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Laura Vesala

Environmental Factors Modulating Cold Tolerance, Gene Expression and Metabolism in *Drosophila montana*

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To the memory of my late grandmother Taimi

ABSTRACT

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Yhteenveto: Ympäristötekijöiden vaikutus *Drosophila montana* -mahlakärpäsen kylmänkestävyyteen, siihen liittyvien geenien toimintaan ja metaboliaan
Diss.

Abstract. The ability of insects to survive and reproduce at high latitudes depends largely on their capability to tolerate high daily and seasonal variation in environmental conditions. The studies included in my thesis show that the northern *Drosophila montana* flies are highly cold tolerant and that they also show considerable plasticity in this trait. Cold-acclimation is induced in these flies by low temperature and short day length, or a combination of these factors. The effects of cold acclimation were detectable both at phenotypic and gene expression level as well as in metabolism. Cold acclimation increased the expression level of *Hsp* genes probably to control the damage caused by low temperature. Also circadian clock genes (e.g. *period* and *vrille*) were either up- or downregulated during cold acclimation indicating pleiotropic function of these genes and/or the involvement of circadian clock in cold acclimation. Expression level of a putative cold-tolerance gene, *regucalcin,* was not affected by cold acclimation or short day length *per se*, but it was found to be more than two times higher in diapausing than in non-diapausing females. Maintaining *D. montana* flies in natural-like thermo- and photoperiods corresponding to seasonal changes in northern Finland showed that the cold tolerance of the flies increased and they began to accumulate sugars glucose and trehalose and amino acid proline in late summer. Highest increase was detected in the concentration of *myo*-inositol in the winter, which is likely to be the main cryoprotectant of *D. montana*. Overall, my studies revealed several mechanisms that enable *D. montana* flies to survive over the winter conditions in the northern temperate zone and emphasized the importance of using feasible study methods and conditions in cold tolerance studies. Information gained can be used, together with molecular tools being developed for the species, to study the evolution of seasonality and overwintering strategies in cold-adapted *Drosophila* species.

Keywords: Cold tolerance; *Drosophila montana; D. virilis;* gene expression; metabolomics; phenotypic plasticity; seasonality.

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LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original papers, which will be referred to in the text by their Roman numerals I-IV. I have been involved in the planning and execution of all studies. I have collected the data for all studies (I – IV) and conducted the lab work in all studies except the microarray part in study II and metabolic analyses in study III. I also conducted the data analyses, except for the microarray data in study II, and participated in qPCR analyses in studies II & IV.

I Vesala, L. & Hoikkala A. 2011. Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of *Drosophila montana*. Journal of Insect Physiology 57: 46-51.

II Vesala, L., Salminen, T.S., Laiho, A., Hoikkala, A. & Kankare, M. Cold tolerance and cold-induced modulation of gene expression in two *Drosophila virilis* group species with different distributions. Insect Molecular Biology, in press.

III Vesala, L., Salminen, T.S., Koštál, V., Zahradníčková, H. & Hoikkala, A. Overwintering-related changes in cold tolerance, metabolomic profiles, and *myo*-inositol accumulation in a cold-adapted drosophilid fly, *D. montana.* Manuscript.

IV Vesala, L., Salminen, T.S. Kankare, M. & Hoikkala, A. Photoperiodic regulation of cold tolerance and expression levels of *regucalcin* gene in *Drosophila montana.* Manuscript.

1 INTRODUCTION

1.1 Low temperature tolerance

Abiotic factors interact with biotic factors in shaping the life forms on this planet. One of the most important abiotic factors affecting species distribution is temperature (Andrewartha & Birch 1954, Chown & Gaston 2000, Calosi et al. 2010), which has pronounced effects especially on species that are not able to regulate their body temperature. These ectothermic organisms are directly exposed to ambient temperature conditions in their environment and have, not surprisingly, evolved several strategies to cope with them. Traditionally, the insects have been divided into two main categories on the basis of their response to sub-zero temperatures: freeze-tolerant and freeze intolerant (Salt 1961). According to this classification, freeze-tolerant insects can withstand freezing of their body fluids to some degree by restricting the formation of ice in the extracellular compartments, while freeze-intolerant (or freeze avoiding) insects utilize different ways to keep their body fluids unfrozen. Although useful, this classification does not give the whole picture on a vast variety of strategies adopted by insects to survive through the unfavorable cold periods that they may encounter. Actually very few species fall directly into either of these categories (e.g. Bale 1993), and new categories have been suggested to better cover the range of cold tolerance types of insects (Bale 1996, Sinclair 1999), mainly by adding new subgroups of freeze-intolerance types to the continuum.

The types of cold injury that the organism may suffer can be divided into three classes according to the length of the cold exposure and the mechanisms behind the injuries (Bale 2002, Lee 2010). *Freezing injury* is caused by internal ice formation, i.e. transition of water to ice inside the insects' body. In addition to freezing injury, a brief exposure to suboptimal low temperature (cold shock) can cause *direct chilling injuries*, and a prolonged exposure to this kind of temperature can lead to accumulation of *indirect chilling injuries*. Also a fourth, less studied, category has been suggested to describe the injuries that are

manifested in later phases in the insect life-cycle, e.g. deficiencies in reproduction or a failure to complete the full life-cycle (Turnock & Bodnaryk 1991).

Cell membranes may suffer from damage caused by both a short and a prolonged exposure to low temperature (i.e. direct and indirect chilling injuries). In both cases the low temperature can cause transition from a natural, liquid crystalline phase to a rigid gel phase (Drobnis et al. 1993, Hazel 1995), which further leads to a loss of the natural selective permeability of the membranes and hinders the functions of membrane-bound enzymes. Cell membrane integrity is essential for the maintenance of ion regulation; for example Koštál et al. (2004, 2006) have found that chilling injury can lead to the loss of ion homeostasis through movement of sodium and potassium ions down their electrochemical gradients in both the fire-colored beetle (*Pyrrhocoris apterus*) and the tropical cockroach (*Nauphoeta cinerea*). The disruption of metabolic functions during cold exposure has also been suggested to lead to an accumulation of reactive oxygen species (oxidative stress) and chilling injury in the house fly (*Musca domestica*) (Rojas & Leopold 1996).

1.2 Physiological adaptations to low temperatures

Adaptations to counter-act the injuries resulting from low temperature are as various as the types of insects encountering them. Freeze-tolerant insects may synthesize ice-nucleating agents (INAs) to survive in cold temperature. These agents initiate ice formation in extracellular spaces, leaving important intracellular compartments ice-free (e.g. Duman et al. 1985, Lee 1989). On the other hand, insects that cannot withstand freezing may try to get rid of all material that could enhance the formation of ice by cessating feeding (Worland & Lukešová 2000) or emptying the contents of their gut (Cannon & Block 1988).

A wide range of organisms, from plants to insects, synthesize and accumulate various kinds of low molecular weight compounds, such as polyhydric alcohols (polyols), sugars and some of the amino acids. These compounds are called cryoprotectants due to their many functions in improving low temperature resistance. Cryoprotectants act, for example, in stabilization of proteins and cell membranes and in keeping the body fluids unfrozen (supercooled) at low temperatures (Storey & Storey 1991). Also the synthesis of specific antifreeze (or thermal hysteresis) proteins, whose main task is to inhibit the growth of ice lattice, has been reported in some insects during unfavorable thermal conditions (Zachariassen & Husby 1982, Duman & Howarth 1983). Another group of proteins, which has been associated with low temperature stress, are heat shock proteins (Hsp). The main task of these proteins is to handle misfolding or aggregation of other proteins caused by extrinsic or intrinsic stress (Parsell & Lindquist 1993). Heat shock reaction, as a general stress response, may be induced by many kinds of environmental stresses, including heat, heavy metal and desiccation stress (reviewed in Feder

& Hofmann 1999). Heat shock response during cold exposure has been reported e.g. in Colorado potato beetle (*Leptinotarsa decemlineata*) and in the mosquito *Culex pipens* (Yocum 2001, Rinehart et al. 2006). Also in *Drosophila melanogaster* several *Hsp* genes have been found to be upregulated during cold stress and/or during recovery from it (Colinet et al. 2010).

To deal with the phase transitions in the cell membranes during low temperature, insects can remodel the fatty acid composition of the membrane (e.g. Hazel 1995). This is achieved perhaps most commonly by increasing the proportion of unsaturated glycerophospholipid fatty acyl side chains to saturated fatty acids, which leads to a broader functional temperature range. Changes in fatty acid composition represent only one of the many ways in which the cell membrane composition may be modified to improve cold tolerance. Other methods include changes in phospholipid headgroups and cholesterol content (Hazel 1995, Overgaard et al. 2005, Tomcala et al. 2006).

1.3 Phenotypic plasticity in cold tolerance

Phenotypic plasticity is an ability of a single genotype to produce multiple phenotypes in response to external stimuli. Phenotypic plasticity can be categorized to different types: developmental plasticity is irreversible change that occurs during the development of an individual in response to environment, but plasticity is also often a reversible response within an individual, which can be referred to as phenotypic flexibility (Piersma & Drent 2003). Overall, phenotypic plasticity can be adaptive, maladaptive or a neutral response to a changing environment (e.g. Ghalambor et al. 2007). When plasticity is adaptive, it allows an individual to cope with a broader spectrum of environmental conditions and thus have a higher fitness across multiple environments (Bradshaw 1965, Levins 1968, Baker 1974, Pigliucci 2001). Along with genetic adaptation (changes in allele frequencies), phenotypic plasticity can also help the organisms to invade new territories and get adapted to new kinds of environmental conditions (Baker 1974, Robinson & Dukas 1999, Ghalambor et al. 2007).

Temperature-induced plasticity in insect cold tolerance has been studied in several species by rearing (developmental acclimation) or pre-exposing the insects to moderately low temperature before testing their cold tolerance. Usually the term "cold acclimation" is used for a response that occurs over a long time span (days, weeks), like seasonal cold acclimation in the autumn. In cold acclimation physiological changes including cryoprotectant accumulation and cell membrane modifications lead to improved cold tolerance (reviewed in Lee 1991). A more rapidly occurring increase in cold tolerance is called rapid cold hardening (RCH), which launches largely unknown physiological chances in minutes to hours leading to an increased cold tolerance. RCH response was first described in flesh flies (*Sarchophaga crassipalpis*), where cooling at 0 °C prior to exposure to -10 °C increased survival almost 100 % (Chen et al. 1987). After

that, RCH response has been described in several insect species (e.g. Czajka & Lee 1990, Kelty & Lee 2001, Worland & Convey 2001, Terblanche et al. 2010), suggesting that it is a relevant factor helping insects to survive at low temperatures. For example, in *S. crassipalpis* rapid cold hardening occurs, at least partly, through increased accumulation of glycerol and in *D. melanogaster* through changes in cell membrane phospholipid composition (Overgaard et al. 2005). In the Antarctic midge (*Belgica antarctica*) rapid cold hardening response involves an increase in calcium flux involved in cold sensing (Teets et al. 2008).

1.4 Forecasting seasonal changes in environmental conditions

Changes induced by the prevailing temperature conditions in the traits increasing insect cold tolerance are immediate or at best predictive to cooler temperature that will shortly follow. However, in a seasonally varying environment the insects may also prepare for a cold period on a longer time scale, when the temperature is still quite high. The shortening day lengths in late summer offer a reliable cue for tracking seasonal changes and forecasting the forthcoming cold period in advance. The type of phenotypic plasticity where a certain phenotype occurs always at a certain time of the year (such as diapause), can be termed life-cycle staging (Jacobs & Wingfield 2002, Piersma & Drent 2003).

Circadian rhythms (24-hour rhythms) in both behavioral and physiological traits occur in almost all organisms and are controlled by specific genes comprising the circadian clock system (Hall 2003). Also stress resistance traits are known to show resistance peaks and pits following roughly the 24-hour rhythm regulated by the circadian clock. Examples of stress traits showing cyclic 24-hour patterns include resistance to oxidative stress in *D. melanogaster* (Krishnan et al. 2008), resistance to the insecticide permethrin in *Aedes aegypti* (Yang et al. 2010) and resistance to osmotic and oxidative stress in *Caenorhabditis elegans* (Simonetta et al. 2008). Accumulation of information on the length of the light or dark period of the day also helps the organisms to keep up with the seasonal cycles (photoperiodism) and to time changes in important life history traits correctly (Bradshaw & Holzapfel 2007). The mechanisms behind the "photoperiodic counter" system are not well understood, and the topic has been under intense debate since Bünning (1936) proposed that the circadian clock forms the basis for the photoperiodic timer.

Adaptive significance of insects' ability to use photoperiod as a token signal to initiate physiological processes is emphasized at high latitudes where correct timing of reproduction, for example, can be critical (Bradshaw & Holzapfel 2007). A well-known example of physiological responses to photoperiod is photoperiodic diapause, which in many species in the northern temperate zone is launched well before winter, as a response to short day lengths (Danks 1987, 2006). Also insect cold tolerance has often been found to increase in autumn along with a shortening day length and a decreasing

temperature, but the mechanisms behind this process may vary largely between the species.

1.5 Aims of the thesis

The main aim of my doctoral thesis was to find out how a *Drosophila* species, which has distributed around the northern hemisphere, has adapted to a seasonally varying environment. More precisely, I studied how the flies of this species can use changes in temperature (I, II) or photoperiod (I, IV), or their combination (III), as real-time or anticipatory cues to forecast a forthcoming cold period and prepare for it. The main study species, *Drosophila montana* (Fig. 1), has a circumpolar distribution range (north from 40°N; Watabe & Higuchi 1979, Throckmorton 1982) and it is well-adapted to live in seasonally varying environmental conditions. As a comparison, I have used *D. virilis,* which belongs to the same *D. virilis* group as *D. montana*, but is mainly a human commencial and has a distribution range south from 35°N (Patterson 1942, Throckmorton 1982). Previously, the effects of cold acclimation, and to some extent also the direct and indirect effects of photoperiod, have been studied using other *Drosophila* species, which are not well-adapted to low temperatures and/or drastically changing photoperiods. Thus this study, together with the studies on other northern insect species, will offer new perspectives on the evolution of insect cold tolerance.

The first aim of the thesis was to develop a basis for studying cold tolerance of *D. montana* flies, as this species tolerates much lower temperatures than most *Drosophila* species studied so far. I also aimed to separate the direct and indirect (through diapause) effects of photoperiodic cues on female cold tolerance by maintaining the flies in an intermediate photoperiod, producing both diapausing and reproducing females. This study was done using isofemale strains from different geographical origins to see whether the cold tolerance is of about the same level in different populations.

The second aim was to trace the expression changes launched by a low temperature exposure in a set of 219 genes during cold treatments among non-diapausing females. In this experiment I used *D. virilis* females in addition to *D. montana* to determine whether the cold tolerance of these species will increase through cold acclimation and rapid cold hardening. I also studied whether the species share common gene expression responses during cold acclimation and RCH and searched for candidate genes for cold acclimation, RCH and the recovery from cold stress.

