (Figure 8). DNMT3L showed an overall trend of increasing expression levels across the time course in *E. scudderiana* but expression levels were only significantly upregulated by 2.77-fold as compared to the control levels during the -15°C 1 Week condition (Figure 8). Moreover, $MeCP_2$ expression levels showed a significant, 63 %, 57% and 51% down regulation compared to the control levels during the 5°C 1 Week, -15°C 4 Hours, and -15°C 1 Week conditions, respectively (Figure 8).

No significant differences in the protein expression levels of DNMT1, DNMT2, MBD1, and MBD2 were found during any of the experimental conditions when compared to the corresponding control values (Figure 9).

4.3.2 DNMT activity in larvae in response to low temperature exposure

Total DNMT activity was measured as the change in optical density (OD) per hour per mg of total soluble protein in *E. scudderiana*., representing methylation of a

cytosine polynucleotide substrate. There was an overall decreasing trend in DNMT activity across the time course in *E. scudderiana* (Figure 10), although a significant decrease to ~51% of control was observed only at the -15°C 4 hour condition ($P<0.05$; Figure 10).

4.3.3 TET protein expression in response to low temperature exposure

Relative protein expression levels of TET1, TET2, and TET3 were measured in samples of *E. scudderiana* larvae (Figure 11). There were no significant differences observed in the expression levels of TET2 across any of the experimental time points (Figure 11). TET1 and TET3 did not show any cross reactivity with the antibodies used and consequently could not be analyzed (Figure 11).

4.3.4 TET activity in larvae in response to low temperature exposure

Total TET activity was measured as the change in OD per hour per mg of total soluble protein in *E. scudderiana,* representing hydroxymethylation of a substrate (Figure 12). There were no significant differences in total activities at any of the experimental sampling times (Figure 12).

4.3.5 HAT activity in larvae in response to low temperature exposure

Total HAT activity was measured as the change in OD per hour per mg of total soluble protein in *E. scudderiana,* representing acetylation of a histone substrate. There was an overall decreasing trend in HAT activity across the time course (Figure 13) with a significant decrease in HAT activity to 57% and 61% of control values in larvae held at 5°C for 4 hours and 1 week, respectively. A further significant reduction to 29% and 26% of control values was observed when larvae were exposed to -15°C for 4 hours and 1

week, respectively ($P<0.05$; Figure 13). However no significant differences existed in HAT activity between the -15°C 4 Hours condition and -15°C 1 Week condition or between the 5°C 4 Hours and 5°C 1 Week conditions. In contrast, a significant reduction in activity of ~50% was observed in both -15°C conditions when compared to both 5°C conditions ($P<0.05$; Figure 13).

4.3.6 HDAC activity in larvae in response to low temperature exposure

Total HDAC activity was measured as the change in (OD per hour per mg of total soluble protein in *E. scudderiana*, representing the deacetylation of a lysine substrate There was an overall decreasing trend in HDAC activity across the time course (Figure 14). HDAC activity was significantly decreased to 46%, 43% and 33 % during the 5°C 1 Week, -15°C 4 Hours, and -15°C 1 Week conditions compared to control values ($P<0.05$; Figure 14). Additionally, HDAC activity was also significantly decreased by 60% during the -15°C 1 Week condition compared to the 5°C 4 Hours condition. However, no significant differences occurred in HDAC activities between the 15°C 3 Weeks condition and the 5°C 4 Hours condition or between the 5°C 4 Hours condition and the 5°C 1 Week and -15°C 4 Hours conditions, respectively (Figure 14).

4.4 Discussion

Relative protein expression levels of DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, MBD1, MBD2 and $MeCP_2$ were measured in samples of larvae from freeze avoiding *E. scudderiana*. The most pronounced changes in DNMT3A expression were seen during the 5°C 4 Hours condition (Figure 8). Expression levels during the -15°C conditions were also lower than the controls values however these decreases were neither statistically nor biologically significant (Figure 8). DNMT3A is responsible for establishing de novo patterns of methylation, most commonly seen in daughter cells after cell division (Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016). It is likely that the upregulation observed here represented a component of a widespread mechanism for transcriptional silencing of nonessential biological functions initiated to prepare for the subzero temperatures during the winter and for redirecting the limited metabolic energy available towards the prevention of freezing.

For example, it may be that the observed upregulation in DNMT3A occurred in order to suppress mitochondrial activity to prepare for subzero temperature exposures common during the winter. The activities of CS, GDH, and NAD-IDH were reduced by 40-70% in *Epiblema* during the winter (Joanisse and Storey 1994b). *Epiblema* also experiences a ~75% reduction in the activity of the Na^+-K^+-ATPase and Ca^{2+}-ATPase as well as a strong suppression of cytochrome c oxidase activity (McMullen 2004; Lyons *et al*., 2015a). It is likely that such a reorganization of key cellular processes involved in energy production/consumption requires some degree of preparation rather than an immediate shut down in order to buffer the physiological effects on the insect. The

upregulation in DNMT3A may be part of a mechanism for gradually shutting down metabolically expensive processes before the onset of subzero temperatures.

DNMT3B showed a similar expression pattern to DNMT3A with a significant 5.52-fold upregulation during the 5°C 1 Week condition compared to the control levels, before returning to control levels during the -15°C conditions (Figure 8). DNMT3B functions the same way as DNMT3A and is also responsible for establishing de novo patterns of methylation in new cells (Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016). Much like DNMT3A, DNMT3B showed a pronounced upregulation during 5°C exposure although the upregulation occurred after being held at 5°C for 1 week in contrast to DNMT3A which showed its upregulation after 4 hours of 5°C exposure. It cannot be determined based on the data presented here whether the time differences in DNMT3A and DNMT3B upregulation in response to 5°C exposure are a stochastic response or if there is some programmed, principled reason for the difference. Because DNMT3A and DNMT3B have essentially the same function it is likely the former. However it is also possible that there are two different DNMT3 isozymes in insects that serve different functions or that DNMT3A and DNMT3B are differentially expressed in different tissues. Additionally, just as for DNMT3A, the role of DNMT3B upregulation is likely involved in transcriptional silencing of nonessential processes in preparation for subzero temperatures during the winter months. Overall, the data for DNMT3A and DNMT3B provide evidence that de novo methylation is likely a prominent epigenetic mechanism for implementing transcriptional repression in larvae in response to decreases in temperature.

DNMT3L showed an overall trend of increasing expression levels across the time course although the only significant upregulation observed was between the -15°C 1 Week and the control condition. However, this was a significant and likely biologically relevant 2.77-fold upregulation (Figure 8). DNMT3L does not possess any inherent methyltransferase activity although it interacts with DNMT3A to help stimulate de novo methylation as well as recruit HDACs to methylated DNA in order to assist transcriptional repression (Aapola *et al*., 2002; Bogdanovic and Veenstra, 2009; Alvarado *et al*., 2014; Howlett and McGee, 2016; Deplus *et al.,* 2002). The low expression levels of DNMT3A present during -15°C exposure suggest that it is unlikely that DNMT3L was interacting with DNMT3A to stimulate de novo methylation given the limited metabolic resources available during freeze avoidance.

It is more likely that the upregulation of DNMT3L was engaged to recruit HDACs and actively suppress transcription of genes involved in nonessential biological processes, such as been observed in other vertebrate and invertebrate species during hypometabolism and prolonged environmental stress exposure (Krivoruchko and Storey, 2010; Morin and Storey 2006; Storey and Storey 2015). Caution must be taken with this interpretation, however, given that $MeCP_2$ expression levels showed a significant, 63 %, 57% and 51% down regulation compared to the control levels during the 5°C 1 Week, -15°C 4 Hours, and -15°C 1 Week conditions, respectively (Figure 8). $MeCP_2$ is also known to bind HDACs in order to assist in transcriptional repression (Howlett and McGee, 2016: Bogdanovic and Veenstra, 2009). Therefore, it would not be metabolically economical for DNMT3L to bind more HDACs while simultaneously having $MeCP_2$ bind less HDACs.

One way of explaining this apparently contradictory result could be that DNMT3L might be responsible for recruiting specific HDACs to specific targets that require transcriptional repression during freeze avoidance. Perhaps the downregulation of $MeCP_2$ results in reduced HDAC mediated silencing of genes that actively oppose the cold stress brought about by subzero temperatures as well. For example, it was mentioned earlier that certain mitochondrial enzymes involved in lipid oxidation (hydroxyacyl-CoA dehydrogenase, carnitine-palmitoyl transferase, and acetoacetyl-CoA thiolase) actually increase their activity during the autumn and winter (Churchill and Storey 1989; Joanisse and Storey 1996; McMullen and Storey 2008). It might be the case here that these or other enzymes involved in lipid oxidation are also upregulated and that expression of their genes are highly dependent on chromatin conformation. The downregulation of $MeCP_2$ observed here could potentially result in a decrease in HDAC activity at relevant gene loci to result in their upregulation. Alternatively it could be that genes regulating AMPK, antioxidant defences and/or chaperone proteins are suppressed by HDACs and that the $MeCP_2$ downregulation results in a reduction of HDAC binding at these genetic loci and an upregulation in their expression to prevent or minimize damage that may arise from extreme subzero temperature exposure during overwintering. Finally, it could be the case that DNMT3L and $MeCP_2$ play very different and currently unknown roles in *Epiblema* compared to what they are known to do in mammalian species. It might be the case that the changes in expression seen here reflect entirely different processes than what is known about their functions in mammals.

No significant differences in the expression levels of DNMT1, DNMT2, MBD1, and MBD2 were found during any of the experimental conditions when compared to the

corresponding control values (Figure 9). However, there was an overall decreasing trend in total DNMT activity across the time course with a significant decrease to ~51% of control was observed at the -15°C 4 hour condition ($P<0.05$; Figure 10). It could be the case that perhaps the active suppression of transcription mediated by DNMTs is more metabolically expensive to *Epiblema* compared to simply "taking the brakes off" of gene transcription and that a net amount of energy savings therefore occurs with a decrease in DNMT activity. However this is fairly speculative. It is difficult to say whether the *Epiblema* larvae examined in this experiment were also in a state of diapause during subzero temperature exposure as would occur in nature. Entry into diapause is signaled by changes in photoperiod which were not manipulated in this study. However it could be that at -15°C the *Epiblema* larvae were in diapause and implementing freeze avoidance. In this case the *Epiblema* larvae metabolic activity would be largely reduced and the decreases observed in DNMT enzyme activity would reflect the fact that *Epiblema* is mostly shut down and the genomic modifications needed to prepare it for the winter have already been implemented. If this were the case then high epigenetic enzyme activity levels would not be necessary and could be reduced either by posttranslational modifications of the proteins or by their catabolism. Additionally the overall levels of DNMT activity are still rather low so the functional consequences and the amount of metabolic energy invested in regulating DNMT activity may still be small in a "big picture" biological context. It's also worth noting that despite potentially being in a state of diapause *Epiblema* must still prevent freezing. The decrease in DNMT activity might reflect the need for an increase in the activity of certain processes required to combat

subzero temperatures (glycogen breakdown, production of glycerol, upregulation of antioxidant defences etc.).

Relative protein expression levels of TET1, TET2, and TET3 were measured in samples of larvae from *E. scudderiana* (Figure 11). No significant differences were present in the expression levels of TET2 during any of the experimental conditions (Figure 11) whereas TET1 and TET3 did not show any cross reactivity. Furthermore, there were no significant differences in total TET activity levels observed across any of the experimental conditions (Figure 12). These results are expected when considered with the results of DNMT expression presented earlier. TETs catalyze the hydroxylation and oxidation of 5-mC to 5-hmC and are responsible for erasing genomic patterns of methylation. It would be unlikely that *Epiblema* would have evolved mechanisms to invest metabolic energy to change patterns of DNA methylation only to have them immediately cancelled by the actions of TETs.

The changes in DNMT expression and activity seen here are consistent with other results reported in the literature observing an involvement of DNA methylation in hypometabolism and stress response. One study examined the role of DNA methylation during estivation in the sea cucumber *Apostichopus japonicas* (Zhao *et al*., 2015). The results showed not only that DNA methylation varied in a tissue specific manner, but that DNA methylation varied depending on the level of metabolism/stage of estivation. Hypermethylation occurred during the deep-estivation stage suggesting that DNA methylation has an important role in organizing global transcriptional suppression during estivation. Moreover, it was found that full-methylation but not hemi-methylation levels showed the most significant increases during the deep-aestivation stage, suggesting a

strong upregulation in DNMT3A/3B expression and/or activity (Zhao *et al*., 2015). Another study showed more specifically that the hibernation-specific protein-27 (HP-27), which is upregulated in the blood of hibernating chipmunks is controlled by DNA methylation (Fujii *et al.,* 2006). Recently, DNA methylation via DNMTs has also been shown to be an important epigenetic mechanism underlying anoxia tolerance and hypometabolism in the red-eared slider turtle (*Trachemys scripta elegans*) (Wijenayake and Storey 2016).

This study showed results similar to those seen in this thesis and lends more evidence to the notion of a common DNMT response to stress that is likely conserved across a wide range of organisms (Wijenayake and Storey 2016). Increases in DNMT expression and activities appears to be responsible for the early stress response, resulting in an increase in the methylation of the genome and likely widespread transcriptional silencing. However prolonged stress exposure seems to result in DNMT expression and activities falling back to normal levels or even lower. However despite a reduction in DNMT expression/activity during prolonged stress exposure, the genome would likely remain in a highly methylated state that would only change following the removal of the stress and a resumption of the normal behavior of the enzymes.

Other studies examining the involvement of DNA methylation in torpor in 13-lined ground squirrels have reported reductions in global DNA methylation in muscle during torpor in conjunction with significant upregulations in DNMT1 and DNMT3B expression in the liver (Alvarado *et al*., 2015). Another study examining the involvement of DNA methylation in brown adipose tissue in 13-lined ground squirrels found that global DNA methylation levels increased significantly by 1.72-fold during torpor. MBD1

expression levels were also observed to increase 1.91-fold during torpor (Biggar and Storey 2014). Although the role of DNA methylation in hypometabolism and stress response has been understudied in general (and not previously in *Epiblema*), the evidence presented here in combination with the studies reported in the literature collectively indicate that DNA methylation appears to be a conserved mechanism for transcriptional silencing during metabolic rate depression and response to stress.

There were significant decreases in total HAT activity in *E. scudderiana* observed across the time course with decreases to 57-61% of control values in larvae at 5°C and a further significant reduction to 26-29% of control values when larvae were exposed to -15°C (Figure 13). Hence, HAT activities fell stepwise as larvae were exposed to lower temperatures. These results are consistent with what is expected. Acetylation of histones results in a more relaxed chromatin structure that is more accessible to transcription factors and generally results in transcriptional activation. It is not likely that higher levels of transcription would be observed under low temperature conditions that are consistent with the winter diapause condition and freeze avoidance.

There was also an overall decreasing trend in HDAC activity across the time course in *E. scudderiana* (Figure 14). HDAC activity was significantly decreased to 46%, 43% and 33 % after 1 week at 5°C, 4 hours at -15°C and 1 week at -15°C, respectively, compared to the control levels. These results were somewhat unexpected. The deacetylation of histones promotes a condensed chromatin conformation and transcriptional silencing. Therefore, it is unexpected to see a decrease in HDAC activity under conditions when transcriptional suppression of nonessential biological processes should be required. However, several interpretations of this result come to mind. The first

is that despite there being significant decreases in HDAC activity, total HDAC activity levels are low in the larvae and may make only have a modest contribution to transcriptional activation over the low temperature acclimation time course. It is also possible that HDACs are focused on only a subset of genes such as those required to promote cryopreservation and combat potential damage caused by subzero temperature exposure (e.g. activation of antioxidant genes, glycerol production, etc.). It may also be the case that the decrease in HDAC activity with decreasing temperature is due to the downregulation of $MeCP_2$ observed (Figure 8). Recall that one of the functions of $MeCP_2$ is to recruit and bind HDACs for transcriptional silencing. Hence, if there is less $MeCP_2$ present, there could be that less HDAC recruitment to deacetylate histones. Finally, as was the case with *Eurosta*, the kit used to measure total HDAC activity here did not measure the activity of Class III HDAC enzymes (SIRTs 1-7) since they require a NAD^+ cofactor to work. As well the manufacturer claims that HDACs 2 and 8 work poorly with the kit used. It might be that the measured HDAC enzyme activities are reduced with low temperature acclimation but that SIRTs and HDACs 2/8 contribute to transcriptional silencing in important ways that were not detectable, resulting in a biased view of the involvement of HDACs in diapause and freeze avoidance.