The third aim was to investigate the combined effects of fluctuating thermo- and photoperiod on the cold tolerance of *D. montana* flies by maintaining the flies around "an artificial year" in conditions that closely resemble the conditions in the flies' natural habitat in northern Finland. Here the aim was not only to study seasonal variation in cold tolerance, but also to

link it with metabolic changes taking place in the flies around the year. Exposing the flies to fluctuating conditions was expected to produce stronger, and also more natural, responses than the basic laboratory-treatments with constant temperatures and photoperiods.

Finally, I aimed to elucidate the role of photoperiodic cues in cold tolerance by maintaining the flies in several constant photoperiods. Two of the photoperiods produced both diapausing and non-diapausing females, which enabled the estimation of the direct effects of diapause on cold tolerance without the effects of day length. The study was performed using two *D. montana* strains, one with a robust photoperiodic diapause and one lacking this response. I also traced changes in one recently identified candidate gene for seasonal cold adaptation, *regucalcin,* under different photoperiods to find out whether the expression of this gene is linked with photoperiod, or with photoperiodic diapause.

FIGURE 1 *Drosophila montana* male (left) and female (right) anesthetized and seen through a microscope lens. (Photo: Tiina S. Salminen)

2 MATERIAL AND METHODS

2.1 Study species

My main study population of *D. montana* is located in Oulanka, northern Finland (66°22'N, 29°19'E). This population is close to the northernmost location (70°10′N), where a natural population of this species has been found (Laaksonen & Lumme 1983, Tyukmaeva et al. 2011). The flies of this population are exposed to constant light for over two months in the summer, and to constant darkness during long, dark and cold winters. The flies overwinter at an adult stage, but their exact overwintering sites are not known. They are likely to seek hiding places under the snow cover, where the temperature does not reach sub-zero, but in the autumn and spring the flies are frequently exposed to freeze-thaw cycles. In late summer *D. montana* females enter reproductive diapause as a response to changes in photoperiodic cues (short day lengths; Lumme 1978), the critical day length for diapause differing among populations from different latitudes (Tyukmaeva et al. 2011). In addition to the main study population in experiment I, also flies from a high-altitude population of *D. montana* collected from Crested Butte (Colorado, USA, 39 ºN, 3000 m altitude) and a low-altitude population of this species collected from Vancouver (Canada, 49 ºN) were used.

Wild populations of *D. virilis* have been found at least in eastern Asia (Tan et al. 1949, Okada 1956) but in Japan, from where the strain used in experiment II originated, *D. virilis* is mainly found in places related to human activities, such as timber yards and breweries (Ohba 1970, Tsuno 1975). *D. virilis* lacks an ability to enter photoperiodic reproductive diapause and is unlikely to be able to overwinter in nature at high latitudes (Watabe 1983).

All of the flies used in the experiments were maintained in constant light, 60 % humidity and at 19 °C since the time they were collected. These conditions were used to prevent the induction of diapause. The flies were maintained in half-pint plastic bottles containing a malt medium (sugar, baker's yeast, malt, semolina, agar and Nipagen as a preservative, according to Lakovaara 1969).

2.2 Phenotypic measurements

2.2.1 Cold tolerance

Since *D. montana* flies overwinter as imagoes, the most relevant stage for measuring their cold tolerance is the adult stage. In my thesis project I have measured adult cold tolerance under different temperatures and photoperiods (see Table 1 for the conditions) using two different metrics: the chill coma recovery time method (studies I, II, III and IV) and the survival after a cold exposure (II and III).

When an insect encounters a sufficiently low, but not yet lethal, temperature they exhibit a state called chill coma, which is defined by an absence of nerve impulses (Goller & Esch 1990). In chill coma an insect is not able to move, but this is recovered when the insect is brought back to room temperature (e.g. David et al. 1998), which gives a possibility to measure the chill coma recovery time (CCRT). After searching for suitable temperature conditions for measuring CCRT in *D. montana*, I ended up with keeping the flies for 16 hours at -6 °C (I, II, III and IV) and at -7 °C (I) in vials containing agar medium for moisture. After 16 hours in the above mentioned temperatures the flies were brought into room temperature and placed on dishes with lids and separate compartments for individual flies, and chill coma recovery times were measured.

Survival/mortality was defined after keeping the flies at -10 °C (I) or at -11 °C (I, III) for 24 hours. The flies were then placed into vials with food medium and left to recover from the cold treatment for another 24 hours at room temperature. If a fly was able to respond to a slight agitation with forceps after the recovery period it was considered as survived from the exposure. The cold exposures used in the experiments are likely to cause both acute and chronic chill injuries (Sinclair & Roberts 2005, Lee 2010).

2.2.2 Reproductive diapause

I defined the diapausing state of female flies based on the classification by Lumme (1978). The females were dissected under a microscope and the development stage of the ovaries was categorized in three groups based on their appearance. Ovaries of a diapausing female are small, no yolk has accumulated and no segments can be seen. When ovaries start to develop, yolk starts to accumulate into the forming egg chambers (intermediate) and finally, vitellogenesis is completed and mature eggs are easily seen (non-diapausing).

TABLE 1 The experimental conditions and treatments used in the studies I-IV. All fly strains have been maintained at 19 °C and constant light since their establishment. Experimental temperature refers to the temperature in which the experimental flies were transferred and maintained after eclosion and in which the different photoperiodic regimes were applied (in study IV the experimental flies were reared already from the egg stage in the experimental temperature). Photoperiod refers to the day length in which the experimental flies were maintained and temperature treatment refers to rapid cold hardening (RCH) or cold acclimation (CA) treatments. In study III the flies were maintained in gradually changing thermo-and photoperiods mimicking the seasonal conditions in northern Finland. RCH = rapid cold hardening, CCRT = chill coma recovery time.

	I	**II**	**III**	**IV**
Rearing temperature	19 °C	19 °C	19 °C	15.5 °C
Experimental temperature	16.5 °C	19 °C	various	16 °C
Photoperiod	LD 22:2, LD 24:0, LD 16:8, LD 12:12	LD 22:2	various	LD 24:0, LD 22:2, LD 19.5:4.5; LD 18.5:5.5; LD 17:7, LD 16:8, LD 14:10
Temperature treatment	RCH 1 hour at 0°C	RCH 1 hour at 0°C; CA 6 days at +5 °C		
Cold tolerance test	CCRT 16 h at -6°C and -7°C; Mortality/survival after 24 h at -10 °C and -11°C	CCRT 16 h at -6 °C	CCRT 16 h at -6 °C Mortality/survival after 24 h at -11°C	CCRT 16 h at -6°C

2.3 Gene expression

2.3.1 RNA extractions

The modulations in gene expression patterns were studied in response to low temperature (II) and between diapausing and non-diapausing individuals in different day lengths (IV). The flies were exposed to experimental conditions, after which the individuals were snap-frozen in liquid nitrogen and stored at -84 °C until the RNA extractions.

In study II the RNA was extracted from pools of 10 female flies using Qiagen RNA extraction kit with RNase-Free DNase treatment according to the manufacturer's protocols (QIAGEN, Hilden, Germany). In study IV RNA was extracted from pools of 5 flies and a different RNA extraction protocol was adopted. The RNA was extracted using a modified Tri-reagent protocol followed by a clean-up step using RNeasy columns (RNeasy mini kit, Qiagen), with DNase treatment. The extraction followed Trizol-RNeasy RNA isolation of cultured cells (Genomics Laboratory, UMC Utrecht/MGK/Version 2.1, 18-7-2007; http://microarrays.holstegelab.nl/modx/assets/files/protocols/RNA-microarrays12.pdf) with a few modifications for using whole tissue as a starting material. After the extractions, the purity of RNA was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity was checked with Agilent's Bioanalyzer (Agilent, Santa Clara, CA, USA).

2.3.2 Primer design

Since there is no genome sequence information available for *D. montana*, primers for the gene sequences were designed using *D. virilis* and *D. melanogaster* genome data. In study II the 60-mer probes for the DNA-microarray were designed using *D. montana* sequences as described in Kankare et al. (2010). Shortly, in cases where the gene in question was not annotated in *D. virilis*, we obtained the *D. melanogaster* gene sequence from Flybase (Tweedie et al. 2009) and aligned it to the *D. virilis* genome using UCSC Genome Bioinformatics database (http://genome.ucsc.edu/). From this alignment, the most conservative regions were chosen and primers were designed to these regions either by eye or using Primer 3 software (version 0.4.0; http://frodo.wi.mit.edu/primer3/). Then, the region was sequenced from *D. montana* and aligned back to *D. virilis* and *D. melanogaster* genomes to ensure that the correct region was obtained and that it did not align to multiple locations in the *D. melanogaster* genome. In addition, cDNA sequences were obtained for every gene region to ensure that they are located in the coding regions of the *D. montana* genome. We constructed species-specific probes for all of the genes also for *D. virilis* and added the probes in both sense and antisense directions according to *D. virilis/D. melanogaster* sequences. In study

IV, qPCR primers were designed manually using *D. montana* sequences (Kankare et al. 2010). The 5′ and 3′ stability values and the presence of hairpins, dimers and repeats were checked using the NetPrimer program (http://www.premierbiosoft. com/netprimer/).

2.3.3 Candidate gene DNA microarray

Gene expression patterns were investigated using a candidate gene microarray constructed specifically for *D. montana*. The probes were designed as described above and synthesized *in situ* with liquid chemistry. The platform used was the Agilent 60-mer Multi-Pack Gene Expression Microarray, with one-color system (details in Kankare et al. 2010). The first version of the microarray included probes for genes from different annotation groups, potentially important in seasonal adaptation. These groups included diapause, phototransduction, courtship behaviour, circadian clock and genes involved in response to cold and heat. In the second version of the microarray (II) 118 genes were added for a total of 219 genes.

2.3.4 cDNA synthesis (RT-PCR)

Complementary DNA was generated from the RNA using iScript Reverse Transcription Supermix for qPCR (Bio-Rad Laboratories, Hercules, CA, USA) following the manufacturer's protocol. Prior to cDNA synthesis the RNA samples were diluted to equal concentrations of 400 ng/µl (II) or 100 ng /µl (IV) and 1 µl of these dilutions was used in the cDNA reactions. In addition to RNA, the 20 µl cDNA reaction included 5 µl of 5 x iScript reaction Mix, 1 µl of a unique mixture of random and oligo-dT primers, 1 µl of reverse transcriptase enzyme and dH_2O. The PCR cycling conditions were: 5 minutes at 25 ^oC, 30 minutes at 42 ^oC and 5 minutes at 85 ^oC.

2.3.5 Quantitative real time PCR (qPCR)

qPCR reaction mixtures contained 10 µl of 2x Power SYBR Green PCR Master Mix solution (Bio-Rad Laboratories), 0.3 µM of each gene-specific primer (10 µM), 1 µl of cDNA solution and dH_2O to fill up the total volume to 20 µl. Cycling conditions in Bio-Rad CFX96 instrument were: initiation at 95 °C for 3 min, followed by denaturation at 95 °C for 10 seconds, annealing at specific T_A (53-55 °C) for 10 seconds and finally extension at 72 °C for 30 seconds. The whole cycle of denaturation, annealing and extension was repeated 40 times, followed by melting curve analysis to check the purity of the qPCR reaction.

2.3.6 Metabolite analyses

The samples (pools of ten adult flies) for analyzing sugars, polyols, free amino acids and free fatty acids (III) were weighed using a Mettler Toledo XS204

analytical balance with precision of 0.1 mg and then homogenized in 70 % ethanol. The metabolite analyses followed protocols explained in detail in Koštal et al. (2007, 2011a and 2011b). After shaking for 10 minutes and centrifugation (22000 g at 25 °C) the supernatant was collected and 10 µl of xylitol was added as an internal standard. The extract was then purified using hexane and evaporated to dryness under a stream of nitrogen. After oximation (with dimethylformamide and *o*-methylhydroxylamine) and silylation (dimethylformamide and trimethylsilylimidazol) the aliquot containing sugars and polyols was re-extracted into 150 µl of isooctane. Other metabolites were analyzed in the same ethanolic extracts after they were treated with ethyl chloroformate under pyridine catalysis and simultaneous extraction into chloroform.

Sugars and polyols were analyzed by gas chromatography coupled to mass spectrometry (GC/MS) and the other metabolites were analyzed using a combination of GC/MS and LC/MS (liquid chromatography/MS) techniques.

3 RESULTS AND DISCUSSION

3.1 Effects of environmental conditions on cold tolerance

In the northern hemisphere, where environmental conditions are characterized by distinct seasons and long winter periods, plastic responses (both phenotypic flexibility and life-cycle staging) to environmental cues play a major role in survival and reproduction. Accordingly, it has been hypothesized that the organisms living in heterogeneous environmental conditions have evolved a high capacity to optimize their performance by fitting it with the prevailing and forthcoming environmental conditions (Chown & Terblanche 2007, Ghalambor et al. 2007). This capability can partly arise from high phenotypic plasticity and partly from genetic adaptation to thermally varying conditions.

The studies included in my thesis showed *D. montana* to be highly cold tolerant as well as highly plastic in their cold tolerance. For example, the chill coma recovery times of tropical populations of cosmopolitan *Drosophila* species after 16 hour at 0 °C fell between 0.6 and 58.8 minutes and those of temperate populations between 0.6 and 43.3 minutes (Gibert et al. 2001), while *D. montana* flies do not exhibit chill coma at all at 0 °C (non-acclimated *D. montana* flies recover on average in 6-10 minutes after 16 hours at -6 °C; I). Among the *Drosophila* species studied so far, only *D. subobscura*'s cold tolerance is of about the same level as that of *D. montana* and other northern *D. virilis* group species (Laaksonen & Lumme 1983, Gibert et al. 2001, David et al. 2003). The basal super cooling point (i.e. the freezing point of body fluids) of adult *D. virilis* group species flies, including *D. montana,* is around -20 °C, but all the (non-acclimated) flies die before reaching this point (Lumme 1980). We found the mortality of non-acclimated flies to be over 90 % after 24 hours at -11 °C (I, III). This indicates that *D. montana* flies survive low temperatures by avoiding freezing. Freezing avoidance strategy is more common among terrestrial insects than the freeze-tolerance strategy in the Northern hemisphere (Block 1991, Sinclair et al. 2003).

At high latitudes, photoperiod provides a reliable cue for forthcoming changes in environmental conditions. Many insects occupying these areas are

able to detect and accumulate information on the day length and use it to adjust e.g. their development time (Nylin 1989) and reproduction (Danks 1987) according to the prevailing seasons. Studies I, III and IV clearly show that the shortening days (corresponding to late summer) enable *D. montana* flies to prepare for the winter by entering reproductive diapause and by increasing their cold tolerance through various kinds of physiological and metabolic changes. In the species of *Drosophila obscura* group, cold tolerance has been found to correlate with the species' overwintering strategy; species possessing adult reproductive diapause being generally the most tolerant (Goto et al. 1999).

The effects of photoperiod on the cold tolerance of *D. montana* flies could be partly due to direct effects of day length on cold tolerance and partly due to photoperiodic reproductive diapause triggered by short day length. We attempted to separate the effects of these two factors by maintaining the flies under intermediate day lengths producing both diapausing and reproducing females (I & IV). In study I the effects of diapause on female chill coma recovery times were significant in three out of six studied *D. montana* strains, while in study IV the increased cold tolerance appeared to be due to the effects of the day length or to an interaction between the day length and diapause rather than to diapause *per se*. However, chill coma recovery times and mortality after a cold exposure may not reveal all aspects of cold tolerance that are linked with diapause. Diapausing females feed less than the non-diapausing flies and they do not have eggs in their ovaries, which decreases the amount of material susceptible to freezing. This could explain why only diapausing females succeed to overwinter in northern Finland (see Aspi et al. 1993).

Several recent studies have focused on the role of temperature-induced phenotypic plasticity (acclimation) in thermal tolerance (Fisher et al. 2011, Nyamukondiwa et al. 2011, Overgaard et al. 2011). Cold acclimation and rapid cold hardening (RCH) can be viewed as two separate mechanisms preparing the insects for longer term (cold acclimation) or short term (RCH) changes in the temperature conditions, but they can also be seen as opposite ends of an acclimation response (Loeschcke & Sørensen 2005, Sinclair & Roberts 2005). Cold acclimation (6 days at 5 °C) decreased the chill coma recovery times of the flies both in *D. montana* and *D. virilis*. Acclimation in short day conditions and/or at low temperature was beneficial at least in promoting survival (I & III) and in enabling the flies to recover faster from the chill coma (I, II, III & IV). The effects of RCH on fly cold tolerance were more unclear (I, II), which could partly be due to RCH treatment (1 hour at 0 °C) being not suitable for eliciting RCH response in *D. montana*. However, the flies that had been maintained in short day conditions and then allowed to cold harden in cool temperature prior to the exposure to cold recovered fastest from chill coma (I), which could refer to cumulative effects of photoperiod and RCH on the cold tolerance.

Experimental conditions used to study thermal thresholds/tolerance may have a large effect on the outcome (Powell & Bale 2006, Terblanche et al. 2007, 2010; Chown et al. 2009), and therefore the most realistic results are likely to be obtained using as natural conditions as possible. In experiment III, I

studied the seasonal acquisition of resistance to low temperature stress by exposing *D. montana* flies to gradually changing thermal- and photoperiodic regime, resembling the ones that the flies face at their home site. The study showed the overall cold tolerance of *D. montana* flies to vary between 19 % survival after a 24-hour cold exposure at -11 °C in summer flies to 100 % survival in the winter-acclimated flies. Chill coma recovery times varied from 19 min in summer flies to almost immediate recovery in the overwintered flies. This study revealed the vast ability of these flies to acquire seasonal cold tolerance needed for winter survival in harsh conditions.

3.2 Changes in gene expression during cold hardening and acclimation and during a recovery from cold stress

The molecular basis of cold resistance is not yet fully understood in any organism. Although new information on genes associated with cold tolerance and thermal adaptation is accumulating in model organisms, not much is known about molecular mechanisms behind cold adaptation e.g. in more cold-adapted *Drosophila* species. I utilized a candidate gene approach to trace the expression patterns of 219 genes during cold acclimation, rapid cold hardening and recovery from a cold shock in *D. montana* and its sister species *D. virilis* (II). Cold acclimation elicited changes in the expression level of several genes, but these changes did not show much overlap between the species. During rapid cold hardening, only one gene in *D. montana* and no genes in *D. virilis* showed modulated expression patterns, which fits with the findings that the rapid cold hardening treatment had only a mild effect (I) or no effect at all (II) on cold tolerance in these species. Gene expression changes during the recovery from a cold shock were studied only in *D. montana*. During recovery form cold shock, only *Hsp68* and a gene coding for a key enzyme in the glycolytic pathway (*Glyseraldehyde-3-phosphate dehydrogenase*; *Gapdh 1*) were upregulated. Also previously the induction of *Hsp* response has been detected during the recovery from cold stress (Burton et al. 1988, Colinet et al. 2010). *Gapdh 1* is expressed mainly in the thorax in *D. melanogaster*, suggesting its high expression in the flight muscles (Sun et al. 1988). Its upregulation probably reflects the need to recover the metabolism after chill coma, but increased expression of *Gadph* in plants has been observed under heat-shock conditions (Yang et al. 1993).

Study II provided a set of cold-responsive genes for *D. montana* and *D. virilis* and showed that the genes are mainly linked with the heat shock response, circadian clock and metabolism. One of the most interesting findings was that the heat shock RNA gene *hsr-omega* was upregulated during cold acclimation in both of the species. This gene has also previously been linked to thermal adaptation in *Drosophila* (McColl et al. 1996, Collinge et al. 2008) and my results further support this. Also other genes belonging to the heat shock protein gene family, showed modulated expression, but these genes were

species-specific. Heat shock response can be induced by either extrinsic or intrinsic stress, and in some cases the changes in *Hsp* gene expression have been found to be adaptive (reviewed in Sørensen et al. 2003). Seasonal increase of Hsp proteins (Hsp 110, 70 and 40) has been documented for example in the freeze-tolerant insect *Eurosta solidaginis* (gall fly) (Zhang et al. 2011) and during cold acclimation (Hsp90) in the rice stem borer *Chilo suppressalis* (Sonoda et al. 2006).

Modulated expression of genes related to circadian rhythms was found in both of the species, referring either to temperature-dependent pleiotropic functions of these genes or to modulation of the function of the circadian clock at low temperatures. During cold acclimation we also detected upregulation in *Thor* (*4E-BP*), which was unique to *D. montana.* The function of this gene in translation regulation and previous findings on upregulation of *Thor* transcription through FOXO (transcriptional activator) during oxidative stress and starvation in *Drosophila* (Teleman et al. 2005, Tettweiler et al. 2005) makes it a prominent candidate involved in coping with low temperatures. However, in this experiment we could not distinguish between the genes responding to damage possibly caused by the cold and the genes, which actually play a role in enhancing cold tolerance through cold acclimation.

3.3 Seasonal changes in metabolic traits

In long-term cold acclimation, both the accumulation of cryoprotectants and modifications in the cell membranes have been found to lead to improved cold tolerance (reviewed in Lee 1991). Exposing the flies to gradually changing (seasonal and daily) thermo- and photoperiods resembling those in northern Finland (III) revealed remarkable increase in cold tolerance, and accumulation of two sugars (glucose, trehalose) and amino acid proline, towards the artificial winter. The main seasonal metabolite, *myo*-inositol was at a very low level during summer and autumn, but rose over 400-fold in the winter. The accumulation of this cyclic carbohydrate synthesized from glucose only during the winter indicates that its synthesis, like that of many other cryoprotectants, is triggered only at sufficiently low temperatures (e.g. Storey & Storey 1991). While *myo*-inositol has been found to accumulate also in many other insect species (study III and citations therein), its accumulation in *D. montana* is exceptionally high. However, even though it seems to be the main winter-related compound in *D. montana*, it does not coincidence with the increase in the cold tolerance in autumn.

3.4 The role of *regucalcin* gene in photoperiodic diapause

One gene that has received much attention lately due to growing knowledge of its role in thermal adaptation in *D. melanogaster* is *Drosophila cold acclimation gene*, also known as *Senescence marker protein 30* (Goto 2000, Qin et al. 2005, Sinclair et al. 2007, Clowers et al. 2010). This cold-adaptive gene is absent in *D. montana*, as well as in other Drosophila subgenus species, where only its ancestral form, *regucalcin* is present (Arboleda-Bustos & Segarra 2011). Based on our previous findings, *regucalcin* is not involved in cold acclimation in *D. montana* (II), but it is upregulated under short day conditions inducing reproductive diapause (Kankare et al. 2010; here called *Dca*). I found that this gene is upregulated in diapausing females, regardless of the day length. This indicates that *regucalcin* is important in seasonal adaptation, and is functionally related to diapause.

4 CONCLUSIONS

Temperature plays a central role in dictation of the distribution, abundance and life-history traits of ectothermic organisms, which are not able to control their body temperature (Chown & Gaston 2000, Calosi et al. 2010). The ability of these organisms to distribute and survive in daily and seasonally varying environments prevailing in the northern temperate zone presumes high phenotypic plasticity in life-history traits linked with reproduction and survival. My studies on a northern fly *D. montana* show that the flies of this species tolerate very low temperatures and that their cold tolerance also shows high phenotypic plasticity induced by photoperiod (I, IV) and temperature (I, II) or a combination of these two factors (III). The time that the flies of this species need for a recovery from chill coma showed positive correlation with day length, while diapause itself was found to play only a minor role in photoperiodic acclimation (IV). However, the metrics that I have used to define cold tolerance are likely to describe mostly acute (chill coma recovery time after 16 h at -6 °C) and partly also chronic (survival after 24 h at -11 °C) chilling injuries and do not give direct information on the accumulation of indirect chilling injuries. Although the differences obtained in cold tolerance of *D. montana* using the two static metrics were clear and repeatable, it should be noted that developing and using other ecologically relevant measures of temperature tolerance (both high and low) has received more attention recently. Especially when defining the upper and lower thermal limits of an insect species, a slow rate of heating / cooling during the temperature tolerance assay most likely gives more natural results than exposing the individuals directly to (static) experimental temperature (see Terblanche et al. 2011)

As a freeze-avoidant species, *D. montana* utilizes various physiological and metabolic tools to overcome the harmful effects of low temperature. The effects of cold acclimation were found to be noticeable both at phenotypic (increased cold tolerance) and gene expression level both in *D. montana* and a more southern species of the same group, *D. virilis* (II). This treatment increased the expression level of *Hsp* genes in both species, possibly to control the damage caused by a low temperature. Also some circadian clock genes (e.g. *period* and

vrille) were either up- or downregulated during cold acclimation, which could be due to pleiotropic effects of these genes and/or the involvement of circadian clock in cold acclimation. One of the genes showing modulated expression during cold acclimation only in *D. montana* was *Thor,* which is involved in translation regulation and has been found to play a role also in stress resistance (Teleman et al. 2005, Tettweiler et al. 2005).

Maintaining *D. montana* flies in artificial thermo- and photoperiodic conditions mimicking seasonal changes in the flies home site in northern Finland enabled me to trace seasonal changes in *D. montana* female reproductive stage and cold tolerance as well as in several metabolic traits (III). The females entered diapause in late summer as a response to short day conditions, as expected. At the same time the cold tolerance of the flies of both sexes started to increase and the flies began to accumulate sugars glucose and trehalose and the amino acid proline (III). In contrast to these metabolites, the concentration of the dominant cryoprotectant in this species, *myo*-inositol, increased only during the winter (III). *Myo*-inositol is likely to be involved in the flies' overwintering ability, even though its accumulation starts too late to explain the increase in cold tolerance during the autumn. In the last part of my thesis I also traced changes in the expression of a putative cold-tolerance gene, *regucalcin* under different photoperiods and found the expression level of this gene to be more than two times higher in diapausing than in non-diapausing females reared under the same day length, but the day length *per se* did not cause significant changes in its expression (IV).

Taken together the studies included in my thesis shed light on both physiological and genetic responses to environment in an insect species adapted to a seasonal environment. In the future, studies on the cold tolerance of *D. montana* flies could be brought forward by 1) determining the thermal limits of *D. montana* using ecologically relevant measures and as natural rearing conditions as possible, 2) tracing possible trade-offs between cold-stress resistance and life-history traits and 3) performing overexpression and knock-down experiments on the putative cold-adaptive genes as well as on *regucalcin* to elucidate their importance in the cold acclimation / robust diapause response in *D. montana*.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Ympäristötekijöiden vaikutus *Drosophila montana* -mahlakärpäsen kylmänkestävyyteen, siihen liittyvien geenien toimintaan ja metaboliaan

Lämpötilalla on keskeinen merkitys lajien levinneisyyttä ja runsautta määrittävänä tekijänä etenkin vaihtolämpöisillä eliöillä, joilla se vaikuttaa useisiin lisääntymisen ja selviytymisen kannalta tärkeisiin elinkiertopiirteisiin. Pohjolan vaihteleviin ympäristöoloihin sopeutuminen vaatii selviytymistä äärilämpötiloista, ennen kaikkea kylmyydestä. Vain harvat hyönteislajit selviytyvät jäätymisestä, joten niille on kehittynyt erilaisia strategioita sen välttämiseksi. Useat hyönteislajit kestävät jäätymättä alle 0 °C lämpötiloja niin sanotussa alijäähtyneessä tilassa, jossa niiden ruumiinnesteet pysyvät sulana lämpötilan laskiessa pakkasen puolelle.

Matala lämpötila vaikuttaa eliöihin monella tavalla myös ennen jäätymistä. Lyhytaikainen altistus hyvin matalille lämpötiloille aiheuttaa niin sanotun kylmäshokin, jossa muun muassa solukalvojen rakenteet saattavat vahingoittua ja solujen sisäinen ionitasapaino järkkyä. Pidempiaikainen altistus lievemmälle kylmyydelle aiheuttaa epäsuoria haittoja, jotka kertautuvat ajan kuluessa. Monet lajit hyönteisistä kasveihin selviytyvät kylmyydestä tuottamalla ja kerryttämällä erilaisia sokereita, sokerialkoholeja ja aminohappoja, jotka ehkäisevät kylmän aiheuttamia vaurioita.

Vaihtelevissa ympäristöoloissa elävien eliöiden on hyödyllistä mukautua (akklimoitua) vallitseviin olosuhteisiin, eikä esimerkiksi ylläpitää korkeaa kylmänkestävyyttä ympäri vuoden. Monet pohjoisen hyönteislajit talvehtivat lepotilassa eli diapaussissa, jolloin niiden elintoiminnot hidastuvat, lisääntyminen siirtyy lämpimämpään vuodenaikaan ja hyönteisissä tapahtuu erilaisia fysiologisia muutoksia. Useimmat pohjoisen hyönteislajit ovat sopeutuneet käyttämään päivänpituuden muutoksia merkkinä tulevista ympäristöoloissa tapahtuvista muutoksista, mikä auttaa niitä valmistautumaan muutoksiin hyvissä ajoin. Miten hyönteiset sitten mittaavat päivänpituutta? Tässä auttaa sisäinen, fysiologinen kellojärjestelmä eli sirkaadinen kello, joka mittaa valoisan ajan pituutta vuorokauden aikana. Lisäksi monet hyönteiset kykenevät kerryttämään tietoa lyhyiden (tai pitkien) päivien lukumäärästä. Tällaisen "valojaksokellon" kehittyminen on ollut tärkeää erityisesti pohjoisessa eläville hyönteislajeille, mutta vielä on epäselvää, toimiiko vuorokautisia rytmejä säätelevä sirkaadinen kello myös vuodenaikaisia vaihteluita mittaavan kellon taustalla, vai pohjautuvatko kellot eri mekanismeihin. Hyönteisten sietokyky monia stressitekijöitä (kuumuus, kylmyys, torjunta-aineet) vastaan vaihtelee vuorokaudenaikojen mukaan juuri sirkaadisen kellon säätelemänä. Valojakson lisäksi myös kylmäakklimaatio voi auttaa hyönteisiä selviämään matalista lämpötiloista. Vuodenaikaisella akklimaatiolla tarkoitetaan kylmänkestävyyden paranemista päivien tai viikkojen kuluessa, kun taas kylmäkaraistuminen on nopea prosessi, joka auttaa selviämään vuorokauden aikana tapahtuvista lämpötilan vaihteluista.