In summary, the aforementioned evidence provides affirmation for hypothesis #2- that *Epiblema* showed differential changes in the expression and activity of DNMT, HAT and HDAC enzymes (although not in TETs) corresponding to low temperature exposure. *Epiblema* appears to rely on a temperature and time dependent upregulation in selected DNMT enzymes despite a simultaneous overall decrease in total DNMT activity across the time course. Moreover, the upregulation of said enzymes appears to be accompanied

by a specific downregulation of $MeCP_2$ possibly to assist in the activation of specific genes despite an overall trend towards transcriptional repression. The expression and activity patterns of TETs were unchanged by decreases in temperature suggesting that TETs do not have an active role to play in hypometabolism or freeze avoidance. Accordingly, total HAT and HDAC activity was observed to decease in a temperature dependent manner. This was again likely used to facilitate suppression of nonessential biological processes during subzero temperature exposure while specifically upregulating genes necessary to combat the stress of subzero temperature exposures. These results are similar to the epigenetic processes observed in other species that serve as models of metabolic rate depression and extreme stress tolerance, suggesting that epigenetic responses may be a conserved mechanism across the animal kingdom for implementing hypometabolism and dealing with extreme environmental stress.

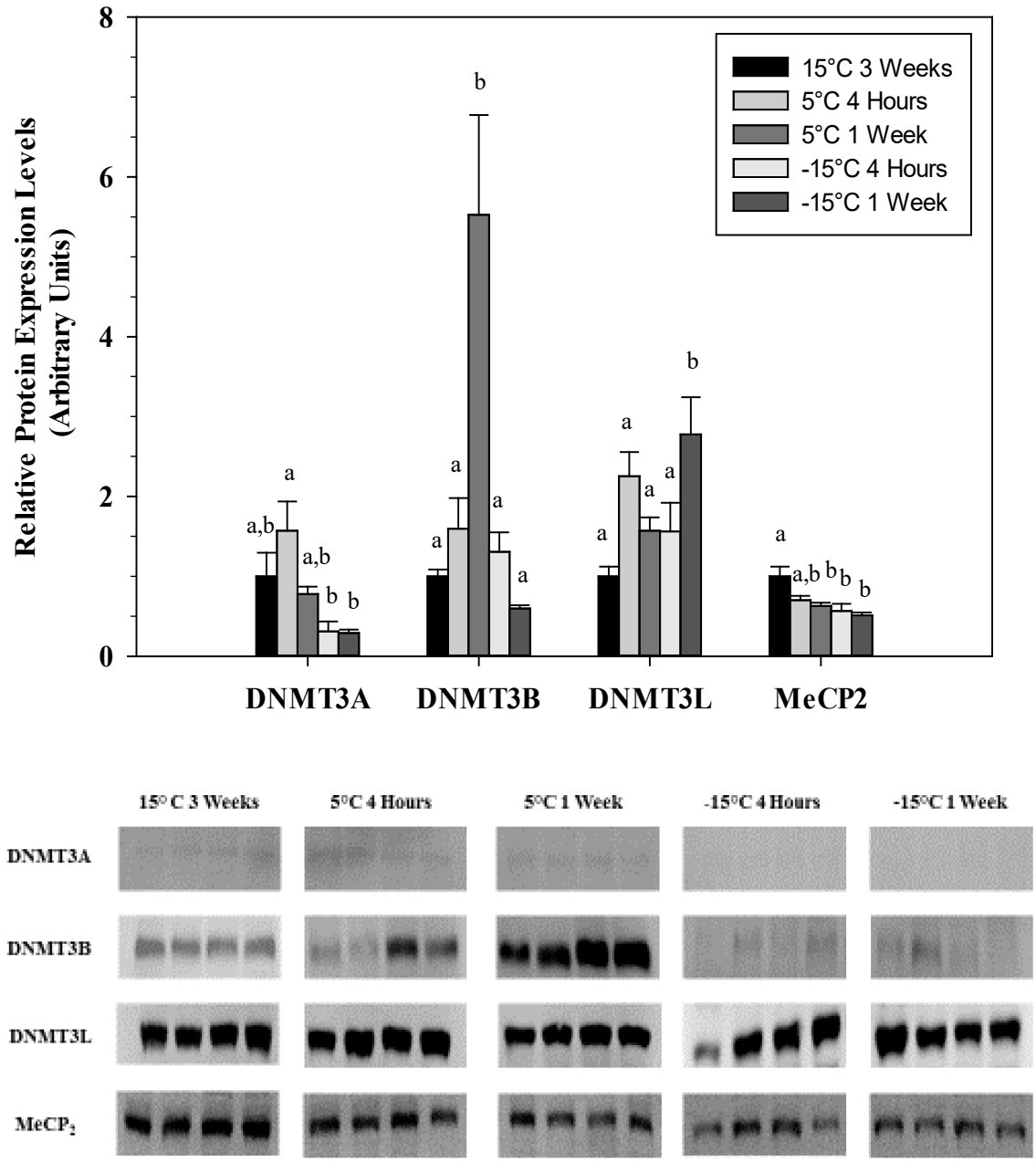

Figure 8: DNMT3A, 3B, 3L and $MeCP_2$ expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

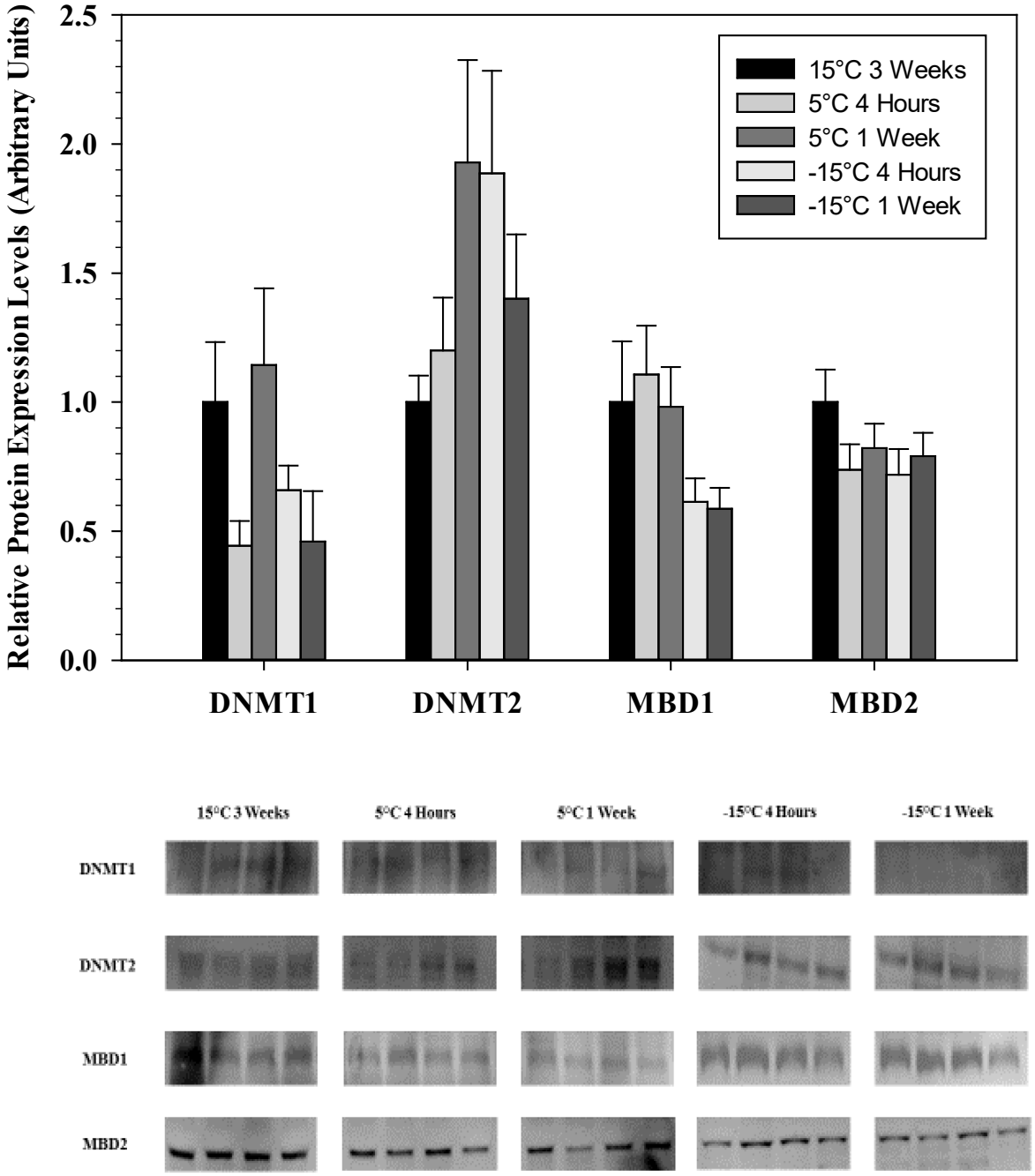

Figure 9: DNMT1, DNMT2, MBD1 and MBD2 expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences in expression levels were found for any of the conditions for each protein.

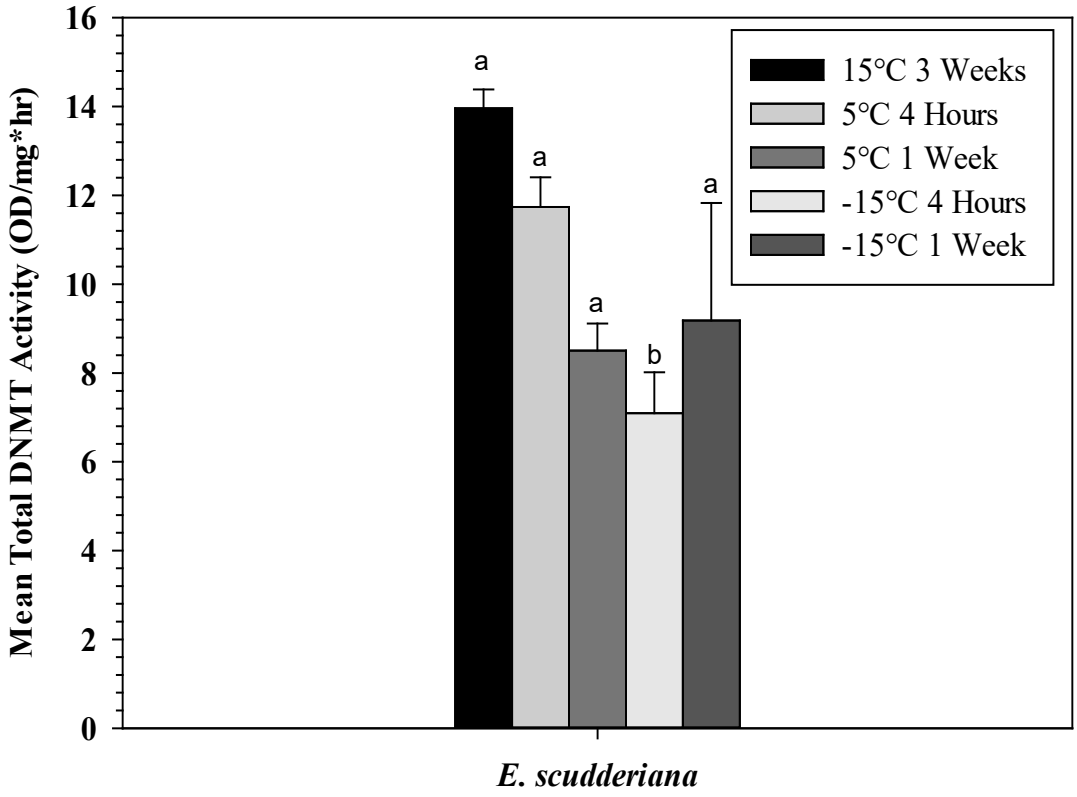

Figure 10: Total DNMT enzyme activity (measured in OD/mg*hr) in *E. scudderiana* larvae at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week. Activity was measured by an EpiQuick DNMT Activity/Inhibition Colorimetric Assay Ultra Kit from Epigentek. Histograms display mean activity levels (±S.E.M. n= 3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

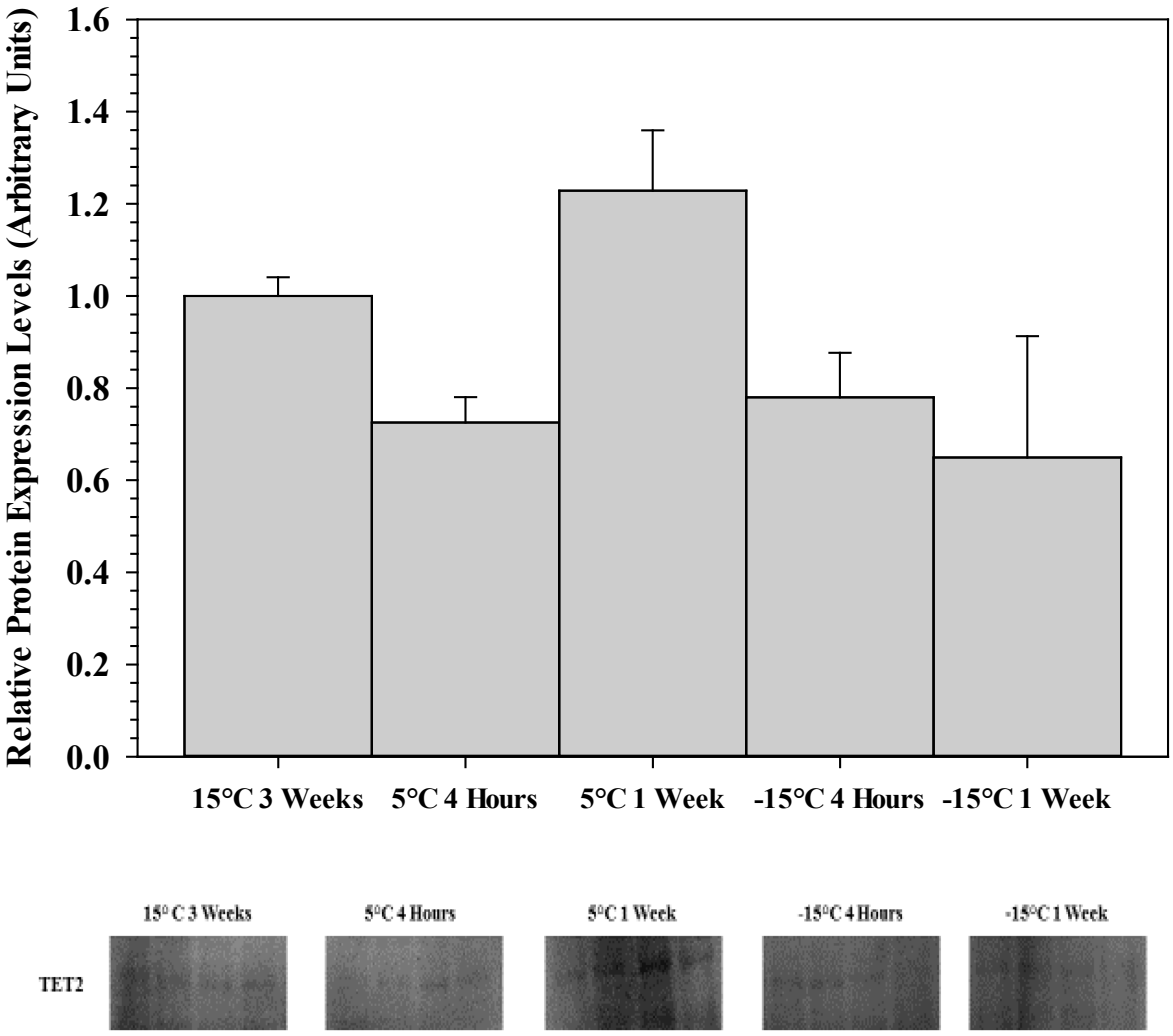

Figure 11: TET2 expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation. No significant differences in expression levels were found for any of the conditions. TET1 and TET3 showed no cross reactivity with the antibodies used.