Väitöskirjatyöni tavoitteena oli selvittää, miten pohjoiseen levinnyt *Drosophila*-laji, *D. montana*, on sopeutunut vuodenaikaisiin vaihteluihin elinolosuhteissa. Lisäksi tavoitteena oli selvittää, miten erilaiset ympäristötekijät vaikuttavat kärpästen kylmänkestävyyteen geenien toiminnassa ja metaboliassa tapahtuvien muutosten kautta.

Tutkimuksissani selvisi, että *D. montana* -mahlakärpäset ovat erittäin kylmänkestäviä verrattuna muihin *Drosophila*-suvun lajeihin. Tämä ei ole yllättävää, koska kyseinen laji on levinneisyydeltään eräs pohjoisimmista *Drosophila*-lajeista, mikä tekee siitä mielenkiintoisen tutkimuskohteen selvitettäessä kylmästressin vaikutuksia vaihtolämpöisillä eliöillä. Tutkimukseni tuloksista voidaan myös päätellä, että fenotyyppisellä plastisuudella, eli yhden genotyypin kyvyllä tuottaa useita ilmiasuja eli fenotyyppejä, on suuri rooli selviytymisessä pohjoisen olosuhteista. *D. montana* -lajin yksilöt ovat sopeutuneet käyttämään päivänpituudesta saamaansa informaatiota hyväkseen ja muuntelemaan kylmänkestävyyttään sen mukaan. Mitä lyhyempi vuorokauden valoisa jakso on, sitä kylmänkestävämpiä kärpäset ovat. Lyhyt päivä laukaisee myös lisääntymisdiapaussin eli aikuisvaiheen diapaussin, jossa naaraiden ovaariot eivät kehity sukukypsiksi. Tämä estää kärpäsiä lisääntymästä liian myöhään syksyllä, jolloin niiden jälkeläisillä ei olisi aikaa valmistautua talveen, ja lisääntymiseen käytetty energia menisi hukkaan. Tutkimukseni perusteella vaikuttaa kuitenkin siltä, että päivänpituus vaikuttaa kylmänkestävyyteen naaraiden lisääntymisvaiheesta riippumatta eli päivänpituus saa aikaan fysiologisia muutoksia, jotka eivät suoraan liity diapaussiin. *regucalcin*-geeni osoittautui tutkimuksissani olevan kytkeytynyt diapaussiin: se toimi aktiivisemmin diapaussissa kuin lisääntymisvaiheessa naarailla, mutta päivänpituus ei yksinään vaikuttanut geenin toimintaan. *regucalcin* saattaa siis olla osatekijänä vuodenaikaiseen vaihteluun mukautumisessa, ja sen osuutta pohjoisen olosuhteisiin sopeutumisessa tulisi selvittää jatko-tutkimuksissa.

Toinen tutkimuslajini, *D. virilis*, joka ei ole levinnyt pohjoiseen, kestää huonommin kylmää kuin *D. montana*. Kylmäakklimaation havaittiin vaikuttavan sekä *D. montanan* että *D. viriliksen* kylmänkestävyyteen, kun taas kylmäkaraistuksella ei ollut suurtakaan vaikutusta kylmänkestävyyteen kummallakaan lajilla. Tutkiessani 219 geenin toimintaa lähetti-RNA:n määrän perusteella havaitsin kylmäaltistuksen aiheuttavan molemmilla lajeilla muutoksia sellaisten geenien ekspressiossa, jotka liittyvät sirkaadisen kellon toimintaan. Tämä viittaa muutoksiin kellon toiminnassa matalissa lämpötiloissa ja/tai kelloa säätelevien geenien muiden toimintojen aktivoitumiseen. Geenit, joiden toiminta muuttui matalissa lämpötiloissa, olivat tutkimuslajeillani suurimmaksi osaksi eri geenejä; ainoastaan *hsr-omega* -geeni aktivoitui molemmilla lajeilla kylmäakklimaation aikana. Tämä geeni kuuluu *heat shock* -geeneihin, jotka muodostavat yleisen vastareaktion erilaisten stressitekijöiden laukaisemana. Myös muita *Hsp*-geenejä aktivoitui matalassa lämpötilassa, mutta ne vaihtelivat lajien ja kylmäkäsittelyjen välillä. Lajien erilaiset vasteet geenien toiminnan tasolla saattavat kuvastaa näiden lajien erilaista kylmänkestävyyttä ja erilaista sopeutumishistoriaa.

Luonnon olosuhteita simuloimalla voidaan saada selville sellaisia muutoksia kylmästressin siedossa ja fysiologiassa, jotka eivät välttämättä tule esiin silloin, kun tutkimuksissa käytetään tasaisia olosuhteita. Jäljittelemällä pohjoisen Suomen lämpötila- ja valojakso-olosuhteita simuloivassa kokeessa löysin suurta vuodenaikaista muuntelua matalien lämpötilojen sietokyvyssä, mikä viittaa siihen, että fenotyyppinen plastisuus on tärkeä tekijä kylmään sopeutumisessa. Syksyn aikana kärpäset kerryttivät glukoosia ja trehaloosia sekä proliinia, joiden kaikkien tiedetään toimivan kryoprotektantteina eli kylmän haitoilta suojaavina aineina. Suurin muutos tapahtui kuitenkin syklisessä hiilihydraatissa *myo*-inositolissa, jota syntyi talvella yli 400-kertainen määrä kesään ja syksyyn verrattuna. *myo*-inositolilla näyttää olevan rooli talveen sopeutumisessa, mahdollisesti pitkäaikaisten, akkumuloituvien kylmähaittojen estäjänä. Kärpästen kylmänkestävyys parani huomattavasti jo ennen *myo*-inositolin biosynteesiä, joten tämä yhdiste ei yksin selittä kylmänkestävyyden paranemista.

Kaiken kaikkiaan väitöskirjatyöni valottaa sekä fysiologisia että geneettisiä mekanismeja, jotka edesauttavat hyönteisten sopeutumista vuoden aikojen mukaan muuttuviin ympäristöihin. Lisäksi työni antaa hyvän pohjan pohjoisten hyönteislajien kylmänkestävyysstrategioita ja niiden evoluutiota koskeville jatkotutkimuksille.

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ORIGINAL PAPERS

I

EFFECTS OF PHOTOPERIODICALLY INDUCED REPRODUCTIVE DIAPAUSE AND COLD HARDENING ON THE COLD TOLERANCE OF *DROSOPHILA MONTANA*

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Effects of photoperiodically induced reproductive diapause and cold hardening on the cold tolerance of *Drosophila montana*

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ABSTRACT

Coping with seasonal and daily variation in environmental conditions requires that organisms are able to adjust their reproduction and stress tolerance according to environmental conditions. Females of *Drosophila montana* populations have adapted to survive over the dark and cold winters at high latitudes and altitudes by spending this season in photoperiodically controlled reproductive diapause and reproducing only in spring/summer. The present study showed that flies of a northern population of this species are quite tolerant of low temperatures and show high seasonal and short-term plasticity in this trait. Culturing the flies in short day length (nearly all females in reproductive diapause), as well as allowing the flies to get cold hardened before the cold treatment, increased the cold tolerance of both sexes both in chill coma recovery time test and in mortality assay. Chill coma recovery time test performed for the females of two additional *D. montana* populations cultured in a day length where about half of the females enter diapause, also showed that diapause can increase female cold tolerance even without a change in day length. Direct linkage between diapause and cold tolerance was found in only two strains representing a high-altitude population of the species, but the phenomenon will certainly be worth of studying in northern and southern populations of the species with larger data sets.

1. Introduction

Adaptation to environments with high seasonal variation requires synchronization of reproduction and stress resistant phases of the life-cycle with changes in environmental conditions (Lumme and Lakovaara, 1983). Insect cold tolerance is often affected by physiological responses to the cues received from the environment; it usually increases towards the cold season (cold acclimation) and it may also show short-term fluctuations following daily changes in temperature (cold hardening). Photoperiodic cues may play an important role in cold acclimation as they enable the insects to predict long-term changes in temperature and adjust their development, physiology and behavior according to the forthcoming season. This is especially important in the temperate zone where winter sets substantial challenges on insect survival. Low temperatures may induce severe damage to an organism even before actual freezing, e.g. by altering the viscosity of cell membranes, disrupting ion homeostasis and hindering protein function (Bale, 2002; Sinclair et al., 2003).

Diapause triggered by changes in day length and/or temperature is one of the most common winter-adaptations among insects occupying areas with distinct seasons (Tauber and Tauber, 1976). The way that photoperiodically induced diapause affects insect survival in cold temperatures has remained somewhat controversial and seems to vary among the species (Hodková and Hodek, 1997; Goto et al., 2001). For example, in the European corn borer (*Ostrinia nubilalis*) larval diapause is induced by short day length, but the diapausing larvae are not more tolerant to cold than non-diapausing larvae. Increase in larval cold tolerance in this species requires a decrease in temperature (reviewed in Denlinger, 1991). On the other hand, in the flesh fly, *Sarcophaga crassipalpis*, diapausing pupae can survive in substantially lower temperatures (up to −23 °C) than non-diapausing pupae (Lee and Denlinger, 1985). The relationship between photoperiodically controlled adult diapause and cold tolerance is especially interesting in the linden bug (*Pyrrhocoris apterus*). Short day length not only evokes diapause but also induces changes in cell membrane phospholipid composition in a similar way as exposure to low temperature (Hodková et al., 1999). Diapausing *P. apterus* have a higher survival rate in cold temperatures as well as a lower super cooling point (the point where spontaneous nucleation of body fluids occurs, (Salt, 1966)). Diapause also has been found to be a prerequisite for further decrease in the super cooling point by temperature mediated cold acclimation (Hodková and Hodek, 1994, 1997). Finally, in some species, such as *Dendroides canadiensis* beetles, photoperiod enhances cold tolerance even without diapause (Howarth and Duman, 1983).

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The ability of insects to improve their cold tolerance when exposed to low temperature shortly before the cold period is important for coping with daily fluctuations in temperature (Kelty and Lee, 2001; Koveos, 2001; Overgaard and Sørensen, 2008). This kind of rapid cold hardening (RCH) has been found to lead to a dramatic reduction in mortality in cold, e.g. in *D. melanogaster* (Czajka and Lee, 1990), and it has also been shown to sustain mating and courtship activity of flies of this species at low temperatures (Shevre et al., 2004). The exact physiological mechanism of rapid cold hardening is not known, but it seems to be enhanced by changes in the phospholipid composition of cell membranes (Overgaard et al., 2005).

Drosophila montana, a species of the *D. virilis* group, has a circumpolar northern distribution range with wild populations living near watersides (Throckmorton, 1982). In the northern parts of their distribution, *D. montana* flies are able to survive through harsh winter conditions with a mean temperature below 0 °C for about half of the year and occasional drops in temperature to −20 °C or even below. Frequent freeze-thaw cycles (daily variation) in autumn and spring cause additional challenges for fly survival and may be detrimental especially for reproducing females. Most, if not all, females of the northern and high-altitude populations of this species spend the winter in photoperiodically controlled adult reproductive diapause characterized by ovarian developmental arrest (Baker, 1975; Lumme, 1978; Watabe, 1983), whereas in milder climate zones the reproductive stage of the females may not be so critical for overwinter survival. The physiological stage and condition of overwintering males has been less well-studied in insects, but short day length seems to affect several physiological traits also in males (Pener, 1992).

In the present study our aim was to measure the cold tolerance of *Drosophila montana* flies and to find out what kind of effects the day length/photoperiodic reproductive diapause and rapid cold hardening have on this trait. In the first part of the study we measured the cold tolerance of the females and males of a northern *D. montana* population using a chill coma recovery time-test and mortality assay with and without prior cold hardening treatment. The tests were performed separately for the flies cultured in short day (most females in reproductive diapause) and long day (most females sexually mature) to see how the day length affects the cold tolerance of the flies of the two sexes and whether it affects the ability of the flies to cold harden. In the second part of the study we measured chill coma recovery times for the females of two additional *D. montana* populations cultured in a day length, where about half of the females enter diapause, to see if diapause can increase female cold tolerance without a change in day length. These experiments showed that *D. montana* flies are very cold tolerant and that they also are able to increase their cold tolerance towards the cold season according to the cues that they perceive from the environment.

2. Material and methods

2.1. Experimental flies

The effects of day length/reproductive diapause and rapid cold hardening on chill coma recovery times (CCRT) of the flies were studied using females and males from a mass bred *D. montana* population established from F2 progenies of 20 females collected in Oulanka (northern Finland, 66°N) in summer 2008, while the mortality test was performed using the flies of one isofemale strain originated from the same population (strain O3F77). The second set of CCRT-tests was performed using females of isofemale strains originated from both Crested Butte (Colorado, USA, 39°N, 3000 meters above sea level; strains C3F2, C3F6 and C3F13) and Vancouver (Canada, 49°N; strains Can3F9, Can3F18 and Can3F24). The numbers of the isofemale strains refer to the stock collections at the University of Jyväskylä. All these strains have been established from the progenies of females collected in the wild in 2003 and maintained in constant light and 60% humidity at 19 °C in bottles containing malt-yeast medium (Lakovaara, 1969) since their establishment.

The experimental flies were sexed within one day after eclosion under light CO_2 anesthesia and transferred to plastic vials containing malt-yeast medium. The cold tolerance tests were performed on 14-day-old flies maintained in climate chambers (Memmert) with different day lengths and with an experimental temperature of 16 °C (±0.5 °C).

2.2. Methods to study the effects of day length and cold hardening on fly cold tolerance

Cold tolerance of the flies cultured in different light:dark regimes was tested using a CCRT-test and mortality assay. The flies for these tests were maintained in a climate chamber at 16 ± 0.5 °C in two wooden, light insulated chambers in short day (light:dark cycle LD16:8) and long day (LD22:2 in CCRT-test and LD24:0 in mortality assay) conditions. For the Oulanka population LD16:8 is a diapause-inducing photoperiod and LD22:2 and LD24:0 are diapause-preventing photoperiods, the latter photoperiods resembling the conditions during the reproductive season of the flies in northern Finland. After 14 days in a given light:dark cycle, the flies from both day length groups were further divided into two groups. The flies of the first group received no pretreatment before the cold tolerance tests, while the flies of the second group were cold hardened by placing them in plastic vials, 10 flies per vial, in a box containing melting ice (0 °C) for

Table 1
Experiments, culturing conditions (LD = light:dark cycle), female reproductive status (D: diapausing and R: reproducing females), treatments (cold hardening or not), cold tolerance tests with test temperatures (°C) and durations (h) and fly material used in cold tolerance experiments.