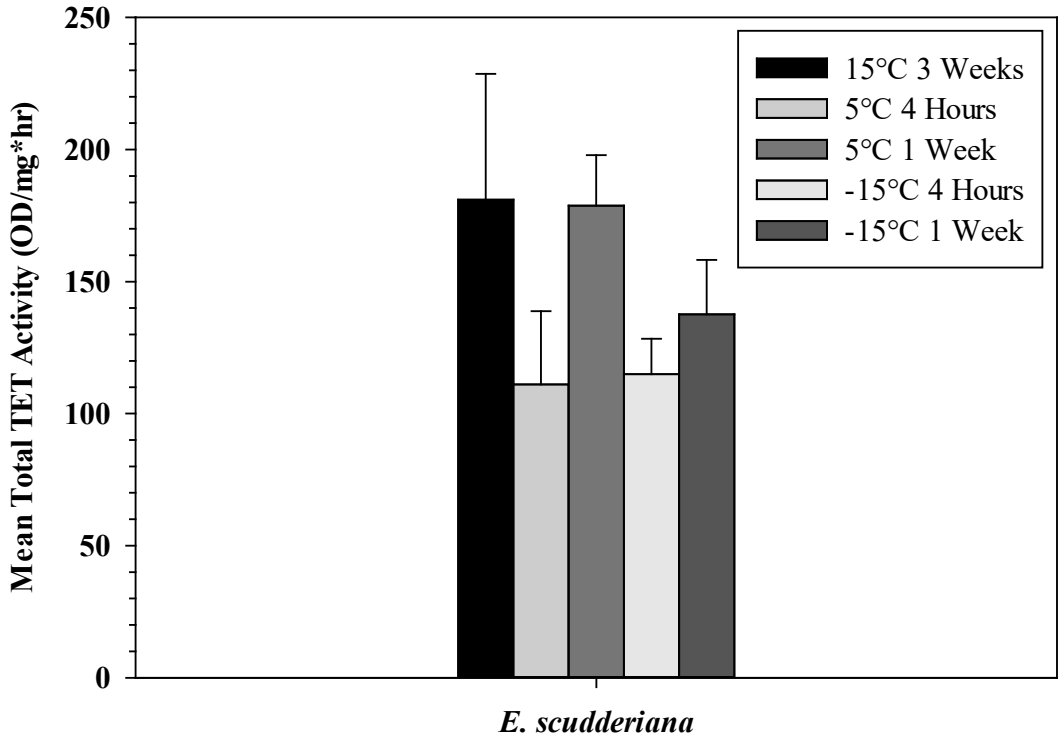

Figure 12: Total TET enzyme activity (measured in OD/mg*hr) in *E. scudderiana* larvae at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week. Activity was measured by an Epigentek Epigenase 5mC Hydroxylase TET Activity/Inhibition Assay Kit. Histograms display mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were observed for any of the experimental conditions.

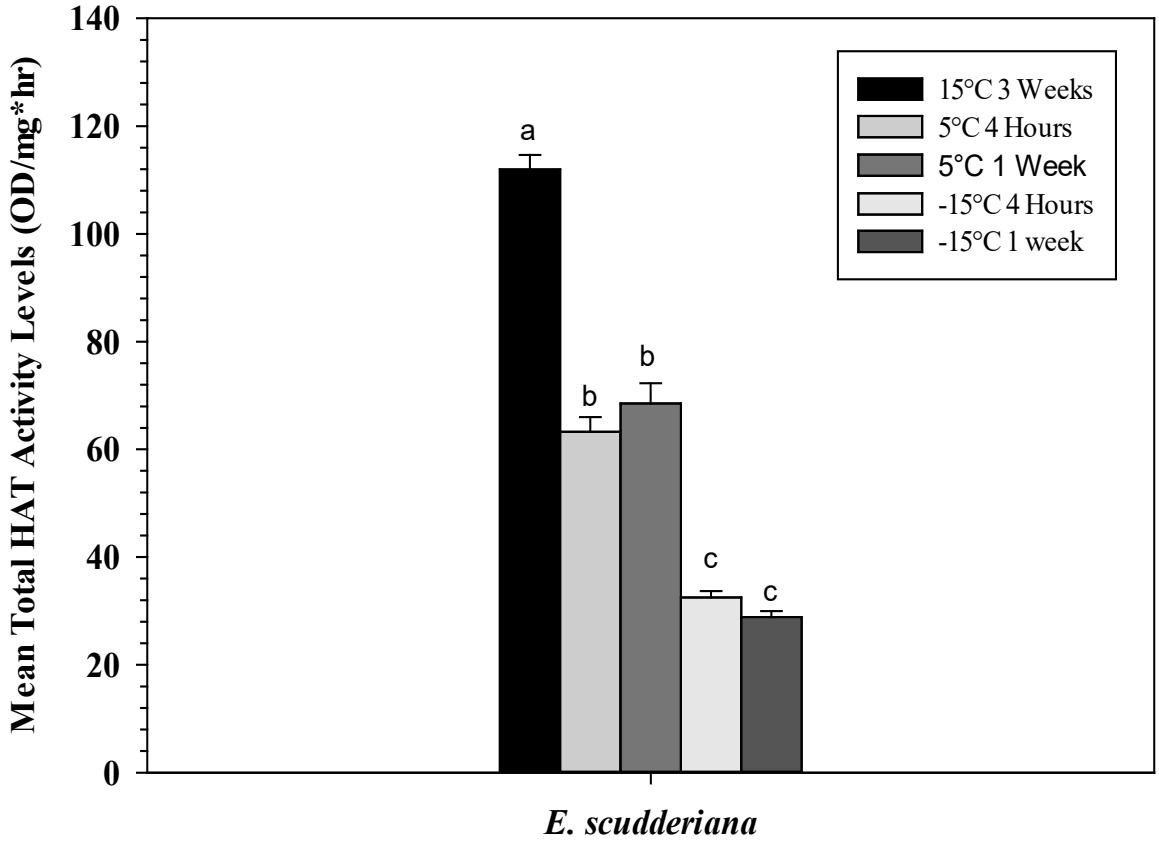

Figure 13: Total HAT enzyme activity (measured in OD/mg*hr) in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week. Activity was measured by an EpiQuick HAT Activity/Inhibition Assay Kit from Epigentek. Histograms display mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

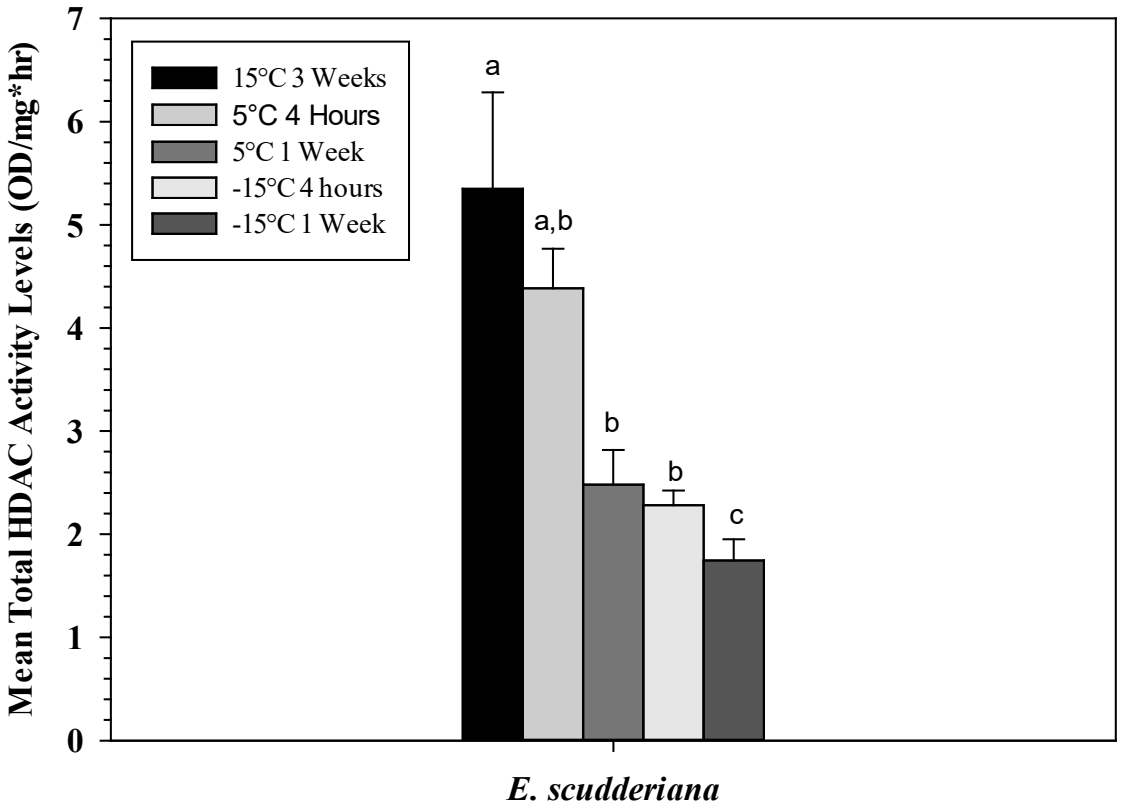

Figure 14: Total HDAC enzyme activity (measured in OD/mg*hr) in *E. scudderiana* larvae at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week. Activity was measured by an Active Motif HDAC Assay Kit (Colorimetric). Histograms display mean activity levels (±S.E.M. n=3-4 samples from total protein isolations). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

Chapter 5
General Discussion

5.1 Freeze Tolerance and Freeze Avoidance in Overwintering Insects

The goldenrod gall fly *Eurosta solidaginis* and gall moth *Epiblema scudderiana* are both cold hardy insects with similar lifestyles. Both insects spend their larval stages inhabiting galls on the goldenrod plant (*Solidago canadensis*). Both species have basically the same distribution range and must survive subzero winter temperatures in northern parts of their ranges (Lee and Costanzo 1998; Morin *et al*., 2005; Courteau *et al*., 2012). Additionally, the final instar larva of both insects is the overwintering stage and enters into a state of diapause to conserve energy during the winter before pupating in the spring. Furthermore, both species implement similar physiological mechanisms to combat subzero temperature exposure, including: (1) the production of very high concentrations of cryoprotectants/low molecular weight antifreeze carbohydrates (glycerol and sorbitol in *Eurosta*; glycerol exclusively in *Epiblema*) (2) glycerol synthesis through glycogen breakdown activated via phosphorylation of glycogen phosphorylase. (3) inhibition of gluconeogenesis through phosphorylation of glycogen synthase (Storey 1997; Pfister and Storey 2006; Rider *et al*., 2011). (4) upregulation of genes involved in antioxidant responses and chaperone protein production. (5) regulated suppression of mitochondrial enzyme activities (Storey 1997; Pfister and Storey 2006; Zhang *et al*., 2011; Courteau *et al*., 2012; Storey and Storey 2012). (6) suppression of the highly energy expensive Na^+/K^+-ATPase and Ca^{2+}-ATPase ion pumps (Storey and Storey 2012; Storey and Storey 2013). Additionally both insects make use of ice nucleating agents. *Epiblema* uses ice nucleating agents that coat ice crystals and prevent them from growing whereas *Eurosta* employs them to control ice formation and restrict it to extracellular spaces. *Eurosta* also uses ice nucleating agents to initiate freezing at high subzero

temperatures to regulate freezing in a slow and controlled manner. (Pfister and Storey 2006; Courteau *et al*., 2012).

Despite the many similarities there are several key differences in the cold hardiness survival strategies used by *Epiblema* and *Eurosta. Eurosta* uses a freeze tolerance strategy where it restricts the formation of ice to extracellular spaces in order to preserve vital intracellular structures and functions to thereby tolerate freezing. By contrast, *Epiblema* allows its body fluids to supercool to temperatures well below 0°C without freezing. Both strategies are enacted while these insects are in a state of diapause. In effect, during the winter *Eurosta* shuts down and freezes whereas *Epiblema* shuts down and uses its metabolic resources to avoid freezing altogether. The acute, but reversible, physiological changes seen in these insect survival strategies suggest that epigenetic regulation might be involved in these survival strategies. Accordingly, the expression and activity patterns of DNMTs and TETs were investigated as well as the activities of HATs and HDACs across a time course of exposure to low temperatures.

In *Eurosta* expression patterns of DNMT3L and $MeCP_2$ were upregulated during 5°C exposure (Figure 1) suggesting that *Eurosta* makes use of a temporary upregulation of these proteins in order to prepare itself for subzero temperature exposure. Since neither of these enzymes have any inherent methyltransferase activity it is more likely that this upregulation occurred to assist in the suppression specific genes in order to save metabolic energy during the winter through their recruitment of and interactions with HDACs. Similarly the upregulation of MBD2 during prolonged exposure to -15°C also suggests that MBD2 might have an active role in suppressing transcription, again likely through interactions with HDACs, while *Eurosta* is frozen. Total DNMT activity was

greatly increased during -15°C exposure again suggesting that *Eurosta* actively suppresses gene transcription while frozen (Figure 3). It is possible that *Eurosta* invests in increasing DNMT activity during -15°C exposure in order to suppress the expression of specific genes that are not necessary for survival in the frozen insect. Increasing enzyme activity via fine control mechanisms (post translational modifications) is a much less metabolically expensive means of altering processes compared to increasing protein expression or rates of protein turnover. This is also likely aided by the fact that TET expression and activity was significantly decreased during 5°C and -15°C exposure (Figures 4-5). Reducing the activity of TETs would reduce the rate of erasure of genomic methylation patterns and, together with elevated DNMT activity, would result in a net increase in genomic methylation and gene silencing. It also seems likely that *Eurosta* selectively increases the transcription of certain genes during 5°C and -15°C exposure by affecting the histones that surround DNA as reflected in the increases in total HAT and decreases in total HDAC activities (Figures 6-7).

Similar to *Eurosta*, *Epiblema* also showed a temporary upregulation of DNMT enzymes during 5°C exposure. DNMT3A and DNMT3B were both upregulated during 5°C exposure, although their upregulations were dependent on the length of time they were held at 5°C, with DNMT3A coming into play earlier than DNMT3B (Figure 8). As was the case in *Eurosta*, these data suggest that temporary upregulation of enzymes involved in gene silencing during 5°C exposure might be a component of preparing *Epiblema* for the conservation of energy via transcriptional suppression during subzero winter temperatures. DNMT3L also showed an upregulation during -15°C exposure, which again suggests that *Epiblema* is actively suppressing gene transcription during

subzero temperature exposure much like *Eurosta* did with MBD2. However, given that the total DNMT activity decreased during -15°C exposure (Figure 10), and since DNMT3A expression levels were at control values at this temperature (Figure 8), it is likely that DNMT3L was exerting its effects on transcription through the recruitment of, and interactions with, HDACs rather than by interacting with DNMT3A to assist in de novo methylation. The changes in DNMT3L expression seen in these insects suggests that cross-talk between DNMT3L and HDACs might be a conserved mechanism in regulating subzero temperature survival in both insects.

This notion of conserved cross talk between DNMT enzymes and HDACs is further supported by analysis of the $MeCP_2$ expression levels. In contrast to *Eurosta*, $MeCP_2$ levels were downregulated during 5°C and -15°C exposure in *Epiblema*. This might be a mechanism to assist in the activation of expression of specific genes during freeze avoidance through the decreased recruitment of HDACs, or it might reflect a decrease in the methylation status of specific genes during subzero temperature exposure and hypometabolism. Hard conclusions cannot be drawn regarding the specific functions of these changes in methylation enzyme expression on the basis of this data alone. However, it does seem plausible that temperature and time dependent changes in DNMT/MBD enzyme-mediated recruitment of HDACs might be a means of coordinating gene expression for hypometabolism and freeze survival. Moreover, it appears that although similar players are involved in these processes in each species of insects, they seem to behave in roughly opposite ways.

The possibility of time-temperature dependent changes in DNMT and HDAC modulations of gene transcription is further supported by the analyses of total DNMT,

HAT and HDAC activities. Total DNMT activity decreased in *Epiblema* during -15°C exposure (Figure 10). This was the opposite pattern as that observed in *Eurosta* (Figure 3). This is may be attributable to the differences in their mechanisms for coping with subzero temperatures. *Eurosta* is frozen and mostly metabolically shut down during -15°C exposure. By contrast, *Epiblema* can supercool far past -15°C (down to near -40°C). It is likely that *Epiblema* reduces the levels of transcriptional repression of certain genes by reducing DNMT activity in order to actively adjust its metabolism as temperatures fall lower and lower despite maintaining an overall global level of metabolic suppression in the diapause state. A reduction in DNMT enzyme activity would also, in itself, help to conserve metabolic energy, potentially through a reduction in DNMT synthesis. This possibility is also supported by the decrease in total HDAC activity seen to correspond with decreases in temperature in *Epiblema* (Figure 14). Although DNMT and HDAC activities both decreased during low temperature exposure, their overall activity levels are low across the time course, suggesting that their overall role in transcriptional activation during freeze avoidance is small. Therefore, it is likely that the transcriptional activation resulting from reduced activities of these enzymes is limited to a select number of genes rather than being a mechanism for widespread transcriptional activation during -15°C exposure. The pattern of HDAC activity across the time course was very similar in both *Epiblema* and *Eurosta* lending evidence to the notion that overall decreases in HDAC activity may be a conserved mechanism between the two insects for activating genes responsible for combating cold stress. Finally, total HAT activity was decreased with 5°C and -15°C exposures. This suggests that a reduction in HAT activity might represent an additional means of achieving

transcriptional repression during freeze avoidance. Importantly, the decrease in HAT activity observed across the time course for *Epiblema* was the opposite pattern to what was observed in *Eurosta*. Also worth noting is that the overall activities of total DNMT, HAT and HDAC across the time course were similar in both *Epiblema* and *Eurosta* despite the fact that patterns of total activity were opposite for DNMTs and HATs between the two insects.

A theme that seems to be emerging in the evidence presented here is that although *Eurosta* and *Epiblema* use similar epigenetic players to regulate subzero temperature survival, they seem to do so in opposite ways. This makes sense given that each insect is essentially in a physiologically opposite state during subzero temperature exposure. *Epiblema* supercools and prevents freezing altogether whereas *Eurosta* freezes and tolerates it. The data also suggests that changes in the patterns of HAT activity are the most prominent epigenetic mechanism underpinning cold hardiness, with DNMTs and HDACs likely playing a more specialized role. Based on the evidence presented here it seems likely that increases in total HAT activity and decreases in total HDAC activity in *Eurosta* occur in order to trigger the widespread activation of genes responsible for combatting damage from ice formation during freezing, while increases in DNMT activity may be responsible for the silencing of genes involved regulating non-essential biological processes. In *Epiblema* a mostly opposite pattern appears to be the case. Overall decreases in total HAT activity are likely responsible for the suppression of genes involved in regulating non-essential biological processes whereas decreases in DNMT and HDAC activities are likely responsible for the selective upregulation of genes for processes involved in the prevention of freezing. However, specific mechanisms cannot

be inferred on this data alone and the activity of individual enzymes cannot be determined based on total levels of activity. Future experiments could potentially purify individual DNMTs, HATs and HDACs for kinetic analysis in order to determine the contribution of specific epigenetic enzymes in each class to changes in total activity. Moreover, by identifying changes in the activity of specific enzymes one could more clearly elucidate the mechanisms and pathways by which freeze tolerance and freeze avoidance are achieved by examining changes in the behavior of upstream and downstream targets of specific enzymes.

5.2 Histone Modifications Supplementary Information

5.2.1 Expression of HDACs, Histone Lysine Acetylation, and HATs in Eurosta solidaginis

Other studies in our lab conducted by a former student (Shostak 2015, data unpublished), examined changes in the expression of specific HDACs, HATs, and acetylated lysine residues on histone targets in both *Eurosta* and *Epiblema* across the same time courses used in this thesis (Appendix A; Figures 15-24). These data were included in this thesis to provide a more comprehensive and complete interpretation of the data presented in this thesis and will also be integrated into joint publications at a later date.

In *Eurosta*, significant suppression was observed in the expression of the protein levels of HDAC3 and HDAC4 as well as in the phosphorylation state of two HDACs, pHDAC4 (Ser632) and pHDAC5 (Ser498) during 5°C to -15°C exposure ($P<0.05$; Figure 15). However, expression levels of HDAC1 and HDAC5 were unaffected by temperature changes (Figure 16). Such changes, especially for the phosphorylated HDACs (pHDAC4

and pHDAC5) correlate with the observed decrease in total HDAC enzyme activity in *Eurosta* (Figure 7). These data potentially represent at least part of a mechanism by which *Eurosta* decreases HDAC activity. It may be that the total HDAC activity is reduced during low temperature exposure as a result of a posttranslational modification (likely catalyzed by a protein phosphatase) that reduces the portion of total HDAC4 and HDAC5 that is phosphorylated during low temperature exposure.

HDAC3 is a Class I HDAC whereas HDAC4 and HDAC5 are Class IIa HDACs which are generally responsible for the recruitment of corepressors and do not possess inherent lysine deacetylase activity themselves (De Ruijter *et al*., 2003; Thiagalingam *et al*., 2003; Lahm *et al*., 2007; Howlett and McGee, 2016). HDAC3 not only deacetylates histones but also shows involvement in responses to oxidative stress via its interaction with targets including the mTOR protein kinase and transcription factor Nrf2 (Martin *et al*., 2014). It also interacts with the p53 transcription factor to influence cell growth, tumor progression and apoptosis (Juan *et al*., 2000). HDAC4 has a critical role in skeletogenesis primarily through associations with the MEF2 transcription factor (Lu *et al*., 2000; Vega *et al*., 2004). HDAC5 is known to be a regulator of the cell cycle and apoptosis (Fan *et al*., 2014). The downregulation of the expression levels of these HDACs with low temperature exposure makes sense. There is likely a global decrease in metabolism resulting directly from the decrease in temperatures to 5°C and even more so during -15°C exposure. Nonessential biological processes such as growth, tissue differentiation, and the cell cycle would also be slowed/shut down in order to conserve metabolic energy. Therefore, it is not surprising to see that the protein expression of

HDACs, that are important regulators of cellular processes, are suppressed along with the decrease in environmental temperature.

Changes in the relative acetylation of lysine residues on selected histone targets were also analyzed in *Eurosta* (Appendix A; Figure 17). Significant suppression of lysine acetylation was observed during 5°C and/or -15°C exposure for histone H3 at lysine residues 9, 18 and 27 (ie. H3.K9, H3.K18, H3.K27) and also for histone H4 at lysine 8 (H4.K8) ($P<0.05$; Figure 17). By contrast, a significant upregulation in acetylation levels were seen for histone 2A at lysine 5 (H2A.K5) during 5°C exposure before returning to control levels during -15°C exposure ($P<0.05$; Figure 17). Expression levels of total H3 acetylation were unaffected by temperature decreases (Figure 18). These results are a bit unusual given that total HAT activity levels increased during low temperature exposure in *Eurosta* It could be that HDAC activity was more prominent at specific histone loci despite the fact that overall HAT activity increased. However, this view could be challenged given that total H3 acetylation levels did not show significant changes across any of the conditions (Figure 18).

Significant changes were observed in the expression levels of three HATs during 5°C to -15°C exposure: MYST1, PCAF, and Tip60 (Figure 19). MYST1 protein expression levels were mostly constant across the time course aside from a significant downregulation observed during -15°C exposure for 4 hours ($P<0.05$; Figure 19). PCAF expression levels were significantly downregulated during -15°C exposure whereas Tip60 expression levels showed a pronounced upregulation during 5°C and -15°C exposure ($P<0.05$; Figure 19). PCAF acetylates K9, K14, and K18 on H3 as well as K8 and K16 on H4 (Poux and Marmorstein 2003; Kouzarides 2007). Tip60 has been

identified as acetylating K5, K8, K12, and K16 on H4, K14 on H3, and K5 on H2A (Kimura and Horikoshi 1998; Kouzarides 2007; Gao *et al*., 2014). MYST1 acetylates K5, K8 and K16 on H4 (Dou *et al*., 2005; Thomas *et al*. 2008; Cai *et al.,* 2010).

The downregulation of PCAF expression during -15°C exposure correlates with the observed decreases in acetylated H3.K9, H3.K18, and H4.K8 expression during -15°C exposure (Figure 17). It is possible that the downregulation of PCAF represents the mechanism underpinning the decrease in acetylated histone targets, although knockout studies would be required to demonstrate cause and effect. It is also possible that HDACs might have the main responsibility for the suppression of histone acetylation at the mentioned targets and that PCAF has a minimal role. Similarly, the increase in Tip60 expression may be part of the mechanism underpinning the increase in H2A.K5 expression (Figure 17). But again caution must be drawn as data here can only be correlated and knockout studies would be needed to demonstrate cause and effect. Furthermore it is likely that nonspecific HDAC activity is responsible for the downregulation of acetylated H3.K27 observed (Figure 17).

PCAF regulates cell growth, differentiation, as well as tumor formation (Modak *et al*., 2013; Yang *et al*., 1996; Watts *et al*., 2004; Okumura *et al*., 2006). The downregulation of PCAF makes sense given that during -15°C exposure *Eurosta* is frozen. Therefore, very minimal, if any, growth or proliferation processes would be taking place. Tip60 is involved in DNA repair and so its upregulation is reasonable given that *Eurosta* would have to cope with various forms of physical and oxidative stress from ice formation and anoxia during -15°C exposure (Legube *et al*., 2004; Murr *et al*., 2006; Van Den Broeck *et al*., 2011; Sun *et al*., 2005). The biological roles of MYST1 remain

unclear but it seems to be involved in the regulation of autophagy (Fullgrabe *et al*., 2013). Therefore, it is not surprisingly that its expression levels show minimal change given that *Eurosta* would likely have little cell turnover during freezing.

The downregulation in expression observed in two out of the three HATs examined should be considered with the observed increase in total HAT activity (Figure 6). Total HAT activity increased with low temperature exposure in *Eurosta* despite a downregulation of MYST1 and PCAF. It is important to note however that only 4 HATs were examined in this study: MYST1, PCAF, and Tip60 were successfully analyzed whereas MYST2 could not be assessed because antibodies did not show cross reactivity). It is possible that, despite a downregulation of protein content, that MYST1 and PCAF increased their activity as a result of posttranslational modifications. Part of the observed increase in total HAT activity across the time course may also be attributable to the increase in Tip60 levels and it is also possible that Tip60 may have also been post-translationally modified to increase activity as well. Finally, it is important to note that the expression levels of only 3 HATs were measured. It could also be the case that the expression levels of numerous important HATs increased and they simply weren't measured in this study.

5.2.2 Expression of HDACs, Histone Lysine Acetylation, and HATs in Epiblema scudderiana

Protein expression levels of HDAC 1, HDAC3, and HDAC5 as well as relative phosphorylation of pHDAC4 (Ser632) and pHDAC5 (Ser498) in *E. scudderiana* across the time course (Figure 20). Significant suppression was noted for both protein levels and enzyme phosphorylation during -15°C exposure for each enzyme, as well as at 5°C for

HDAC1 ($P<0.05$). These expression patterns were very similar to the data for *Eurosta* (Figure 15) suggesting that commonalities in HDAC regulation are a conserved mechanism for regulating subzero survival in both insects. These changes also correlate with the observed decrease in total HDAC activity across the time course (Figure 14). Again, especially for the phosphorylated HDACs (pHDAC4 and pHDAC5) the observed suppression of phosphorylation might partially provide a mechanism for decreasing total HDAC activity with low temperature exposure. It may be that the total HDAC activity decreases during low temperature exposure result from a decrease in the total amount of phosphorylated HDACs expressed during low temperature exposure. No significant differences were observed in the expression levels of HDAC4 for any of the conditions examined (Figure 21).

HDAC1, HDAC3, and HDAC5 are involved in the regulation of cell growth/proliferation, cell cycle and apoptosis (Magnaghi-Jaulin *et al*., 1998; Juan *et al*., 2000; (Fan *et al*., 2014). The downregulation in the expression levels of these HDACs with low temperature exposure in *Epiblema* is a reasonable result. During the transition to 5°C and -15°C exposure, nonessential biological processes such as growth, tissue differentiation, and the cell cycle would be slowed/shut down in order to conserve metabolic energy. *Epiblema* appears to use the same approach to regulating HDAC expression and activity as *Eurosta* does during diapause and subzero temperature exposure.

The expression levels of several acetylated lysine residues on selected histone targets were also analyzed in *Epiblema* (Appendix A; Figure 22). Total H3, global H3 acetylation, and acetylation at H2B.K5, H3.K9, H3.K18, H3.K27, and H4.K8 all showed

significant suppression in *E. scudderiana* with exposure to low temperatures. ($P<0.05$, Figure 22). Acetylation patterns of specific histone lysine residues (H3.K9, H3.K18, H3.K27, H4.K8) showed virtually identical expression patterns to those seen in *Eurosta* under the same conditions. This again implies that condensation of chromatin as a means of repressing gene transcription is likely a conserved mechanism in both these species for regulating low temperature survival. These expression patterns also correlate strongly with the observed decrease in total HAT activity seen in *Epiblema* across the time course (Figure 13). The combination of these data on acetylation patterns and the decrease in total HAT activity lends support to the idea that widespread decreases in HAT expression and activity might be responsible for the promotion of a condensed chromatin structure and transcriptional repression of nonessential genes as part of the freeze avoidance strategy. This further highlights what is likely the prominent role of HATs in epigenetic modulations of metabolic rate depression and stress response in *Epiblema*.

There were significant changes in the expression levels of MYST2 across the time course in *Epiblema scudderiana*. MYST2 was upregulated during 5°C exposure before decreasing during -15°C exposure. However, expression levels were still upregulated as compared to the controls during the -15°C 4 hours condition ($P<0.05$; Figure 23). No significant differences were observed in the expression levels of PCAF or Tip60 under any of the experimental conditions (Figure 24). The changes in acetylation patterns of specific histone lysine residues seen in Figure 22 do not correlate with the expression patterns of PCAF or Tip60. It may be the case that PCAF and Tip60 alter their activity via posttranslational modifications in *Epiblema* during 5°C and -15°C exposure without altering their protein expression levels. Future experiments could look at expression

levels of PCAF and Tip60 with posttranslational modifications or could purify them for enzyme kinetics experiments to determine whether or not changes in their activity affect histone acetylation status. MYST2 acetylates lysine 5, 8, 12, 16 on H4 (Doyon *et al*., 2006; Kouzarides 2007). There was no apparent correlation between MYST2 expression and expression of acetylated H4.K8 (Figures 22-23). However, again it is possible that MYST2 modifies its activity through posttranslational modifications independently of changes in its expression patterns. MYST2 is understudied, although it's believed to be involved in the regulation of cell growth and DNA replication (Doyon *et al*., 2006; Lizuka *et al*., 2016). It may be that the transient upregulation observed in MYST2 expression during 5°C exposure have some involvement in slowing down/shutting off the cell cycle during in preparation for later subzero temperature exposures that would occur during the winter. Finally, as was the case with *Eurosta*, it is important to note that the expression levels of only 3 HATs were measured in this study. It could also be the case that the expression of numerous other HATs changed along with the decrease in total HAT activity and their expression levels simply weren't measured in this study.

In summary, the data presented in this section suggest that chromatin modifications mediated by the behavior of HAT and HDAC enzymes are a key component in the epigenetic regulation of subzero temperature survival in both *Epiblema* and *Eurosta*. Furthermore, the mechanisms by which they alter chromatin structure seem to be largely conserved in both species. Both insects demonstrated an overall decrease in total HDAC activity across the time course as well as common downregulation of the expression of multiple HDAC enzymes between the two insects. Furthermore, changes in acetylated histone lysine residue targets also shared much in common between the two

species. Finally, both insects altered their total HAT activity across the time course albeit in opposite ways, *Eurosta* increasing total HAT activity across the time course whereas *Epiblema* decreased HAT activity.

5.3 Future Directions

The aforementioned results provide evidence suggesting that epigenetic responses are an important component of cold hardiness in both freeze tolerant (*E. solidaginis*) and freeze avoiding (*E. scudderiana*) insect species. However, the data presented here are by no means a comprehensive explanation of all the physiological mechanisms employed by these insects to survive low winter temperatures. Moreover, the time course used in this study provides only a snapshot of the epigenetic responses to temperature changes across a specific range of temperatures and time frames. This study could be expanded in order to test how robust these insect adaptations are to a range of conditions.