Study question/Test	LD	Female reproductive status	Cold hardening	Test	Temperature	Duration	Study flies
Effects of day length and cold hardening on fly cold tolerance/CCRT test	16:8	D	Yes	CCRT	−6 °C and −7 °C	16 h	Oulanka/mass bred pop.
	16:8	D	No	CCRT	−6 °C and −7 °C	16 h	Oulanka/mass bred pop.
	22:2	R	Yes	CCRT	−6 °C and −7 °C	16 h	Oulanka/mass bred pop.
	22:2	R	No	CCRT	−6 °C and −7 °C	16 h	Oulanka/mass bred pop.
Effects of day length and cold hardening on cold tolerance/Mortality assay	16:8	D	Yes	Mortality	−10 °C and −11 °C	24 h	Oulanka/isofemale strain
	16:8	D	No	Mortality	−10 °C and −11 °C	24 h	Oulanka/isofemale strain
	24:0	R	Yes	Mortality	−10 °C and −11 °C	24 h	Oulanka/isofemale strain
	24:0	R	No	Mortality	−10 °C and −11 °C	24 h	Oulanka/isofemale strain
Effects of reproductive diapause on female cold tolerance/CCRT test	12:12	D/R	No	CCRT	−6 °C	16 h	Crested Butte/three isofemale strains
	12:12	D/R	No	CCRT	−6 °C	16 h	Vancouver/three isofemale strains

1 h prior to the tests. Consequently, both the CCRT-tests and mortality assays involved flies from four treatment groups: (1) short day, (2) short day + cold hardening, (3) long day and (4) long day + cold hardening (Table 1). In CCRT-tests each treatment group involved 15–20 females and males per test and temperature, the tests being replicated at each temperature three times on different days. In the mortality assay the treatment groups involved 10–20 females and males per test and temperature and these tests were replicated twice during different weeks.

The CCRT-test is based on the disruption of electrical activity in the muscles at low temperature, which leads to immobilization (Goller and Esch, 1990). Chill coma is a reversible state, i.e. the insects usually regain their ability to move when brought back to room temperature, and the time it takes for an insect to stand on its legs after the treatment gives a chill-coma recovery time (David et al., 1998). In CCRT-tests the flies were given a 'cold shock' by placing them in a cold chamber (Termaks, KB 8400F) at −6 °C or −7 °C for 16 h. The flies were kept in small plastic vials containing agar medium for moisture. After the cold shock, the motionless flies were placed in transparent dishes with lids (4 cm × 4 cm, 12 compartments for individual flies per dish) at room temperature (21 ± 1 °C). The time required for each of the flies to stand on its legs was recorded. The females were dissected under a microscope immediately after the experiment to find out whether they were diapausing (small ovaries, no egg chambers, transparent or some yolk accumulating) or whether they had mature ovaries (clear egg chambers and mature or almost mature eggs; Lumme and Lakovaara, 1983).

In the mortality assay the females and males of the four experimental groups were placed without anesthesia in empty glass vials (about 10 flies per vial) in a cold bath (Haake, DC50-K35) containing 35% ethylene glycol solution at −10 °C or −11 °C for 24 h. After the cold treatment the flies were transferred to vials containing food medium and left to recover at room temperature for 24 h. The flies were considered dead if they did not move after this time period (even after slight agitation). The mortality assay was originally performed also at −9 °C, but since only about 5% of the flies died at this temperature, the data were excluded from the analysis.

2.3. Methods to study the effect of reproductive diapause on female cold tolerance

In *D. montana* populations from Crested Butte (Colorado, USA) and Vancouver (Canada) only part of the females enter reproductive diapause in LD12:12 at 16 °C (A. Lehtovaara, personal communication), which gave us an opportunity to compare the cold tolerance of diapausing and reproducing females without the possible effect of day length. Freshly emerged females of three isofemale strains from both populations were maintained in LD12:12 for 14 days. The chill coma recovery times of the females were tested as explained above after keeping them at −6 °C for 16 h. Each isofemale strain was tested two times, the number of tested females per strain ranged from 41 to 59. The developmental stage of the females was checked (see above) after which the females were divided into two groups (diapausing/reproducing).

2.4. Statistical analyses

Data analyses were performed using SPSS 16.0 software. The recovery time data were log transformed to achieve homogeneity of variances and normality. Normalized data were analyzed with one or two-way analysis of variance (ANOVA) with cold hardening, day length, sex, strain and/or replicate as fixed factors, depending on the case. Data on fly mortality were analyzed using logistic regression with sex, cold hardening and day length as independent variables.

3. Results

3.1. Effects of day length and cold hardening on fly chill coma recovery time and mortality after the cold treatment

Chill coma recovery time tests were made for four groups of flies from a mass-bred population from Oulanka at −6 °C and −7 °C (flies reared in short or long day, with or without cold hardening; see Table 1). The recovery times of the three replicates for the four groups showed no variation in either of the temperatures (1-ANOVA −6 °C and 7 °C: $F_{2,430} = 0.07$, $p = 0.93$; and $F_{2,436} = 0.11$, $p = 0.90$, respectively) and the data were pooled for subsequent analysis. As expected, the rearing day length had a major effect on female sexual maturity: in LD 16:8 97% of the females entered reproductive diapause, whereas in LD 22:2 the respective percentage was only 7.5%. The reproductive status of the males could not be determined and the data for females and males were analyzed separately.

As Fig. 1 shows, both the females and the males on average recovered from chill coma in 6–10 min after a cold treatment at −6 °C and in 9–12 min after a treatment at −7 °C. At −6 °C, the females reared in short day length and cold hardened prior to cold treatment had the shortest recovery times, female cold tolerance showing significant interaction between the day length and cold hardening (2-ANOVA: $F_{1,208} = 9.18$, $p = 0.013$; Fig. 1A). However, further analyses showed that the cold hardened females recovered faster than the non-hardened females regardless of the day length (1-ANOVA: LD16:8: $F_{1,99} = 4.36$, $p = 0.04$; LD22:2: $F_{1,109} = 40.21$, $p < 0.001$) and that the females reared in short day recovered faster than those reared in long day regardless of whether or not they had been cold hardened (1-ANOVA: cold hardened: $F_{1,101} = 6.30$, $p = 0.013$, non-hardened: $F_{1,107} = 43.91$, $p < 0.001$). Also, the males recovered faster from the cold treatment at −6 °C when reared in short vs. long day (2-ANOVA: $F_{1,217} = 24.06$, $p < 0.001$) or when cold hardened vs. non-hardened prior to the treatment (2-ANOVA:

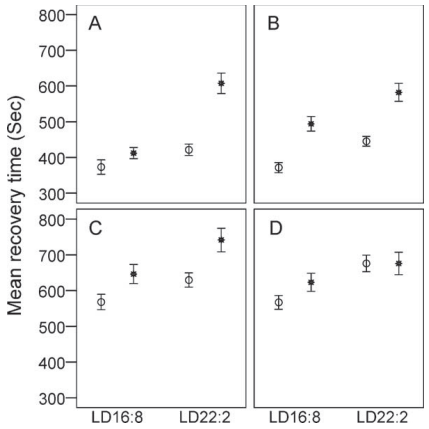

Fig. 1. Mean (±1 SE) chill coma recovery times of the flies reared in LD16:8 or LD22:2. (A) and (B) show the recovery times of females and males after 16 h cold treatment at −6 °C and (C) and (D) the recovery times of females and males at −7 °C, respectively. Bars marked with a circle (○) represent cold hardened flies and the ones marked with a star (*) represent the flies that did not receive cold hardening treatment.

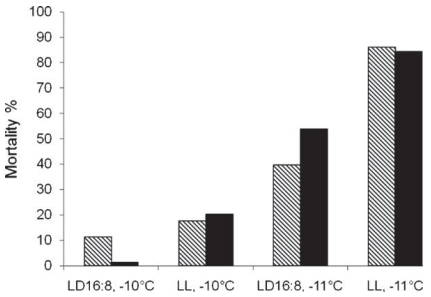

Fig. 2. Mortality of cold hardened (striped columns) and non-hardened (black columns) flies cultured in LD16:8 or continuous light (LL) after 24 h cold treatment at −10 °C and −11 °C. Data for females and males combined.

$F_{1,217} = 53.15$, $p < 0.001$; Fig. 1B). At −7 °C the females from the short day length group again recovered faster than the females from the long day length group (2-ANOVA: $F_{1,216} = 11.00$, $p = 0.001$) and also cold hardening shortened their recovery times (2-ANOVA: $F_{1,216} = 14.06$, $p < 0.001$; Fig. 1C). In males, only the day length had a significant effect on recovery time (2-ANOVA: $F_{1,215} = 12.54$, $p < 0.001$; Fig. 1D) at this temperature.

The cold treatment temperatures used in the mortality assay had a major impact on the percentage of flies dying during the experiment, the overall mortality of the flies being 12.8% at −10 °C and 65% at −11 °C (see Fig. 2). At both temperatures the full model containing all of the predictors was significant (−10 °C: $N = 304$, $\chi^2_3 = 15.47$, $p < 0.001$; −11 °C: $N = 300$, $\chi^2_3 = 55.25$, $p < 0.001$), indicating that the model used was able to distinguish between survived and dead flies. Among the three independent variables (sex, cold hardening, day length) only day length had an effect on mortality by decreasing it at both temperatures (−10 °C: $\text{Wald}_1 = 11.05$, $p < 0.001$; −11 °C: $\text{Wald}_1 = 41.2$, $p < 0.001$; Fig. 2). Odds ratios for staying alive were 3.8 and 6.4 at −10 °C and −11 °C, respectively, indicating that flies reared in short day length were about 4 and 6 times more likely to survive the cold treatment than flies reared in long day length.

3.2. Effects of photoperiodic reproductive diapause on female chill coma recovery time

Females of the three isofemale strains from Crested Butte and Vancouver were reared in a critical day length, at which about half of the females are expected to enter diapause. This allowed us to trace the effects of diapause on female cold tolerance without a change in day length. In Crested Butte strains about 48%, and in Vancouver strains about 78% of the females entered diapause when reared in LD12:12. Consequently our experimental data included 80 diapausing and 85 reproducing females from Crested Butte and 112 diapausing and 32 reproducing females from Vancouver. There was significant variation between the two replicates in both of these data sets (1-ANOVA: Crested Butte: $F_{1,164} = 25.78$, $p < 0.01$; Vancouver: $F_{1,142} = 6.5$, $p < 0.01$). However, since there were no significant interactions, the main effects of diapause and strain on female cold tolerance could be examined. In Crested Butte, the chill coma recovery time of the females depended both on the strain (2-ANOVA: $F_{2,153} = 7.28$, $p < 0.001$) and on the reproductive stage of the females ($F_{1,153} = 5.22$, $p < 0.05$; Fig. 3). Closer inspection revealed that diapausing females recovered faster in two of the strains (1-ANOVA: C3F2: $F_{1,51} = 4.07$, $p < 0.05$, C3F13: $F_{1,57} = 6.71$, $p < 0.05$), whereas in one strain the difference between diapausing and reproducing females was not significant (C3F6: $F_{1,51} = 3.05$, $p = 0.09$). In Vancouver, only the strain (2-ANOVA: $F_{2,132} = 6.17$, $p < 0.01$), but not the reproductive stage of the females ($F_{1,132} = 0.19$, $p = 0.66$, Fig. 3), had an effect on recovery times.

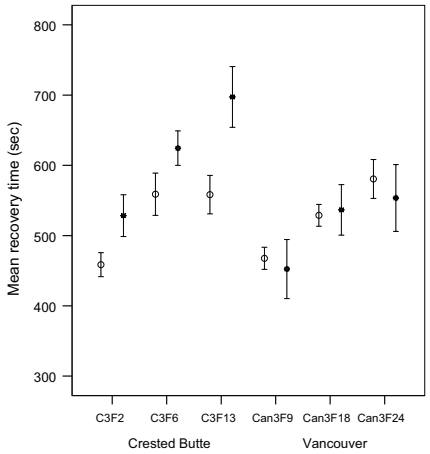

Fig. 3. Mean (±1 SE) chill coma recovery times of diapausing (marked with a circle) and reproducing females (marked with a star) for isofemale strains originating from Crested Butte (C3F2, C3F6, C3F13) and Vancouver (Can3F9, Can3F18, Can3F24).

4. Discussion

Adaptation of insect populations to seasonal variation at high latitudes and altitudes has required changes in several life-history traits including reproductive strategies, cold tolerance and developmental rate. Our study on one of the northernmost *Drosophila* species, *D. montana,* demonstrates that the flies of this species are able to tolerate low temperatures and that they also show high seasonal (day length/reproductive diapause) and short-term (cold hardening) plasticity in their cold tolerance.

Cold tolerance of several *Drosophila* species has been studied by measuring their chill coma recovery time (CCRT) and/or by observing their mortality at low temperatures. Gibert et al. (2001) compared the CCRTs of the flies of 26 temperate and 48 tropical species recovered after a 16 h cold treatment at 0 °C and found large differences between the groups: the flies of temperate species recovered from chill coma in 1.8 min (range 0.15–4.9 min) and the flies of tropical species recovered in 56 min (range 24–120 min), on average. The present study showed *D. montana* flies to recover from chill coma in 6–12 min after a much more severe cold treatment (−6 °C and −7 °C). Among other *Drosophila* species studied so far, only *D. subobscura*'s cold tolerance has been found to be of about the same level as that of *D. montana,* even though the females of this species do not enter reproductive diapause during the cold season (Goto et al., 1999; David et al., 2003). Comparisons between species showing high variation in their cold tolerance may be easier to do when measuring the temperatures, at which half of the flies die in 24 h (Lt50), rather than by performing chill coma recovery time tests. Our mortality assay tests showed the Lt50 temperature for *D. montana* (Oulanka population) to be near −11 °C, which is quite low when compared, e.g. to −8 °C and +7 °C measured for temperate and subtropical species of *D. takahashii* and *D. montinum* subgroups (Goto et al., 2000).

One of the most reliable seasonally changing environmental cues helping the insects to predict future changes in climatic

conditions is the day length, which has been found to affect fly cold tolerance both alone and through changes linked with photoperiodic diapause (see Pullin, 1996). For example, Higuchi and Kimura (1985) have found that *D. triauraria* adults survive better from the cold treatment when they are reared in diapause inducing (LD14:10) rather than when reared in diapause preventing (LD16:8) day length. In the present study rearing *D. montana* flies from the Oulanka population in short day length (LD16:8) increased the ability of both females and males to tolerate low temperatures with similar rates. The fact that the cold tolerance of the flies of both sexes changes in the same way could be due to the possibility that the critical factor increasing fly cold tolerance is the day length per se, and not the reproductive diapause, or that also the males enter some kind of diapause state when exposed to short day length. Diapause is generally characterized by a low level of metabolism and higher stress resistance (Tauber et al., 1986) and, as Pullin (1996) states, the relationship between diapause related metabolic suppression and carbohydrate cryoprotectant synthesis is supported by an increasing number of studies. Even though male reproductive diapause has been described only in a few *Drosophila* species (e.g. *D. triauraria*; Kimura, 1988) and only in the form of decreased mating activity, metabolic changes could take place also in males.