For starters, *E. solidaginis* and *E. scudderiana* are both known to survive temperatures well below -15°C. In the Ottawa region, winter temperatures can often decline to at least -30°C for several days at a time. It would be interesting to experiment in order to see what the lowest temperature is that each insect could survive as well as look at potential epigenetic or other biochemical changes that occur at the furthest margin of the freeze survival capacity. Additionally, the duration that each insect is held at a given temperature could also be expanded in order to determine what the upper limit is on the length of time they could survive when held at a given subzero temperature (e.g. perhaps they can survive being held at -15°C for one week but not three weeks). Additionally, as mentioned earlier these insects often have to contend with temperature fluctuations that can range as great as 25-35°C both over the winter and sometimes even

during a day-night cycle (Lee and Costanzo 1998; Morin *et al*., 2005). The robustness of their epigenetic responses could also be tested by examining how fast of a temperature drop they could survive and how quickly epigenetic modifications could occur (both a decrease and increase in temperature. Experiments using gradients of temperature decreases of shorter and shorter durations could be performed. Furthermore, a related study could be performed where insects are sampled from different months of the year and their epigenetic mechanisms analyzed according to month or season in order to relate the changes seen in this experiment to those that occur under natural conditions.

Subzero temperatures often introduce an anoxic and dehydration element since the insects are in a state of diapause and not actively feeding/drinking. Experiments on models of dehydration stress, such as *Xenopus laevis*, often involve monitoring water loss over several days as a percentage of total body weight (Biggar *et al*., 2015). Similar experiments could be done to examine the impact of dehydration on the expression and/or activity of epigenetic enzymes as well as test the limit of dehydration that these insects can tolerate. Lower temperatures can also limit the diffusion of oxygen and impair breathing. Oxygen deprivation chambers could also be employed to test the extent of the anoxia tolerance of both species as well as examine the epigenetic responses to discern if the same mechanisms involved in freeze survival also regulate their ability to tolerate reduced availability of oxygen.

All of the aforementioned potential studies could be examined at both the DNA and chromatin level by examining changes in the expression and activity of DNMTs, HATs, and HDACs under the different conditions as was done in this thesis. The epigenetic responses could also be examined at the microRNA level by using qRT-PCR.

Work elsewhere has examined the role of microRNAs in subzero survival in both *Eurosta* (Lyons *et al*., 2016) and *Epiblema* (Lyons et al., 2015a) as well as in as their role in anoxia tolerance (Lyons et al., 2015b). All of the above mentioned studies could be expanded to examine the role of microRNAs responses in these insects to larger and more varied ranges of temperatures, oxygen availability and hydration, or to faster gradients of temperature change.

Other studies could broaden the examination of HATs and HDACs. Only four HATs were examined in this study. The expression and activities of other HATs such as GCN5, P300-CPB, TFIIC, and CLOCK could be examined in future studies (Howlett and McGee 2016). Moreover, immunoprecipitation experiments could be conducted in order to study their posttranslational modifications. Additionally, another entire class of HDACs known as SIRTs were not evaluated at all in the current thesis. Similar to other HATs, SIRTs 1-7 expression and activity could be measured directly or their transcript levels could be measured using qPCR.

One final element of epigenetics that has yet to be examined in *E. solidaginis* and *E. scudderiana* is the role of Histone Methyltransferases (HMTs). HMTs catalyze the methylation of lysine and arginine residues on histone tails to promote activation or suppression of gene transcription depending on the HMT and their target residue (Di Lorenzo and Bedford 2011; Sims and Reinberg 2006; Sims et al., 2003). Future studies could measure expression and activity levels of HMTs, or could employ q-PCR to measure levels of methylation or acetylation at genes encoding HMTs. Other studies could expand on these to measure the expression levels of downstream H3 and H4 lysine and arginine residue methylation levels.

In addition to expanding on the conditions and parameters already examined. The downstream targets of DNMTs, HATs, and HDACs could also be investigated in future experiments. Although the genomes of *E. solidaginis* and *E. scudderiana* are not sequenced, it would be possible to take specific gene sequences from their genetic cousins the fruit fly *Drosophila. melanogaster* and *the silk moth Bombyx mori* that are sequenced and develop primers for genes expected to be methylated during the winter (e.g. genes regulating the cell cycle) using bi-sulfite sequencing and methylation specific PCR (MSq-PCR). Similarly, one could correlate states of chromatin accessibility with the expression and/or activity of specific HATs and HDACs by using nucleases to digest strands of chromatin in genes suspected to be involved in regulating responses to subzero temperature exposure and then use q-PCR or sequencing methods to analyze the level of histone acetylation or deacetylation. Moreover, one could also simultaneously measure chromatin modifications and DNA methylation status by isolating chromatin and treating them with DNMTs that will only methylate CpG sites unprotected by nucleosomes or protein complexes. DNA is then subjected to bisulfite sequencing followed by q-PCR to assess chromatin accessibility and methylation status. The C to T conversion in the GpC sites indicates that chromatin structure is in a relaxed conformation, accessible, and could potentially be correlated to specific HAT expression and/or activity. Similarly, unmodified CpG sites indicate chromatin regions that are condensed and inaccessible which could also be correlated to specific HDAC expression/activity. The overall expression and/or activity of DNMTs, HATs, HDACs, euchromatin and heterochromain could be correlated to provide evidence of interactions between the enzymes.

The above mentioned methods could be applied to a number of specific pathways to gain a broader understanding of the physiological mechanisms involved in regulating hypometabolism and cold/freeze survival in these insects. For example, changes in levels and activity of members of thePI3K/Akt/mTOR signaling pathway during periods of metabolic rate depression have been observed in other organisms (Tessier *et al*., 2015; Wu and Storey 2012; Ramnanan *et al*., 2007). Given the involvement of this pathway in protein synthesis and autophagy it is likely that the pathway is differentially regulated during diapause and freeze avoidance/tolerance. Future experiments could measure levels of methylation and/or acetylation of the genes/chromatin encoding for components of this pathway or look directly at the levels of expression and activity of these proteins themselves. A similar line of thinking could be applied to the p53 pathway. It has been seen to be altered in other organisms during periods of stress and hypometabolism (Hefler *et al*., 2015; Zhang et al., 2013) and given its role in regulating the cell cycle, apoptosis and DNA repair it is likely that it is highly involved in the cold-hardiness of these insects as well. Finally, the same approach could be taken for specific proteins involved in regulating the cell cycle such as Cyclin D, Cdc 2, Cdks (2, 4, 6), and the cyclins (A, B1, D1, E) as well as the phosphorylation states of these proteins. These targets have shown changes in other organisms and would likely show changes in *Eurosta* and *Epiblema* as well, particularly as part of a transition into diapause over the winter months (Biggar and Storey 2012; Zhang and Storey 2012; Roufayel *et al.,* 2011).

Finally, these approaches could all be applied to the study of upstream regulators of DNMTs, HATs, and HDACs. Upstream regulators of these enzymes are understudied, but examining their role in regulating epigenetic enzyme behavior would provide a more

expansive view of the processes involved in regulating diapause and subzero temperature survival. For example, DNMT1 has been shown to be regulated by UHRF1, elsewhere there has been a strong inverse correlation between levels of DNA methylation and H3.K4 methylation. By contrast, H3K36me3, H3K9me3 and H3K27me3 have been correlated with more DNA methylation, and microRNAs from the microRNA 29 family have been strongly associated with DNA methylation regulation. Micro-RNA 126, 148 and 152 specifically have been identified as regulating DNMT1 (Dennis *et al*., 2011). DNMT expression can also be upregulated by the Ras-c-Jun pathway as well as by p53 and FOXO3a (Lin and Wang 2014). Similarly members of the ING family of tumor suppressor proteins (e.g. ING 1-4) are involved in regulating HATs and HDACs and USP19 has been shown to regulate HDAC1 and HDAC2 specifically (Wu et al. 2016; Doyon *et al*., 2006).

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Appendices

Appendix A: Supplementary Expression Data for Histone Modifications

A.1 HDAC expression in response to low temperature exposure in Eurosta solidaginis

Relative protein expression levels of HDAC 1, HDAC3, HDAC4, pHDAC4 (Ser 632), HDAC5 and pHDAC5 (Ser498) were measured in larvae of freeze tolerant *E. solidaginis* (Figure 15-16). HDAC3 showed a significant 50% downregulation during the -15°C 4 hours condition compared to the 5°C 4 hours condition and a significant 54% downregulation compared to the 5°C 1 Week condition ($P<0.05$; Figure 15). HDAC4 showed significant 54% and 38% downregulations during the 5°C 1 Week condition compared to the control and 5°C 4 Hour condition, respectively ($P<0.05$; Figure 15). Similar significant 56% and 41% downregulations were also observed during the -15°C 4 Hours condition compared to the control and 5°C 4 hours condition ($P<0.05$; Figure 15). HDAC4 was also significantly suppressed during the -15°C 1 Week condition, showing a 81% and 74% downregulation compared to the control and 5°C 4 Hours condition, respectively ($P<0.05$; Figure 15). The phosphorylated forms of HDAC4 and HDAC5 showed similar patterns. pHDAC4 showed a significant 49% and 51% downregulation compared to the control and 5°C 4 hours condition ($P<0.05$; Figure 15). pHDAC4 was also significantly downregulated during the -15°C 4 hours condition, showing a 34% and 36% downregulation compared to the controls and 5°C 4 Hours condition ($P<0.05$; Figure 15). pHDAC4 also showed a significant 59% and 61% downregulation compared to the controls ($P<0.05$; Figure 15). pHDAC5 showed a significant 57% and 53% downregulation compared to the controls during the -15°C 1 Week condition ($P<0.05$; Figure 15). No significant differences were observed in the expression levels of HDAC1 and HDAC5 during any of the experimental conditions (Figure 16).

A.2 Histone acetylation expression during low temperature exposure in Eurosta solidaginis

Relative acetylation at specific lysine sites on histones was examined in larvae of freeze tolerant *E. solidaginis*: H3.K9, H3.K18, H3.K27, H4.K8, H2A.K5 (Figure 17). Acetylated H3.K9 levels were significantly downregulated during both 5°C and -15°C exposure. Expression levels showed a significant 48% suppression compared to the controls during the 5°C 4 hours condition and a 67% downregulation during the 5°C 1 Week condition compared to the controls ($P<0.05$; Figure 17). During the -15°C 4 Hours condition there was a significant 80% and 62% downregulation compared to the controls and 5°C 4 Hours condition. This was mirrored during the -15°C 1 Week condition with a 84% and 70% downregulation compared to the control and 5°C 4 Hours condition respectively ($P<0.05$; Figure 17). H3.K18 acetylation levels showed a significant 10% decrease during the 5°C 4 Hours condition compared to the controls before returning to control levels. They were again downregulated during the -15°C 4 Hours condition by 40%, 33%, and 36% compared to the control, 5°C 4 hours and 5°C 1 Week conditions respectively ($P<0.05$; Figure 17). Similarly, suppression was seen during the -15°C 1 Week condition with 49%, 43%, and 46% decreases compared to the control, 5°C 4 hours and 5°C 1 Week conditions respectively ($P<0.05$; Figure 17). H3.K27 acetylation levels showed a significant 67%, 73%, and 74% downregulation during the -15°C 4 Hours condition when compared to the control, 5°C 4 hours and 5°C 1 Week conditions respectively ($P<0.05$; Figure 17). A similar trend was observed during the -15°C 1 Week condition with a 69%, 74% and 75% suppression compared to the control, 5°C 4 hours and 5°C 1 Week conditions, respectively ($P<0.05$; Figure 17). H4.K8 expression levels

showed a significant downregulation during the -15°C 4 hours condition, with a 72% and 70% reduction compared to the control and 5°C 4 Hours condition ($P<0.05$; Figure 17). Similarly, a 82% and 81% downregulation was observed during the -15°C 1 Week condition compared to the control and 5°C 4 Hours condition ($P<0.05$; Figure 17). H2A.K5 acetylation levels showed a significant 59% downregulation during the -15°C 1 Week condition compared to the 5°C 1 Week condition ($P<0.05$; Figure 17). However, despite this variety of changes in acetylation of specific histone 3 lysine sites, no significant changes in global H3 acetylation were detected under any of the experimental conditions (Figure 18).

A.3 HAT expression during low temperature exposure in Eurosta solidaginis

Relative protein expression levels of MYST1, P300/CBP-associated factor (PCAF), and Tip60 were measured in larvae of freeze tolerant *E. solidaginis* (Figure 19). MYST1 showed a significant 50% and 49% downregulation during the -15°C 4 Hours condition compared to the 5°C 1 Week and -15°C 1 Week conditions ($P<0.05$; Figure 19). PCAF showed significant 59%, 49%, and 48% suppression during the -15°C 4 hours condition when compared to the controls, 5°C 4 hours, and 5°C 1 week conditions, respectively. Similarly, during the -15°C 1 Week condition, downregulations of 50%, 40%, and 48% were observed compared to the controls, 5°C 4 hours, and 5°C 1 week conditions, respectively ($P<0.05$; Figure 19). In contrast. Tip60 protein levels showed overall upwards trends. A 1.52 fold and 1.63 fold upregulation was observed during the 5°C 1 Week condition compared to the control and 5°C 4 Hours condition ($P<0.05$; Figure 19). A 1.55 fold and 1.65 fold upregulation was seen during the -15°C 4 hours condition compared to the control and 5°C 4 Hours condition ($P<0.05$; Figure 19).

Finally, expression levels in the -15°C 1 Week condition showed a significant upregulation compared to all other conditions with a 2.16 fold, 2.31 fold, 1.41 fold, and 1.40 fold upregulation compared to the control, 5°C 4 hours, 5°C 1 week, and -15°C 4 hours conditions, respectively ($P<0.05$; Figure 19).

A.4 HDAC expression in response to low temperature exposure in Epiblema scudderiana

Relative protein expression levels of HDAC 1, HDAC3, pHDAC4 (Ser632), HDAC5, and pHDAC5 (Ser498) were measured in larvae of freeze avoiding *E. scudderiana* (Figure 20). Significant downregulation was observed at all experimental point as compared with controls with levels reduced by 74%, 77%, 77,% and 79%, respectively, for at the 5°C 4 hours, 5°C 1 week, -15°C 4 hours, and -15°C 1 week conditions ($P<0.05$; Figure 20). However, there were no significant differences between any of the low temperature conditions. HDAC3 showed a significant downregulation during the -15°C 4 hours condition of 64%, 54%, and 66% compared to the control, 5°C 4 hours, and 5°C 1 week conditions, respectively. Similarly, during the -15°C 1 Week condition pHDAC4 was significantly reduced by 54%, 42%, and 63% compared to the control, 5°C 4 hours, and 5°C 1 week conditions, respectively ($P<0.05$; Figure 20). pHDAC4 showed significant downregulations of 80%, 86%, and 84% during the -15°C 4 hours condition compared to the control, 5°C 4 hours, and 5°C 1 week conditions, respectively. Similarly, expression levels were downregulated during the -15°C 1 Week condition by 87%, 91%, and 90% compared to the control, 5°C 4 hours, and 5°C 1 week conditions, respectively ($P<0.05$; Figure 20). HDAC5 levels were significantly reduced during the -15°C 1 Week condition by 31% and 35% compared to the control and 5°C 4 hours condition. pHDAC5 was significantly downregulated during the -15°C 4 Hours

condition by 45% and 55% compared to the controls and 5°C 1 Week conditions. Moreover, the -15°C 1 Week condition was significantly downregulated by 44% compared to the 5°C 1 Week condition ($P<0.05$; Figure 20). No significant differences were observed in expression levels for HDAC4 for any of the conditions tested (Figure 21).

A.5 Histone acetylation expression during low temperature exposure in Epiblema scudderiana

Relative expression levels of total H3 protein, total H3 acetylation, and acetylation on specific histone residues (H2B.K5, H3.K9, H3.K18, H3.K27, H4.K8) were measured in freeze avoiding *E. scudderiana* (Figure 22). There was a significant 70%, 62%, 93%, and 97% reduction in acetyl-H3 content between the control and the 5°C 4 hours, 5°C 1 week, -15°C 4 hours, and -15°C 1 week conditions, respectively ($P<0.05$; Figure 22). However, comparable changes in total H3 protein were substantially less in all cases; H3 levels showed a significant downregulation of 68% during the -15°C 4 hours condition compared to the control. Moreover, there was a significant downregulation of 90%, 85%, 86%, and 69% during the -15° 1 week condition compared to the controls, 5°C 4 hours, 5°C 1 week, and -15°C 4 hours conditions, respectively ($P<0.05$; Figure 22). Acetylation of H2B.K5 was significantly reduced by 76%, 75%, 61%, and 70% between the controls and the 5°C 4 hours, 5°C 1 week, -15°C 4 hours, and -15°C 1 week conditions, respectively ($P<0.05$; Figure 22). H3.K9 acetylation after -15°C 4 hours was also significantly reduced by 71%, 63%, and 73% compared to the control and the 5°C 4 hours, and 5°C 1 week conditions, respectively ($P<0.05$; Figure 22). Similarly, there were significant 92%, 90%, and 94% reductions in the -15°C 1 Week

condition compared to the control, 5°C 4 hours, and 5°C 1 week conditions, respectively ($P<0.05$; Figure 22). H3K18 acetylation was significantly reduced by 59% and 56% during the 5°C 4 Hours condition compared to the control and 5°C 1 Week conditions ($P<0.05$; Figure 22). Similarly, there were significant downregulations of 83% and 81% observed during the -15°C 4 Hours condition when compared to the control and 5°C 1 Week condition, respectively ($P<0.05$; Figure 22). As well, acetylation levels during the -15°C 1 Week condition were significantly suppressed by 91% and 90%, respectively, when compared with the control and 5°C 1 Week condition ($P<0.05$; Figure 22). Significant reductions in H3K27 acetylation of 77%, 87%, 96% and 98% were seen between the control and the 5°C 4 hours, 5°C 1 week, -15°C 4 hours, and -15°C 1 week conditions, respectively ($P<0.05$; Figure 22). Similarly, reductions of 65%, 90%, ~100% and ~100% in the expression levels of H4.K8 were observed between the controls and the 5°C 4 hours, 5°C 1 week, -15°C 4 hours, and -15°C 1 week conditions, respectively ($P<0.05$; Figure 22).

A.6 HAT expression during low temperature exposure in Epiblema scudderiana

Relative protein expression levels of three histone acetyltransferases (MYST, PCAF, and Tip60) were measured in *Epiblema scudderiana* (Figures 23-24). MYST protein levels showed significant 9.04 and 9.28 fold upregulations at the 5°C 4 hours and the 5°C 1 Week conditions, as compared to the controls ($P<0.05$; Figure 23). At –15°C, however, protein levels were strongly reduced again although still elevated compared with controls. . Furthermore, the -15°C 4 hours condition showed a significant 3.58 fold upregulation compared to the control ($P<0.05$; Figure 23). No significant differences in

protein expression levels were observed for PCAF or Tip60 during any of the experimental conditions (Figure 24).

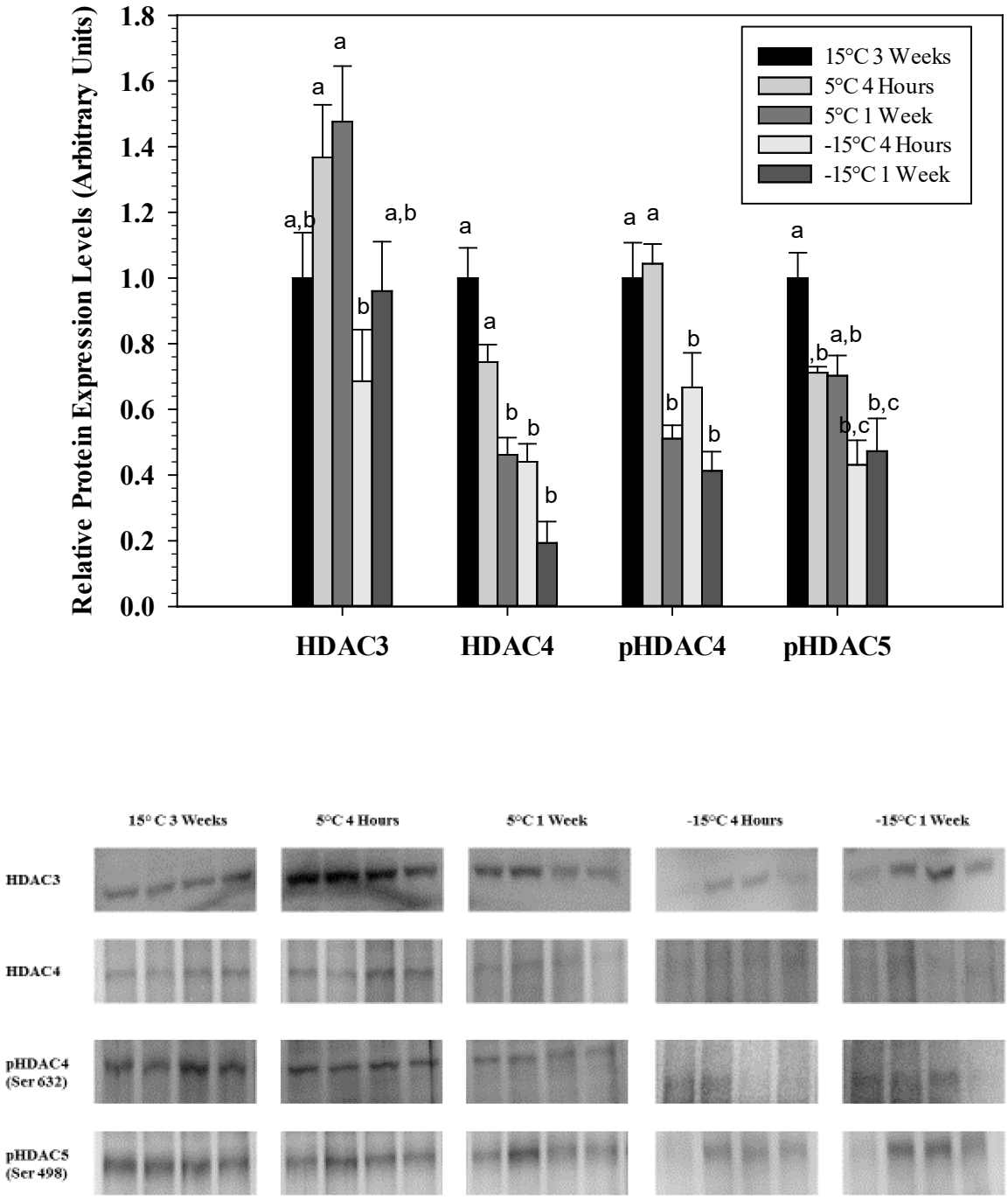

Figure 15: HDAC3, HDAC4, pHDAC4 (Ser 632), and pHDAC5 (Ser498) expression levels in *E. solidaginis* under five conditions: 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

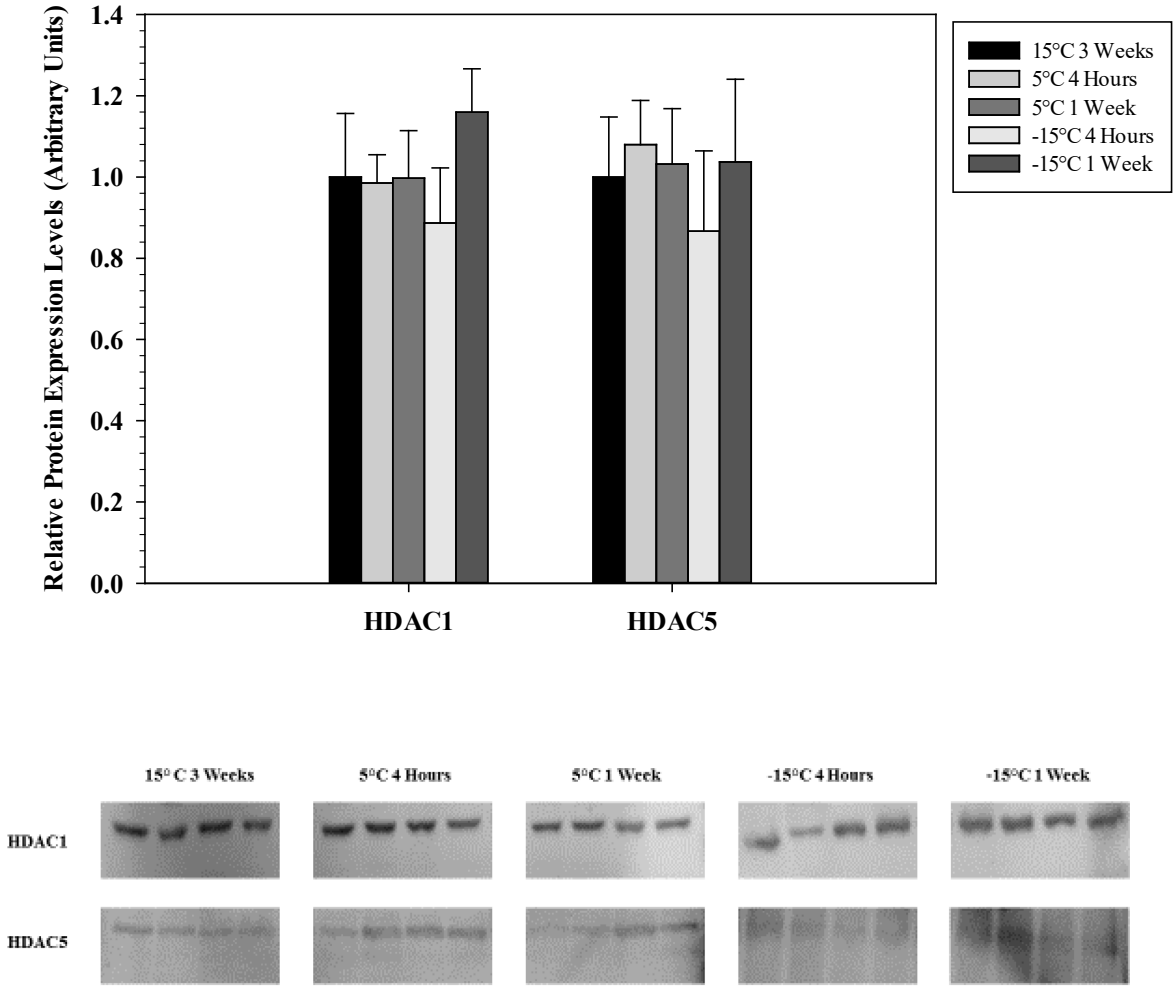

Figure 16: HDAC1 and HDAC5 expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were observed during any experimental condition.

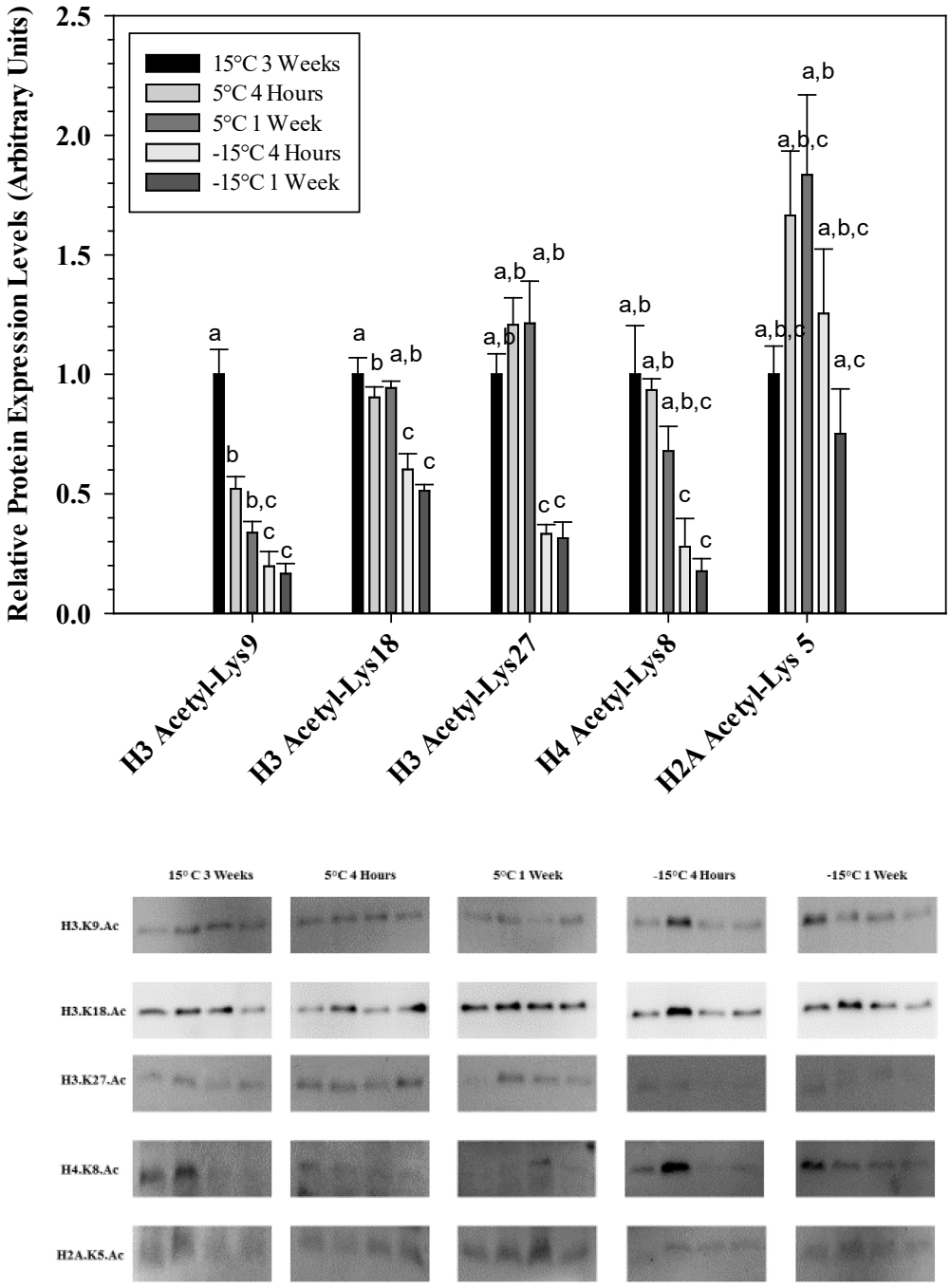

Figure 17: Relative acetylation levels of histones in *E. solidaginis* comparing H3.K9, H3.K18, H3.K27, H4.K18, and H2A.K5 in the larvae at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

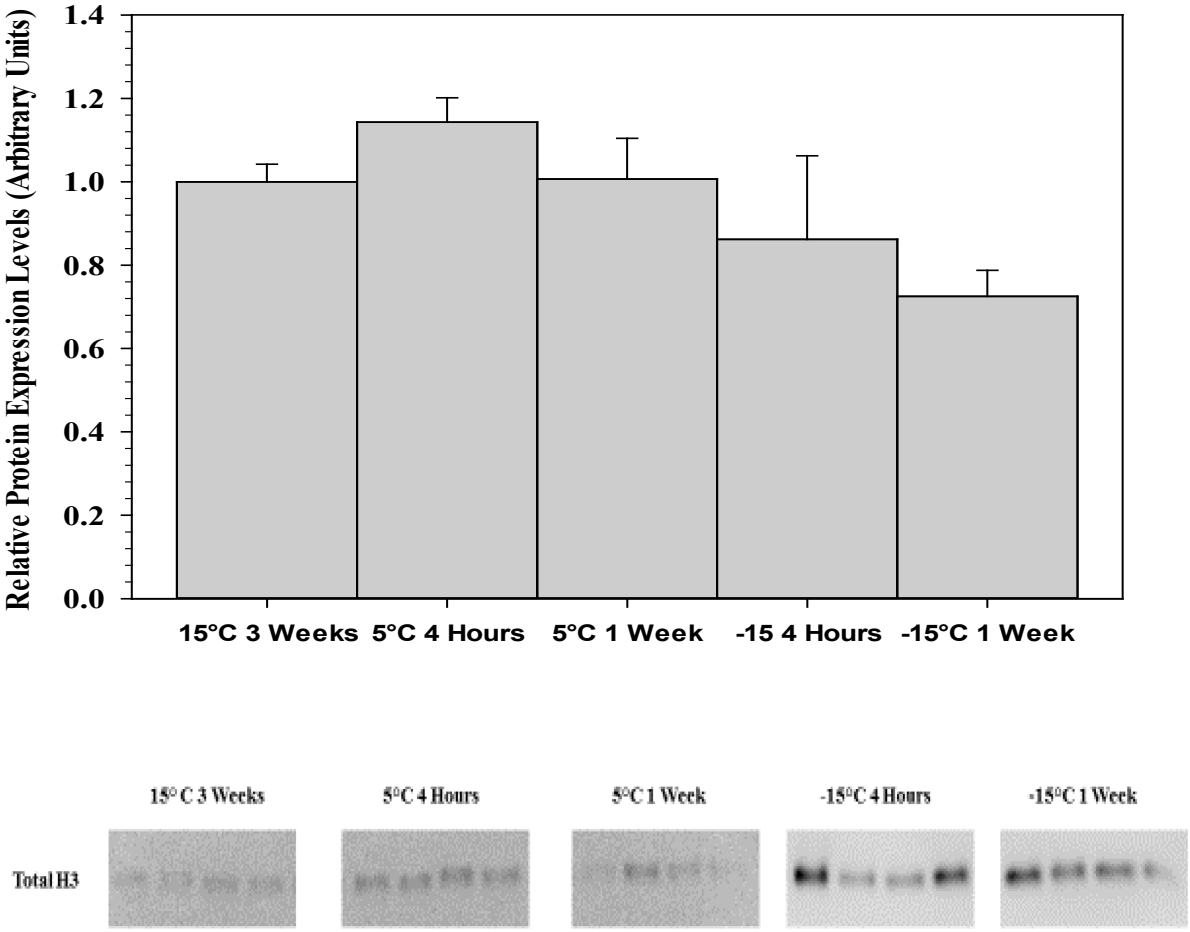

Figure 18: Total H3 acetylation expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were found between any of the experimental groups.