The most obvious factors increasing the cold tolerance of *Drosophila* flies in fluctuating environmental temperatures are cold hardening and cold acclimation. The effectiveness of cold hardening, which was also tested in the present study, depends largely on the pace at which the temperature is lowered (Kelty and Lee, 1999) and it may be of special importance in chill-sensitive species (Doucet et al., 2009). For example Hoffmann et al. (2005) have shown that rearing flies in a fluctuating thermal regime decreases the chill coma recovery times compared to those of the flies reared in constant conditions. In northern latitudes cold hardening is most important in autumn, when the temperatures may show high daily fluctuations and when the flies or pupae of several *Drosophila* species are already in diapause. In the present study cold hardening was found to decrease CCRT of both females and males at −6 °C and of the females also at −7 °C. However, it did not have an effect on fly cold tolerance when measured with the mortality assay, possibly due to the low temperatures used. The exact mechanism of cold hardening is not known, but Overgaard et al. (2007) have found that cold hardening of *D. melanogaster* flies is followed by elevated levels of glucose and trehalose and that the onset and magnitude of increased sugar levels correlates tightly with the improved chill tolerance of flies.

In the present study we also checked the CCRTs of flies from two additional *D. montana* populations (Vancouver, Canada, and Crested Butte, CO, USA). In this part of the study we wanted to separate the effects of day length and diapause and so the females from these populations were cultured in a day length where only about half of the females enter diapause. Interestingly, the effect of diapause varied among the studied strains: females of two of the three isofemale lines from Crested Butte had a shorter CCRT when in diapause, whereas in the three Vancouver strains, diapause had no effect on CCRT. Crested Butte is located at high altitude (nearly 3000 m) with winters about as long and cold as in Oulanka, and the fly populations in these two areas have practically only one generation per year (Baker, 1975; Lumme, 1978). Vancouver has a much milder climate and the flies from this area have two partly overlapping generations per year (Moorhead, 1954). Studies on fresh population samples from these sites (preferably latitudinal clines) could allow tracing selection pressures on the reproductive strategies and cold tolerance of the flies in different environments and detection of the links between day length, reproductive diapause and cold tolerance.

Variation in traits like insect cold tolerance are quite complex to study in wild populations, as they exhibit both plasticity and genetic variation, show costs and trade-offs with other life-history traits and may be affected by different kinds of selection pressures during different seasons. While laboratory experiments in constant temperatures and lighting conditions are important for determining the basic cold tolerance levels and underlying physiological mechanisms in our study species, also experiments in fluctuating environmental conditions both in the laboratory and the wild will be important because of the plastic nature of responses to low temperatures.

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II

COLD TOLERANCE AND COLD-INDUCED MODULATION OF GENE EXPRESSION IN TWO *DROSOPHILA VIRILIS* GROUP SPECIES WITH DIFFERENT DISTRIBUTIONS

by

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ABSTRACT

The importance of high and low temperature tolerance in adaptation to changing environmental conditions has evoked new interest in modulations in gene expression and metabolism linked with stress tolerance. We investigated the effects of rapid cold hardening and cold acclimation on the chill coma recovery times of two *Drosophila virilis* group species, *Drosophila montana* and *D. virilis*, with different distributions and utilized a candidate gene approach to trace changes in their gene expression during and after the cold treatments. The study showed that cold acclimation clearly decreases chill coma recovery times in both species, while rapid cold hardening did not have a significant effect. Microarray analysis revealed several genes showing expression changes during different stages of cold response. Among the 219 genes studied, two genes showed rather consistent expression changes: *hsr-omega*, which was upregulated in both study species during cold acclimation, and *Eip71CD*, which was downregulated in nearly all of the cold treatments. In addition, 29 genes showed expression changes that were more treatment- and/or species specific. Overall, different stages of cold response elicited changes mainly in genes involved in heat shock response, circadian rhythm and metabolism.

INTRODUCTION

Adaptation of insects to fluctuating temperature conditions prevailing at high latitudes includes a capability to survive and remain active at lower than optimal temperatures. This has evoked interest in the physiological and genetic mechanisms helping the insects to cope with low temperatures (Hoffmann *et al.*, 2003; Sinclair *et al.*, 2003; Danks, 2005). Most insect species are able to adjust their tolerance levels according to ambient temperature, which is important

especially in fluctuating temperature conditions. Short-term exposure to non-lethal low temperatures preceding a more severe cold period has been found to sustain insects' courtship and mating activity (Shevre *et al*., 2004) and to improve their survival (Czajka & Lee, 1990) at low temperatures. This phenomenon, termed rapid cold hardening, has been suggested to be at least partly based on the mechanisms that prevent phase transitions in the membrane phospholipids and help to preserve membrane fluidity (Overgaard *et al*., 2005). Yi *et al*. (2007) found rapid cold hardening to decrease the incidence of apoptosis by 38% after a cold shock, when compared to a non-hardened control group in *Drosophila melanogaster*. Cold acclimation, on the other hand, usually refers to a process that occurs over a longer time span (days or weeks). It is accompanied with qualitative and quantitative changes in sugars, polyols and amino acids, which act as cryoprotectants shielding an organism against injurious effects of low temperature (reviewed in Denlinger & Lee, 1998).

Many insect species exhibit chill coma when the temperature decreases below a certain point, but still remains above the lethal temperature. In chill coma the electrical activity of muscles is disrupted, which leads to immobilization (Goller & Esch, 1990). This is a reversible state, if not prolonged, and an insect regains its ability to move when the temperature rises. The time it takes for an insect to recover from the chill coma can be used as a measure of its cold tolerance (David *et al*., 1998), and it has been found to show latitudinal variation in several *Drosophila* species (Gibert *et al*., 2001). Morgan and Mackay (2006) and Norry *et al*. (2007) found that thermotolerance and slow recovery from chill coma is associated with a quantitative trait locus (QTL) region on chromosome 2 in *D. melanogaster*, and later on Norry *et al*. (2008) also reported large-effect QTLs for chill coma recovery time on chromosomes 2 and 3. Candidate genes for chill coma recovery time located on the areas of QTL include *hsr-omega*, *hsc70*, *Sialic acid phosphatase synthase* (*Sas*), *desat* and *Frost* (Morgan & Mackay, 2006; Norry *et al*., 2008).

The majority of studies on the genetic basis of insect cold tolerance have been conducted on chill susceptible *Drosophila* species, mainly *D. melanogaster*. Although using model organisms in this kind of studies is well justified, studying these phenomena in species adapted to more demanding environments may bring new perspectives on adaptation to low temperatures. We examined the expression patterns of a set of candidate genes during different stages of cold stress response in two *Drosophila virilis* group species with different distribution ranges. *D. montana* has a wide natural distribution range in the northern hemisphere (30°N-70°N; Throckmorton, 1982) and it is adapted to survive over the long winters in the north with a mean temperature below 0°C for about half of the year. *D. virilis* has a more southern distribution range (south from 35°N); it has been found in the wild in eastern Asia, but in most parts of its distribution area it is a human commensal found, e.g., in market places and breweries (Throckmorton, 1982).

We began the experiment by measuring the basal chill coma recovery times of *D. montana* and *D. virilis* flies after keeping them at -6 °C for 16 hours, as well as by determining the effects of rapid cold hardening and cold

acclimation on chill coma recovery times. We then quantified the expression patterns of 219 genes during the rapid cold hardening and cold acclimation treatments utilizing a candidate gene microarray specifically prepared for these two species. In *D. montana* we also investigated changes in gene expression during recovery from chill coma to obtain a wider picture of genetic processes during cold exposure. The genes were chosen according to their annotations in other insect species (mainly *D. melanogaster*) and they include genes affecting several life-history traits that are potentially important in adaptation to northern environmental conditions (see Kankare *et al*., 2010). The study showed that cold acclimation has a clear effect on chill coma recovery times in both species, while cold hardening did not have any detectable effects. The study also revealed several genes showing expression changes at different stages of cold stress, some of which have not been previously connected to cold stress-response in insects. However, only a few genes were upregulated during the same treatments in both species.

RESULTS AND DISCUSSION

In the present study we examined cold tolerance and traced changes in gene expression after rapid cold hardening and cold acclimation in two *D. virilis* group species, *D. montana* and *D. virilis*. We also examined the modulation of gene expression during the recovery from chill coma in *D. montana* (see treatments in Table 1). Our microarray consisting of 219 candidate genes clearly showed that cold response includes changes in genes involved at least in heat shock response, circadian rhythm and metabolism. Only a few genes showing expression changes during rapid cold hardening or cold acclimation overlapped between the two species, which is most likely due to better adaptation of *D. montana* to low temperature conditions.

Fly cold tolerance measured as chill coma recovery time

The chill coma recovery times of 20-day-old control, cold hardened and cold acclimated females were measured after keeping the flies for 16 hours at -6°C. In general, *D. montana* females recovered considerably faster from the chill coma than *D. virilis* females (ANOVA: $F_{1,95} = 232.5$, $P = 0.004$). In the control treatment the recovery times of *D. montana* were on average 10 minutes and those of *D. virilis* were about 23 minutes. The difference in cold tolerance between the two species was expected as the recovery times typically show a negative correlation with the latitude of the species and population distribution (Gibert *et al*., 2001; Hoffmann *et al*., 2002). *D. virilis* females also differ from *D. montana* females in their inability to enter reproductive diapause (Watabe, 1983), which is a common overwintering strategy in northern *D. virilis* group species (e.g. Lumme 1978). In this study, the possible effects of diapause on female cold hardening, acclimation and overall cold tolerance were excluded by maintaining the flies in diapause preventing conditions.

Maintenance of female flies at moderately low temperatures (0 °C and +5 °C) before the cold treatment had a significant effect on their chill coma recovery times in both species (*D. mon*: $F_{2,159} = 58.8$, P = 0.001; *D. vir*: $F_{2,127} = 22.1$, P = 0.006; Fig. 1). However, post-hoc tests revealed that rapid cold hardening did not have any detectable effects on recovery times in either of the species (Tukey HSD: *D. mon*: P = 0.439; *D. vir*: P = 0.921), contrary to the findings in our previous study, where cold hardening had a small but significant effect on chill coma recovery time in *D. montana* (Vesala & Hoikkala, 2011). The result of the present study is in concordance with the study by Rako and Hoffmann (2006), where rapid cold hardening was found to have no effect on chill coma recovery times in *D. melanogaster*. However, it has been postulated that even if the effects of rapid cold hardening may be too transient to cause changes in recovery times (MacMillan & Sinclair, 2011), they may still be beneficial for insect survival (Lee *et al*., 1987) and/or activity (Kelty & Lee, 2001). While the rapid cold hardening treatment might have been too weak to elicit responses in recovery times in our study species, the cold acclimation treatment appeared to be more effective as it shortened the chill coma recovery times significantly both in *D. montana* (Tukey HSD: P < 0.001) and *D. virilis* (P < 0.001).

Changes in gene expression during rapid cold hardening

The maintenance of female flies for an hour at 0 °C (rapid cold hardening treatment) elicited expression differences in only three genes, and only in *D. montana*. These genes, as well as other genes showing significant up- or downregulation under this or other treatments, are shown in Table 2 with their corresponding fold changes compared to control. Transcriptional changes during the rapid cold hardening have previously been examined by Qin *et al*. (2005), who also found only few genes (37 out of 12 000 transcripts on the array) to show expression changes after cold hardening in *D. melanogaster* males. In their study the upregulated genes involved many membrane protein coding genes, as well as heat shock genes.

Our study revealed only one gene, *Pyrroline 5-carboxylate reductase* (*P5cr*) that was upregulated during rapid cold hardening in *D. montana*, while none of the genes studied showed expression changes in *D. virilis*. *P5cr* codes for an enzyme important in proline biosynthesis, which, in turn is found to be more abundant in cold-acclimated than non-acclimated insects (e.g. Fields *et al*., 1998). In addition, recent findings in *Chymomyza costata* show that proline is an important compound in extreme cold tolerance (Koštál *et al*., 2011). Our finding implies that the cryoprotectant production may be initiated in *D. montana* already after a relatively short cold exposure.

Downregulation during rapid cold hardening occured in two genes in *D. montana*: *Ecdysone-induced protein 28/29kD* (*Eip71CD,* also known as *MsrA*) and *clockwork orange* (*cwo*). The transcription of *Eip71CD* is activated by the presence of the hormone ecdysone in *Drosophila* (Savakis *et al*., 1980) and it codes for the enzyme methionine sulfoxide reductase, which functions in defense against oxidative stress (Sun *et al*., 1999; Weissbach *et al*., 2005). Ecdysone belongs to the

ecdysteroid hormones, which play a crucial role at various stages during post-embryonic development in insects (Riddiford, 1993). In adult *D. melanogaster* females ecdysone functions in the regulation of vitellogenesis and yolk protein uptake by oocytes (Richard *et al*., 1998; 2001). Low levels of ecdysone at low temperatures could halt the yolk protein uptake, which leads to lower levels of *Eip71CD* transcription. The second downregulated gene in *D. montana*, *clockwork orange* (*cwo*), is involved in the regulation of circadian rhythms (Matsumoto *et al*., 2007); changes in its expression could partly be induced by the transfer of the flies from light:dark cycle to darkness during the cold hardening treatment (see methods).

Changes in gene expression during cold acclimation

Acclimation at +5 °C for six days induced upregulation in 8 and downregulation in 6 of the 219 genes in *D. montana*, when compared to the control. Respective numbers for *D. virilis* were 11 and 7 (Table 2). Below we have grouped the genes into three main categories according to their function in heat shock response, circadian rhythm and metabolism.

Several probes corresponding one of the heat shock genes in *D. melanogaster*, *hsr-omega* (*Hsr*ω), showed upregulation during cold acclimation in both *D. montana* and *D. virilis*. Sequence variation in *Hsr*ω has been associated to heat tolerance in *D. melanogaster* (McColl *et al*., 1996). Moreover, Collinge *et al*. (2008) found a correlation between DNA repeat number and cold resistance in this species, as well as about 6-fold upregulation of *Hsr*ω transcription in flies after a 6-hour cold acclimation at 0 °C (followed by a 20-minute recovery period). Other heat shock genes on our array showed species-specific changes in their expression. Upregulated heat shock genes included *Hsp68* in *D. montana* and *Hsp67bc, Hsp20* and *Hsc70-2* in *D. virilis.* In addition, a transcript corresponding to the CG7182 gene, which has a predicted function of response to heat (Flybase, FB2011_09; http://flybase.org/; Tweedie *et al*., 2009), was upregulated in *D. montana*. Downregulation occurred in *Dna-J-like-1* (*DnaJ-1*; also known as *Hsp40*) in *D. montana* and *Hsp70/Hsp90 organizing protein homologue* (*Hop*) in *D. virilis*. Heat shock response is a general stress response (reviewed in Feder & Hoffmann, 1999), which can be induced by many kinds of stressors, including low temperatures in insects (Rinehart *et al*., 2007). Consequently, various *Hsp* genes have been found to show modulated expression during the recovery from cold stress (Colinet *et al*., 2010), even though the specific heat shock genes activated seem to vary among the species.