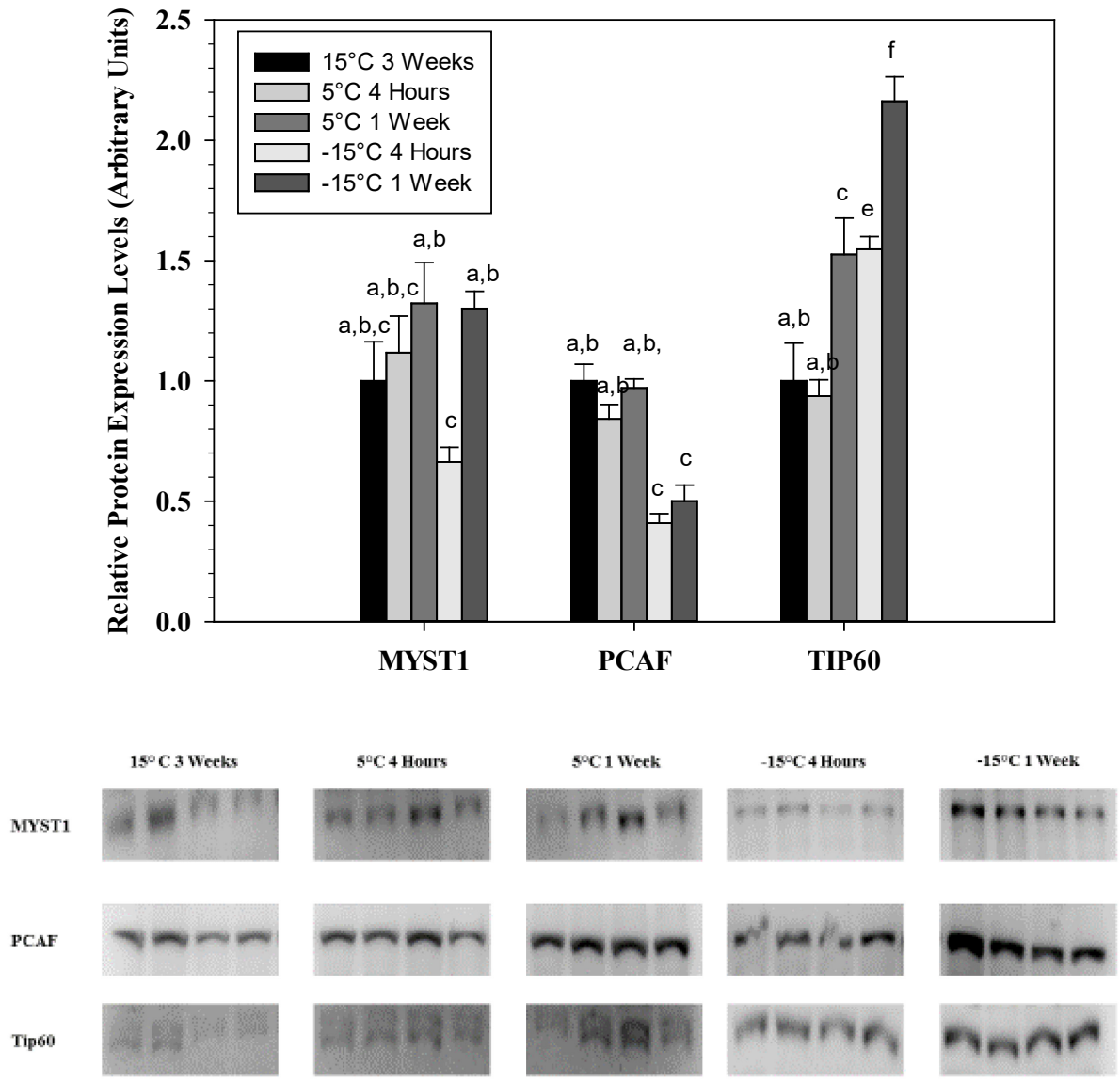

Figure 19: MYST1, PCAF, and Tip60 expression levels in *E. solidaginis* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

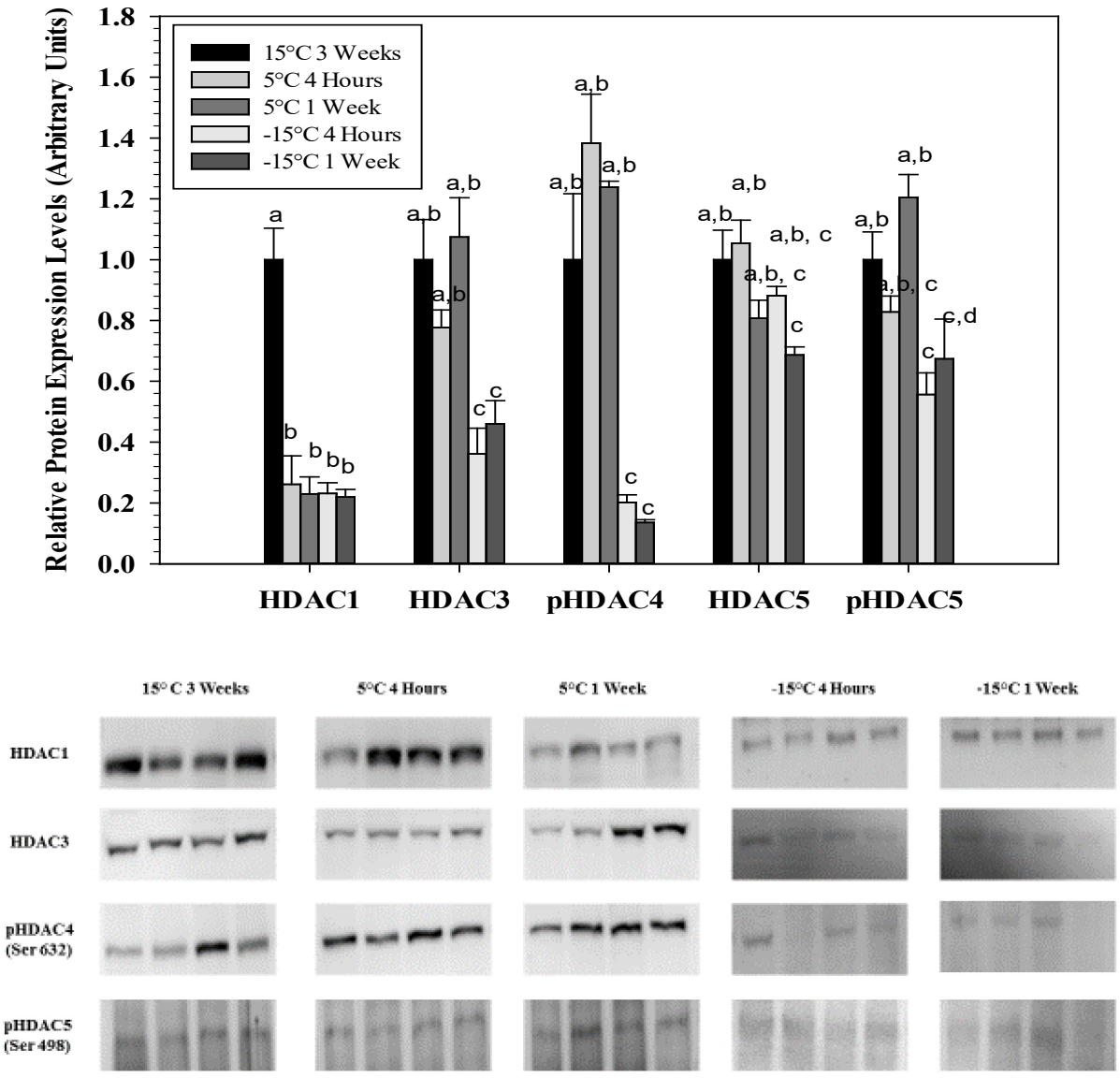

Figure 20: HDAC 1, HDAC3, pHDAC4 (Ser632), HDAC5, and pHDAC5(Ser498) expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

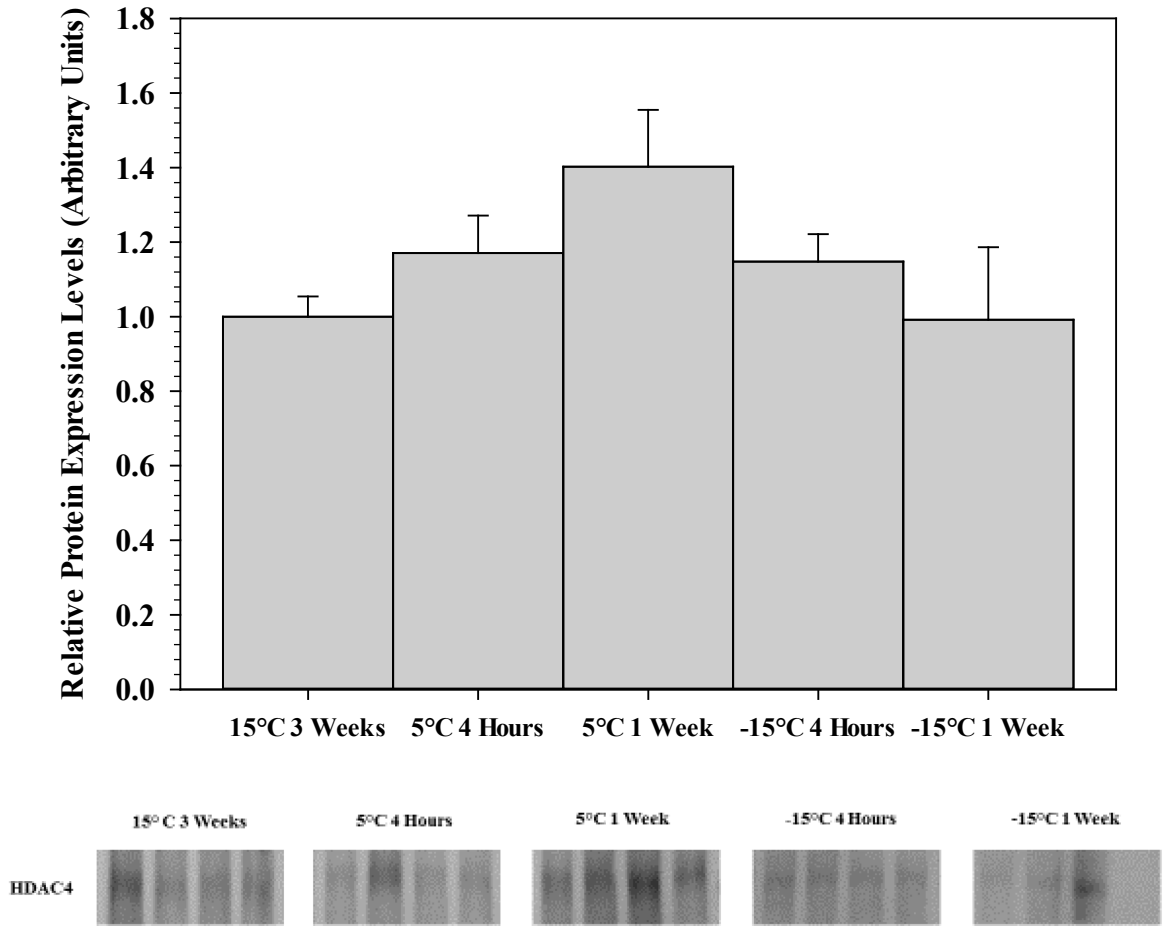

Figure 21: HDAC4 expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were found between any of the experimental groups.

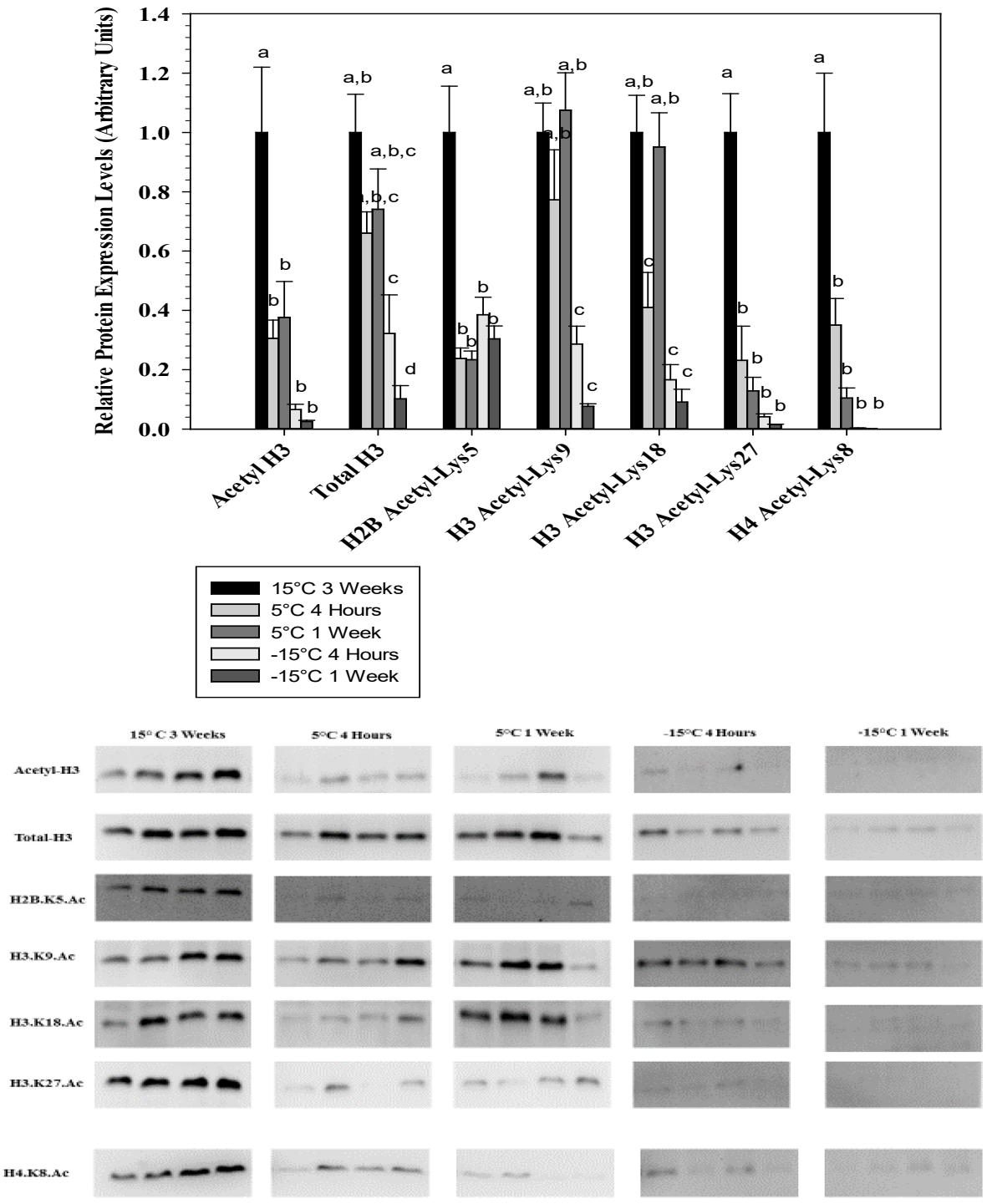

Figure 22: Relative expression levels of Acetyl-H3, Total H3 and acetylation at specific lysine residues (H2B.K5, H3.K9, H3.K18, H3.K27, H4.K8) in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

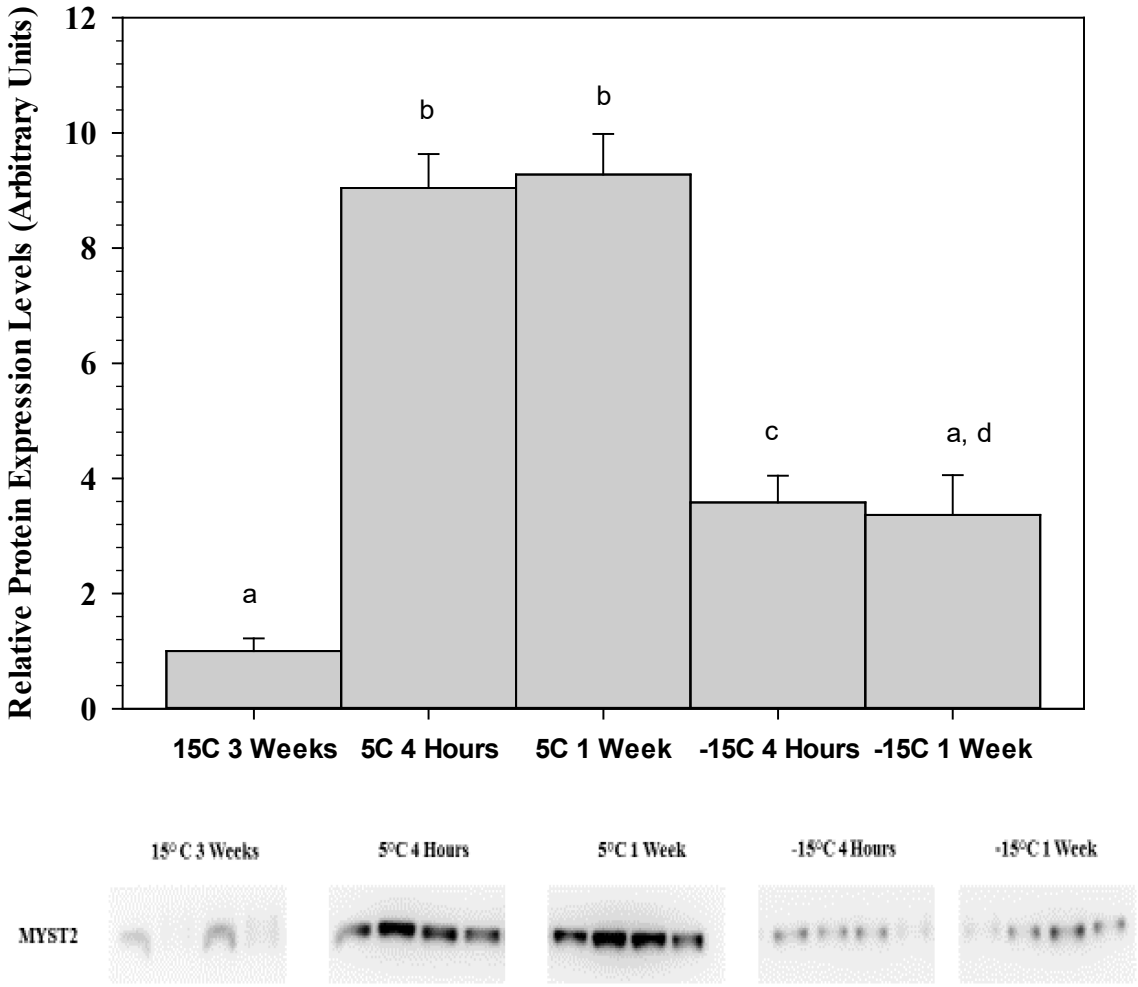

Figure 23: Relative MYST2 expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data were analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). Values that are not statistically different from each other share the same letter notation.

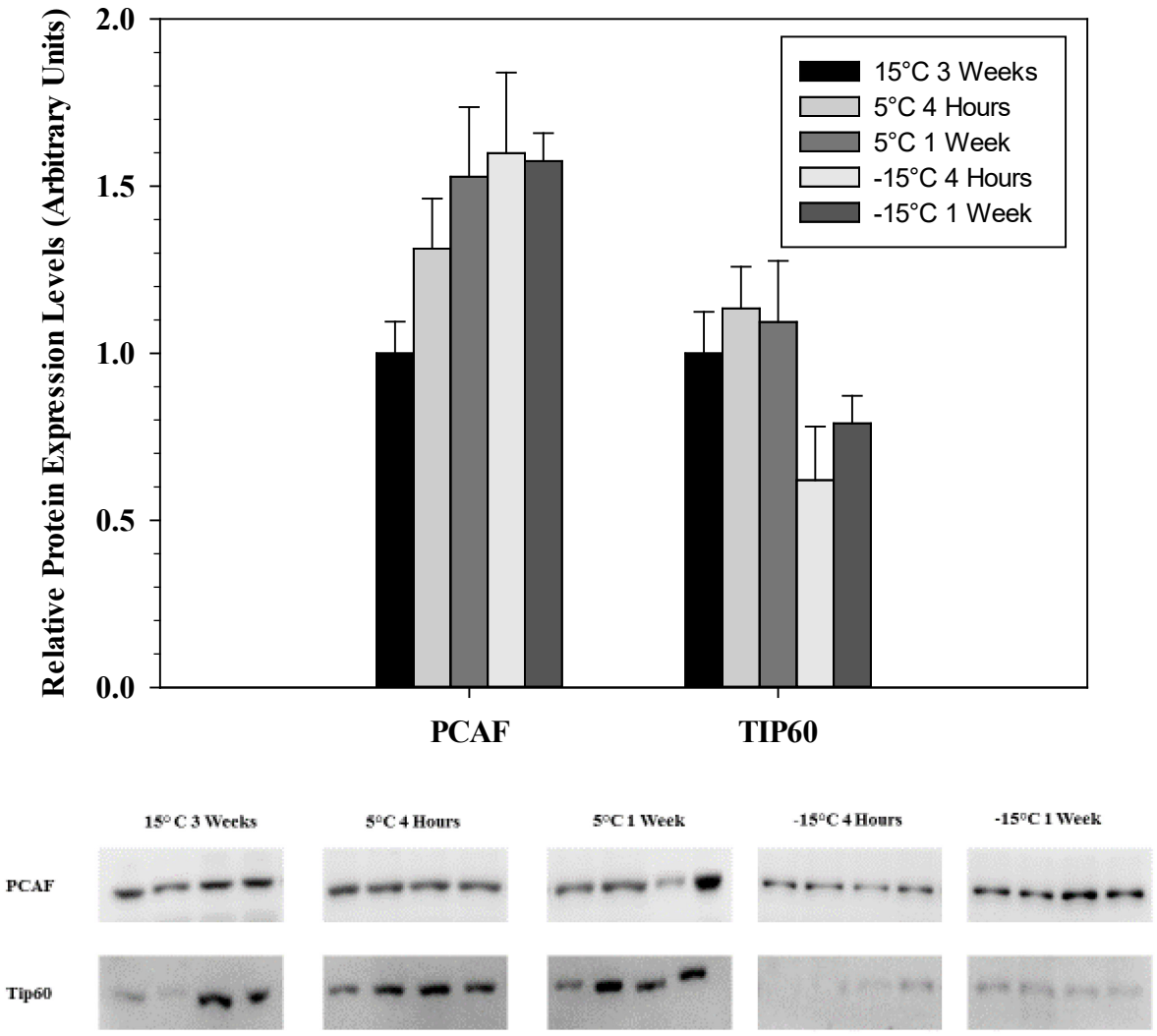

Figure 24: PCAF and Tip60 expression levels in *E. scudderiana* at 15°C 3 Weeks, 5°C 4 Hours, 5°C 1 Week, -15°C 4 Hours and -15°C 1 Week as determined by Western Immunoblotting. Histograms display mean band densities (±S.E.M. n=4). Data was analyzed using an analysis of variance with a post hoc Tukey's test ($p < 0.05$). No significant differences were found between any of the experimental groups.

Appendix B: Western blotting conditions

Insect	Protein	µg	Gel %	Block	[1°]	Reactivity	[2°]
Epi	DNMT1	20	6	None	1:1000	Anti-Rabbit	1:8000
Epi	DNMT2	40	10	None	1:1000	Anti-Rabbit	1:8000
Epi	DNMT3A	25	8	None	1:1000	Anti-Rabbit	1:8000
Epi	DNMT3B	40	8	None	1:1000	Anti-Rabbit	1:8000
Epi	DNMT3L	20	10	None	1:1000	Anti-Rabbit	1:8000
Epi	MBD1	15	10	None	1:1000	Anti-Rabbit	1:8000
Epi	MBD2	20	10	None	1:1000	Anti-Rabbit	1:8000
Epi	$MeCP_2$	20	10	2.5% Milk	1:1000	Anti-Rabbit	1:8000
Eur	DNMT1	20	6	1% Milk	1:1000	Anti-Rabbit	1:8000
Eur	DNMT2	20	10	None	1:1000	Anti-Rabbit	1:8000
Eur	DNMT3B	25	8	1.25% Milk	1:1000	Anti-Rabbit	1:8000
Eur	DNMT3L	20	10	0.75% Milk	1:1000	Anti-Rabbit	1:8000
Eur	MBD1	30	8	0.5% Milk	1:1000	Anti-Rabbit	1:8000
Eur	MBD2	20	10	None	1:1000	Anti-Rabbit	1:8000
Eur	$MeCP_2$	20	10	None	1:1000	Anti-Rabbit	1:8000
Epi	TET2	20	8	None	1:1000	Anti-Rabbit	1:8000
Eur	TET2	20	8	None	1:1000	Anti-Rabbit	1:8000
Eur	TET3	40	6	1.00% Milk	1:1000	Anti-Rabbit	1:8000

Appendix C: Loading control for western blotting

The method for standardizing loading for western blots in this thesis is different from conventional methods that measure the expression of single housekeeping genes. The method here uses multiple protein bands from Coomassie-stained blots. The summed intensity of multiple bands from a single lane are used as representative measurement of the total amount of protein loaded in each lane. Electrochemical luminescence data for each protein expression is then standardized against the summed intensity of Coomassie bands in each lane. This method controls for the possibility of uneven protein loading, and the possibility that changes in expression levels are due to changes in the amount of protein loaded in each lane.

The figure shown below is a representative Western Blot plus its Coomassie-stained blot (**Fig. C1**) which was used to standardize protein loading for the *Eurosta solidaginis* DNMT3L 15°C 3 Weeks-5°C 4 hours-5°C 1 week protein condition shown in **Chapter 3, Figure 1.**

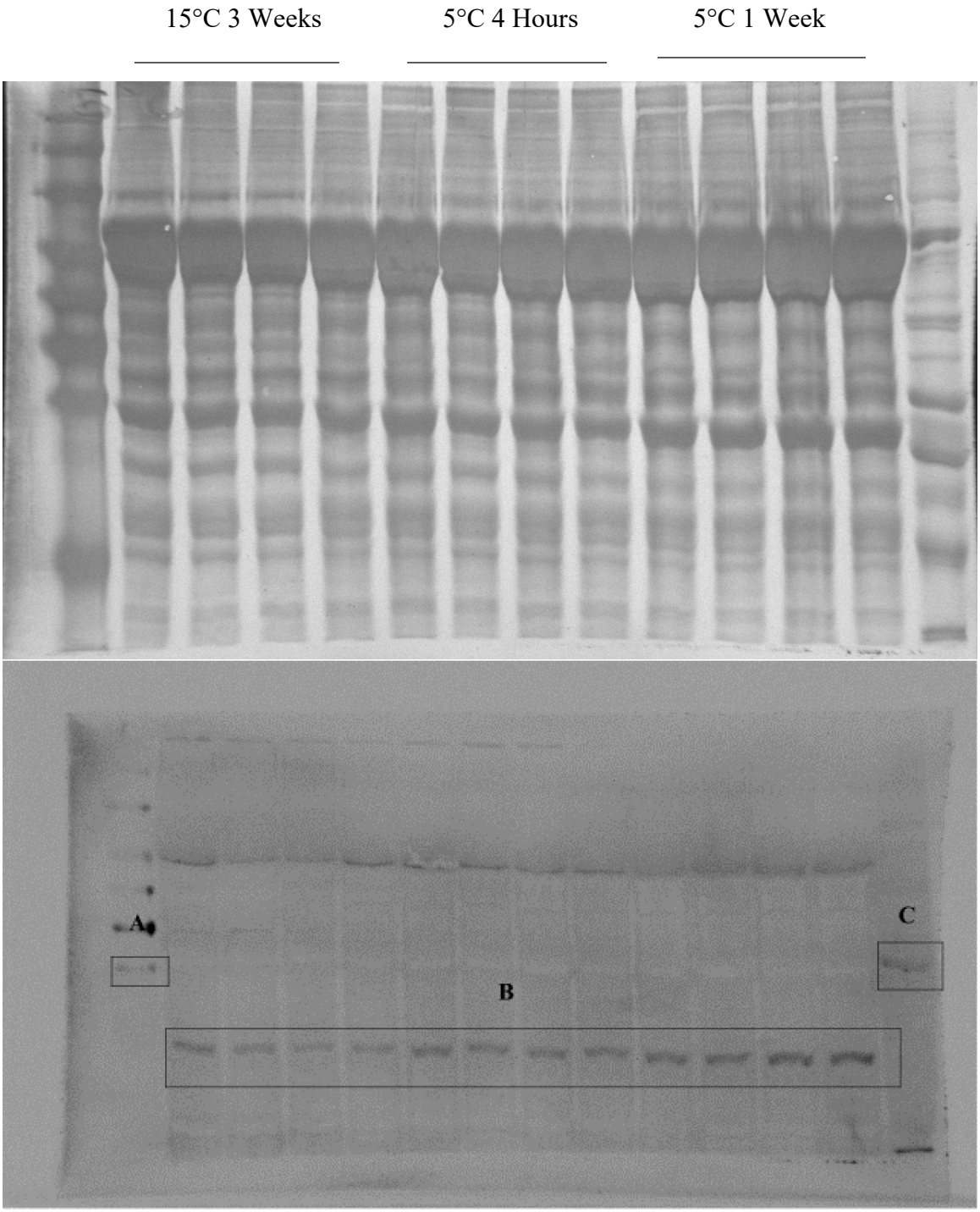

Figure C1. Coomassie stained blot (top) and corresponding western blot (bottom) for DNMT3L from *Eurosta solidaginis*. A) Molecular weight ladder 42 kDa. B) DNMT3L expression bands for 15°C 3 Weeks, 5°C 4 Hours, and 5°C 1 Week. C) Positive Control 13 Lined Ground Squirrel Liver Extract. Blocking was done with 5ml of 0.75% skim milk powder for 20 minutes.

Appendix D: Ensuring antibody specificity for western blotting.

Numerous steps were taken to ensure antibody specificity when performing western blotting (See Appendix C, page 147 for an example):

1. If necessary (when multiple bands were present in the molecular weight region where the protein was suspected to be present) blocking with skim milk powder in 0.5X TBST prior to antibody probing was used in order to ensure that only one band appeared at the molecular weight of the protein being probed.
2. Blots were run (at least once) with a mammalian positive control extract (13-lined ground squirrel) to ensure that the antibodies were crossreacting.
3. If the antibody company supplied the sequence of the epitope that the antibody recognizes, that sequence was aligned in multiple species to see whether the sequence is expected to be conserved in *Eurosta solidaginis* and *Epiblema scudderiana.*