Cold acclimation also elicited changes in genes connected to circadian rhythmicity. Out of the seven main genes involved in the circadian clock in *Drosophila* (*period, timeless, double-time, Clock, cycle, vrille, shaggy* and *cryptochrome*) two, *period* (*per*) and *vrille* (*vri*), were upregulated during cold acclimation in both species on our array. In our study L:D cycle was kept the same (22:2) for the flies of cold acclimation and control groups, and the RNA samples were collected within 5-6 hours after the lights on (Zeitgeber Time, ZT 5-6) to diminish the expression changes due to light-mediated oscillations of the

circadian clock. Similar results to ours have been found in a study on the ruin lizard (*Podarcis sicular*), where *Period 2*, a central gene in the lizard circadian clock system, was found to be expressed at a higher level during acclimation at 6°C than at a high temperature (29 °C), when the light:dark cycle (and ZT) was kept the same between the treatments (Magnone *et al*., 2005).

We also found expression changes in four other genes which have been shown to exhibit rhythmic expression patterns: *eyeless* (*ey*) was upregulated in both species, *jetlag* (*jet*) in *D. montana* and *black* (*b*) and *Rhodopsin 3* (*Rh3*) in *D. virilis; ey* is involved in regulation of transcription (Clements *et al*., 2008) and *jet* in entrainment of the circadian clock by photoperiod (Koh *et al*., 2006). *Black* has a glutamate decarboxylase activity, and typically shows an expression peak in the dark phase of the light:dark cycle in *D. melanogaster* (Ueda *et al*., 2002). *Rh3* codes for one of the rhodopsins involved in phototransduction (Feiler *et al*., 1992), and it has also been found to show rhythmic expression (Ueda *et al*., 2002). Interestingly, recent evidence shows that rhodopsins are required, along with light sensation, for thermosensation in *Drosophila* (Shen *et al*., 2011).

The third group of genes showing expression changes (mainly downregulation) during cold acclimation consisted of genes affecting metabolism. Two genes involved in carbohydrate metabolism, *Phosphogluconate mutase* (*Pgm*) and *Trehalase* (*Treh*), were downregulated only in *D. montana* and one gene, *UDP-galactose 4'-epimerase* (*Gale*) was downregulated in both species. *Pgm* codes for an enzyme important in glycogen synthesis (Ray & Roscelli, 1964), while *Treh* codes an enzyme which catalyzes the conversion of trehalose to glucose. These changes may be related to regulation of glycogen and trehalose concentrations during low temperature exposure: glycogen content has been shown to decrease at low temperature in the arctic collembolan *Onychiurus arcticus* (Worland *et al*., 1998). Accumulation of trehalose, on the other hand, has been found to take place during cold acclimation in many insect species, e.g. in the granary weevil (*Sitophilus granarius*), in the rusty grain beetle (*Cryptolestes ferrugineus*; Fields *et al*., 1998) and in the cabbage root fly (*Delia radicum*; Koštál & Simek, 1995). *Gale* is linked to galactose metabolic process (Sanders *et al*., 2010). Cold acclimation also induced downregulation in two genes coding stearoyl-CoA 9 desaturases: *desaturase 1* (*desat 1*) in *D. montana* and *desaturase 2* (*desat 2*) in *D. virilis*. Both of these genes function in cuticle hydrocarbon (mating pheromones in many *Drosophila*; Jallon, 1984) biosynthesis in *D. melanogaster*, but there is also evidence that desaturases function in stress resistance (e.g. Greenberg *et al*., 2003). Furthermore, *Cytochrome-b5-related* (*Cyt-b5-r*), linked to the lipid metabolic process, was downregulated in *D. virilis*.

The only upregulated gene related to metabolism in *D. montana* was *Thor* (also known as *4E-BP*). This gene has multiple noteworthy functions: it is known to act as a translation inhibitor in cells through binding translation initiation factor eIF4E (Haghighat *et al*., 1995) and increased activity of 4E-BP has also been reported to cause fat accumulation in *Drosophila* (Teleman *et al*., 2005). Furthermore, the transcription of *Thor*, as well as the amount of the 4E-BP protein, has been found to be upregulated under oxidative stress (Teleman *et al*., 2005; Tettweiler *et al*., 2005). Oxidative stress is responsible for some of the

injuries brought upon by low temperatures, a phenomenon that is studied especially in plants (e.g. Prasad *et al*., 1994). Thus the upregulation of *Thor* may play an important role in coping with low temperatures.

Among the genes involved in neurotransmitter (amine) metabolism and transport, *Serotonin receptor 7* (*5-HT7*) and *Serotonin transporter* (*SerT*), were upregulated and α *methyl dopa resistant* (*amd*) downregulated in *D. virilis*. Studies, for example, in the honey bee (Harris & Woodring, 1992) and *Drosophila* (Rauschenbach *et al*., 1993) have shown that stress conditions may cause an increase in biogenic amine content, and injections of the biogenic amine octopamine has been shown to increase the levels of sugars and lipids in insect hemolymph (Candy 1979, Orchard *et al*., 1982). However, based on this study only, we cannot say whether the changes in the expression of these genes in *D. virilis* are linked with the amine content in the flies. In addition to above mentioned changes, also *Shibire* (*Shi*) was upregulated in *D. virilis*. *Shi* encodes a dynamin-like protein in *Drosophila* and function in endocytosis (Chen *et al*., 1991).

Changes in gene expression during recovery from chill coma in D. montana

Gene expression changes during recovery from chill coma were traced at two time points in *D. montana*: when the flies had just recovered from chill coma (15 minutes after the cold treatment) and when the flies had spent one hour at room temperature. The physiological and genetic changes taking place during the chill coma and recovery from it are still somewhat unclear. Chill coma could be due to the loss of the ability to generate muscle action potentials, disruption of ion homeostasis and/or changes in cell membrane fluidity (reviewed in MacMillan & Sinclair, 2011). Chill coma recovery time, on the other hand, depends for example on the duration and severity of the cold shock and it is not simply a reversal of chill coma (MacMillan & Sinclair, 2011).

In our study, we found only transcripts corresponding to *Glyceraldehyde 3 phosphate dehydrogenase 1* (*Gapdh1*) to be upregulated during both recovery periods. *Gapdh 1* encodes an enzyme involved in the glycolytic pathway, where glucose is broken down and energy and pyruvate produced. Pyruvate is then directed to tricarboxylic acid cycle (citric acid cycle) to harvest energy from glucose and other organic fuel molecules. The upregulation of this enzyme-coding gene is probably caused by the need to compensate slowed down / ceased enzyme activity at low temperature. Michaud and Denlinger (2007) found that the amount of pyruvate increased in rapid cold hardening in *S. crassipalpis* and suggested that the increase is due to inactivation of enzymes downstream of pyruvate. In addition to *Gadph 1*, *Hsp68*, *Hsrω*, *Thor* and *derailed* (*drl*), a gene coding a protein kinase, were upregulated during the 1-hour recovery period. *Eip71CD* and *MLF1-adaptor molecule* (*Madm*) were downregulated during recovery periods, and *Open rectifier K+ channel 1* (*Ork*), a potassium channel protein, during 15-min recovery. *Madm* codes for a protein with serine/threonine kinase activity (Flybase, FB2011_09) and it has

previously been found to be downregulated during rapid cold hardening in *D. melanogaster* (Qin *et al*., 2005).

Validation of expression changes with quantitative real time PCR (qPCR)

We tested eight different gene-treatment combinations with qPCR. All of the tested cases showed expression changes in the same direction as detected in the microarray analysis, although in one case the expression difference between the treatment and the control sample was not significant in qPCR analysis. Expression values of tested genes relative to the control are shown in Fig. 2 (see also Table 2 for comparisons of fold changes). *Eip71CD*, tested during rapid cold hardening and 1-hour recovery in *D. montana* and during cold acclimation in *D. virilis*, was significantly downregulated in both microarray and qPCR in all of these treatments ($P < 0.001$). *P5cr* was downregulated during rapid cold hardening in *D. montana* according to microarray and qPCR analysis, but in qPCR the expression change was not significant ($P = 0.100$). *Hsr*ω was tested during cold acclimation in both species and during 1-hour recovery in *D. montana*, and was significantly upregulated also in qPCR analysis ($P < 0.001$). *Hsp68* was tested during 1-hour recovery, and showed upregulation in both microarray and qPCR analyses ($P < 0.001$). Altogether, the results indicate good congruence between the microarray and qPCR methods.

Conclusions

The present study provides a set of cold-responsive genes in *D. montana* and *D. virilis* and shows that the genes are mainly linked with the heat shock response, circadian clock and metabolism. The two species differed in their chill coma recovery times, as well as in the gene expression patterns. The lack of overlap in genes expressed during cold treatments supports the statement made by Sarup *et al*. (2011), that some candidate genes obtained for certain phenomenon may not overlap even in studies on the same or closely-related species. Our results may also reflect species' adaptation to different environmental conditions, as *D. montana* is adapted to northern conditions and is able to overwinter in reproductive diapause, whereas *D. virilis* lacks this ability. We found only two genes to show consistent expression changes in both study species: *Eip71CD*, which was downregulated in nearly all of the treatments, and *Hsr*ω, which was upregulated during cold acclimation. Heat shock response was evident during most of the cold treatments, but the expression changes in specific heat shock genes varied depending on the treatment and species.

While the amount of data on the effects of circadian clock genes on insect life-history traits is increasing, there is debate concerning whether the circadian clock itself underpins seasonal photoperiodicity or whether the observations supporting this idea are due to the pleiotropic effects of the circadian clock genes (e.g. Bradshaw & Holzapfel, 2010). Accordingly, upregulation of circadian rhythm-related genes during cold treatments in *D. montana* may suggest that circadian clock is involved in cold acclimation or that the detected

expression changes are due to the pleiotropic functions of these genes. In *D. melanogaster* cold temperature has been found to affect the function of the circadian clock at least through thermosensitive splicing in the *per* gene, resulting in higher levels of *per* transcript, earlier accumulation of the PER protein and advanced evening activity of the flies (Majercak *et al*., 1999). In plants, the cycling patterns of many circadian-controlled genes are known to change at low temperatures, either to show low-amplitude cycles in diurnal conditions or to stop cycling in continuous light (Bieniawska *et al*., 2008).

Information obtained in this study provides a good basis for further studies, e.g. on the expression changes and differential splicing of 'cold tolerance genes' at different times of year as well as on clinal variation in their structure and function in *D. montana*. When linked with physiological changes, these studies will help to understand processes involved in adaptation to the seasonally varying temperature conditions of high latitudes.

EXPERIMENTAL PROCEDURES

Fly material

We used female flies from *D. montana* strain (O3F77) from Oulanka, Finland (65°N) and *D. virilis* strain (V-Toyama-09) from Toyama, Japan (36°N). Strain names refer to the stock collections at the University of Jyväskylä, where the experiment was conducted. Both strains have been established in 2003 from the progeny of a single wild-caught female and maintained in 500 ml bottles containing malt-yeast medium in continuous light and at 19ºC since their establishment until the study was conducted in 2010. Experimental flies were sexed within one day after eclosion under light CO_2 anesthesia and the females were transferred into vials containing food medium. The vials were placed in a light:dark (LD) cycle of 22:2 at 19ºC and the females were transferred into fresh food vials once a week until they were used in the experiments at the age of 20 days. The 22 hours of light represent normal summer conditions in northern Finland, from where the *D. montana* strain originates. In these conditions all *D. montana* females develop ovaries, i.e., do not diapause (Salminen, T.S., unpublished), and thus *D. montana* and *D. virilis* females were in the same physiological state when the experiment was conducted.

Chill coma recovery times of control, cold hardened and cold acclimated flies

The chill coma recovery times of sexually mature *D. montana* and *D. virilis* females were measured after keeping the flies in a cold chamber at -6°C for 16 hours. To test the effects of rapid cold hardening and cold acclimation on fly recovery times, the females were exposed to no pretreatment (control group) or one of the two pretreatments (cold hardening and acclimation groups) prior to the cold treatment. Females of the control and cold hardening groups were transferred from culturing conditions (LD 22:2, 19°C) at the age of 20 days and

the latter were kept at 0ºC for one hour (in darkness) prior to the cold treatment. Flies of the cold acclimation group were transferred from 19°C to 5ºC (LD 22:2) at the age of 14 days to allow them to acclimate for six days. The females of all groups were kept at -6ºC for 16 hours in vials containing agar for moisture, after which they were placed on dishes with lids and separate compartments for individual flies. The time when a fly was able to stand up after the cold shock was marked as its chill coma recovery time. We tested simultaneously 15-20 females per group for both species, repeating the test three times during a two-week time period. Altogether 45-55 individuals were tested for each group.

The chill coma recovery time data were log-transformed to obtain normal distribution and homoscedasticity and analyzed using analysis of variance (ANOVA) with PASW Statistics 18.0 (SPSS Inc.). In these tests the chill coma recovery time was used as a dependent variable and species and pretreatments as independent variables (factors). Replicate number was included in the test as a random factor.

Gene expression

Preparation of RNA samples

We collected samples (female flies) for RNA extractions from the control, cold hardening and cold acclimation groups (Table 1) to trace changes in the expression patterns of a set of candidate genes during the treatments. For *D. montana*, we also collected flies that had been allowed to recover after the cold treatment (16 hours at -6°C) either for 15 minutes (recovery 1) or 1 hour (recovery 2) at 21 +/- 1°C. These samples enabled us to trace changes in gene expression right after the flies had recovered from chill coma and after they had spent a longer period at a favorable temperature. Because of the limited space on the array plate, the recovery samples were collected only for *D. montana*. All the samples were collected 5-6 hours after the lights had been turned on in the chamber and the flies were immediately immersed in liquid nitrogen, after which they were stored at -84ºC. Ten individual flies were pooled to form a sample, and several samples were collected for each control and pretreatment group. Subsequently, three samples (pools of ten flies) were used for microarray and five (including the ones used for microarray) for qPCR as replicates.

Microarray analysis

Our original candidate gene microarray (Agilent 60-mer Multi-Pack Gene Expression Microarray with One Color-system) included probes for 101 genes designed specifically for *D. montana*, with no genome sequence available (for more information on probe design, see Kankare *et al*., 2010). Prior to the present experiment we added additional probes for 118 genes, including several genes found to affect heat and cold tolerance, using a protocol described in Kankare *et al*. (2010). We also constructed species-specific probes for all the genes for *D. virilis* and added the probes in both sense and antisense directions according to

D. virilis/D. melanogaster sequences. The *D. virilis* probes were designed using genome data available for this species, independently from *D. montana* probes. The final microarray contained probes for 219 genes, and 2-5 probes per gene, for both species (see Supporting information for the complete list of genes).

Three pools of ten flies per sample were ground, transferred into 1.5 ml tubes and mixed with 600 µl of lysis buffer. Total RNA was extracted using Qiagen RNA extraction kit with RNase-Free DNase treatment according to manufacturer's protocols (QIAGEN, Hilden, Germany). The purity of each RNA sample was measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the integrity of these samples was checked with Agilent's Bioanalyzer (Agilent, Santa Clara, CA, USA). 600 ng of total RNA from each replicate was amplified and Cy3-labeled with Agilent's Quick Amp Labeling (One Color) Kit and processed together with Agilent's One-Colour RNA Spike-in Kit. The cRNA concentration (and also specific activity for cRNA) was checked with Nanodrop ND-1000 and the cRNA quality with Agilent 2100 bioanalyzer using RNA 6000 Nano kit, both before and after the amplifications.

In hybridization step 600 ng of each cRNA sample was hybridized to Agilent's 8x15K *D. montana* / *D. virilis* custom arrays at 65˚C. Hybridization was performed overnight using Agilent's Gene Expression Hybridization Kit and the plates were washed with Agilent's Gene Expression Wash Pack and Stabilization and Drying solution. Arrays were scanned with Agilent Technologies Scanner (model G2565CA) and numerical results were extracted with Feature Extraction software version 10.7.1 using 026691_D_F_20091221grid, GE1_107_Sep09 protocol and GE1_QCMT_Sep09 metric set.

The downstream analysis of the microarray data was carried out using R (R Foundation for Statistical Computing) and Bioconductor software (Gentleman *et al.*, 2004). A Probe level quantile normalization method was used for between sample normalization, after which the signals were summarized for each probe type by taking a median value. The data was found to be of high quality and the Pearson's correlations between the samples were between 0.86 and 0.99 in the different sample groups indicating high reproducibility. The statistical analyses were carried out using Bioconductor's Limma package. For filtering out differentially expressed genes, the minimum fold change limit was set at 2 and false discovery rate (FDR) adjusted significance level at 0.05. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Edgar *et al.*, 2002) and is accessible through GEO series accession number GSE31103 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31103).

Quantitative real time PCR analyses

The microarray results were confirmed with quantitative real time PCR (qPCR). We chose eight different gene-treatment combinations to be validated with qPCR, some of the genes being checked in more than one group (Table 3).

Complementary DNA (cDNA) for qPCR was generated from the RNA extracted from the experimental females as described above by reverse transcription with iScript cDNA kit (Bio-Rad Laboratories, Hercules, CA, USA), using a unique mixture of random and oligo-dT primers. RNA (400 ng) from five separate pools of ten flies per each treatment was used to generate cDNA and each of these samples was used in triplicate in qPCR. 20 µl reactions contained 10 µl of 2x Power SYBR Green PCR Master Mix (Bio-Rad Laboratories), 0.3 µM of each gene-specific primer and 1 µl of concentrated cDNA solution. Cycling conditions in Bio-Rad CFX96 instrument were initiated at 95°C for 3 min, followed by denaturing at 95°C for 10 seconds, annealing at annealing temperature (Ta) for 10 seconds and extension at 72°C for 30 seconds. The whole cycle of denaturing, annealing and extension was repeated 40 times, and was followed by melting curve analysis to check the purity of qPCR reaction.

Reference genes for qPCR comparisons were selected among the control genes on the basis of their expression stability in the microarray analysis (marked with a star in Supporting information), using the geNorm program (Vandesompele *et al*., 2002). Three of the most stable genes were first selected for the pilot study and based on these results two of them were used in the qPCR analysis (Table 3). The only exception to this was *D. virilis* cold acclimation sample, where only the most suitable control gene, *Gapdh 1*, was used in the analysis. Primers were designed using Primer 3 program (version 0.4.0; http://frodo.wi.mit.edu/primer3/) or manually. Amplification efficiencies of all primers were calculated using serial dilutions of cDNA (pool of five control sample replicates) and the efficiency values were included in the analysis (Table 3). Relative expression values as well as the statistical significance of the results were calculated using the normalized expression method (ΔΔ(Ct)) with default threshold values using CFX Manager Software 2.0 (Bio-Rad Laboratories) and the REST 2009 program (with 10 000 iterations) (http://www.gene-quantification.de/rest-2009.html), respectively. REST 2009 Software applies a mathematic model that takes into account the different PCR efficiencies of the gene of interest and the reference genes and the statistical significance of the calculated expression ratios was determined using randomization and bootstrapping methods.

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TABLE 1 Descriptions of the pretreatments and chill coma recovery periods during which the RNA samples were collected. Control, cold hardening and cold acclimation samples were collected for both species, recovery samples only for *D. montana*.

Species	Treatment	Description
D. montana & D. virilis	Control	20 days at 19°C
D. montana & D. virilis	Cold acclimation	14 days at 19°C + 6 days at + 5°C
D. montana & D. virilis	Rapid cold hardening	20 days at 19°C + 1 hour at 0°C
D. montana	Recovery 1	16 hours at -6°C, 15-min recovery at 20 +/- 0.5°C
D. montana	Recovery 2	16 hours at -6°C , 1-hour recovery at 20 +/- 0.5°C

TABLE 2 List of 31 genes that showed significant expression level differences during the pretreatments or chill coma recovery periods compared to control in *D. montana* and *D. virilis* females. Gene ontologies (Biological processes, levels 3 and 4) were gathered using DAVID 6.7. (Huang *et al.*, 2009). RCH = Rapid cold hardening, CG number = annotation symbol. Corresponding fold changes obtained from qPCR analysis are presented for the genes, which were validated using this method.

	CG number	**Gene name**	**Gene symbol**	**FC**		**Biological process**
				array	qPCR	
RCH/ *D. mon*	6009	*Pyrroline 5-carboxylate reductase*	*P5cr*	2.5	1.3	Proline metabolic process
	7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*	-4.7	-6.0	Oxidation reduction
	17100	*clockwork orange*	*cwo*	-2.0		Regulation of circadian rhythm
RCH/ *D. vir*	No genes					
Cold acclimation/*D. mon*	CR31400	*hsr-omega*	*Hsrω*	4.8	3.0	Response to heat
	1464	*eyeless*	*ey*	3.2		Adult locomotory behavior
	5436	*Heat shock protein 68*	*Hsp68*	2.8		Response to heat
	7182	*CG7182*	*CG7182*	2.4		Response to heat
	14029	*vrille*	*vri*	2.4		Circadian rhythm
	8846	*Thor*	*Thor*	2.2		Regulation of transcription
	8873	*jetlag*	*jet*	2.1		Circadian rhythm
	2647	*period*	*per*	2.1		Circadian rhythm
	5165	*Phosphogluconate mutase*	*Pgm*	-5.0		Carbohydrate metabolic process
	7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*	-3.7		Oxidation reduction
	5887	*desaturase 1*	*desat 1*	-3.3		Lipid metabolic process
	12030	*UDP-galactose 4'-epimerase*	*Gale*	-3.2		Carbohydrate metabolic process
	10578	*DnaJ-like-1*	*DnaJ-1*	-2.2		Response to heat
	9364	*Trehalase*	*Treh*	-2.1		Carbohydrate metabolic process
Cold acclimation/ *D. vir*	CR31400	*hsr-omega*	*Hsrω*	8.0	8.3	Response to heat
	7811	*black*	*b*	6.3		Cellular amino acid metabolic process
	4190	*Heat shock protein 67Bc*	*Hsp67Bc*	5.9		Response to heat
	12073	*Serotonin receptor 7*	*5-HT7*	3.4		Signal transduction
	4545	*Serotonin transporter*	*SerT*	2.6		Neurotransmitter transport
	18102	*shibire*	*shi*	2.4		Response to heat
	2647	*period*	*per*	2.2		Circadian rhythm
	1464	*eyeless*	*ey*	2.2		Locomotory behavior

	10888	*Rhodopsin 3*	*Rh3*	2.1		Signal transduction, response to light
	4461	*Heat shock protein 20*	*Hsp20*	2.0		Response to heat
	14029	*vrille*	*vri*	2.0		Circadian rhythm
	6009	*Pyrroline 5-carboxylate reductase*	*P5cr*	-5.5		Proline metabolic process
	2720	*Hsp70/Hsp90 organizing*	*Hop*	-4.3		Protein metabolic process
	5925	*desaturase 2*	*desat 2*	-3.8		Lipid metabolic process
	13279	*Cytochrome b5-related*	*Cyt-b5-r*	-3.3		Lipid metabolic process
	7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*	-2.7	-3.5	Oxidation reduction
	12030	*UDP-galactose 4'-epimerase*	*Gale*	-2.4		Carbohydrate metabolic process
	10501	*α methyl dopa-resistant*	*amd*	-2.4		Amine metabolic process
Recovery 15min/ *D. mon*	12055	*Glyceraldehyde 3 phosph.deh. 1**	*Gapdh 1*	2.2		Carbohydrate metabolic process
	7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*	-6.6		Oxidation reduction
	1098	*MLF1-adaptor molecule*	*Madm*	-3.1		Phosphate metabolic process
	1615	*Open rectifier K^+ channel 1*	*Ork1*	-2.1		Ion transport, circadian rhythm
Recovery 1 hour/ *D. mon*	5436	*Heat shock protein 68*	*Hsp68*	2.7	5.2	Response to heat
	CR31400	*hsr-omega*	*Hsrω*	2.7	2.2	Response to heat
	8846	*Thor*	*Thor*	2.5		Regulation of transcription
	17348	*derailed*	*drl*	2.3		Signal transduction
	12055	*Glyceraldehyde 3 phosph.deh. 1**	*Gapdh 1*	2.0		Carbohydrate metabolic process
	7266	*Ecdysone-induced protein 28/29kD*	*Eip71CD*	-10.2	-10.3	Oxidation reduction
	1098	*MLF1-adaptor molecule*	*Madm*	3.1		Phosphate metabolic process

* Glyseraldehyde 3 phosphate dehydrogenase 1

TABLE 3 Primer sequences and efficiencies (%) for genes validated with quantitative PCR and control genes used in the analysis.

Treatment	Gene (study/control)	Primer Sequence F/R	E %	R^2
Rapid cold hardening/ *D. montana*	*P5cr* (study)	5'CAGTGACTGCGTCGATCATT3' 5'CTAGAGGAGACATTCGCGTT3'	90.7	0.995
	Eip71CD (study)	5'ACATTTGGCATGGGCTGCTT3' 5'TGTTCCAGAACAGATCCAGC3'	99.6	0.987
	RpL11 (control)	5'GGTTTTAGAGCAGCTGACTG3' 5'CCATAGATACCGATTGAGGG3'	99.4	0.988
	RpL40 (control)	5'ATGCCAAGCTGTCGCACA3' 5'GAAGCCCGTGGGTAGCAT3'	99.4	0.988
Cold acclimation/ *D. montana*	*Hsrω* (study)	5'GGCGTTGAATAGTTGATACGC3' 5'AGTACCCCTGTGTAGGTTAG3'	98.0	0.997
	His3.3A (control)	5'GGCACGTACCAAGCAAACAG3' 5'TGGAATGGCAGCTTGCGGAT3'	110.7	0.985
	Gadph1 (control)	5'GGGCTGTATGCATCCAAGTT3' 5'GTTGTCAACGGCCAGAAGAT3'	98.9	0.987
1-hour recovery / *D. montana*	*Hsp68* (study)	5'GCCATTTGATGGCTTCACTG3' 5'CCGGCAATGCCAAGAACATA3'	91.0	0.978
	Hsrω (study)	5'GGCGTTGAATAGTTGATACGC 5'AGTACCCCTGTGTAGGTTAG3'	98.0	0.997
	Eip71CD (study)	5'ACATTTGGCATGGGCTGCTT3' 5'TGTTCCAGAACAGATCCAGC3'	99.6	0.987
	Gapdh 2 (control)	5'CCTGTTCAAGTTCGACTCCA3' 5'GTGCCGAAATGATGACCTTC3'	92.5	0.988
	RpL40 (control)	5'ATGCCAAGCTGTCGCACA3' 5'GAAGCCCGTGGGTAGCAT3'	99.4	0.988
Cold acclimation/ *D. virilis*	*Hsrω* (study)	5'GGCGTTGAATAGTTGATACGC3' 5'AGTACCCCTGTGTAGGTTAG3'	98.0	0.997
	Eip71CD (study)	5'ACATTTGGCATGGGCTGCTT3' 5'TGTTCCAGAACAGATCCAGC3'	92.9	0.997
	Gapdh1 (control)	5'GGGCTGTATGCATCCAAGTT3' 5'GTTGTCAACGGCCAGAAGAT3'	96.5	0.972

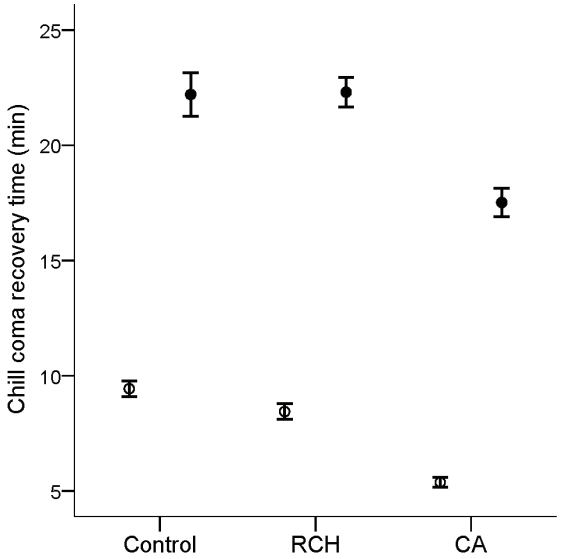

FIGURE 1 The mean recovery times of females (+/- 1 SE) after a 16-hour cold treatment at -6°C in *D. montana* (marked with an open circle) and *D. virilis* (marked with a filled circle). The flies had no pretreatment (control) or were either allowed to cold harden (1 hour at 0°C) or cold acclimate (6 days at +5°C) prior to the cold treatment. RCH = Rapid cold hardening, CA = Cold acclimation.

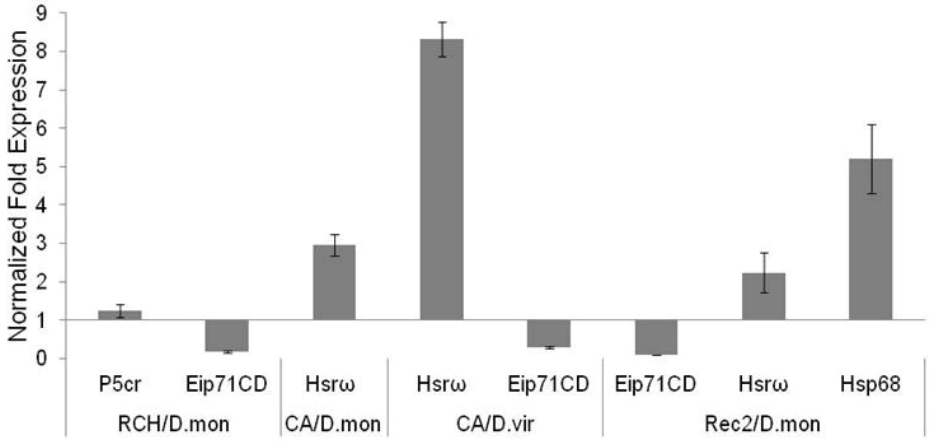

FIGURE 2 Normalized expression values of the study genes (relative to control) validated with quantitative real time PCR (qPCR). Error bars represent standard errors of mean expression based on five biological replicates. Genes that show a ratio under 1 compared to control are downregulated, and those with a ratio above 1 are upregulated. RCH = Rapid cold hardening, CA = Cold acclimation, REC2 = 1-hour recovery after chill coma.

SUPPORTING INFORMATION 1 The complete list of genes on *D. montana* / *D. virilis* microarray.

Gene ID	CG number	Gene name/synonym	FBgn
5-HT7	12073	*Serotonin receptor 7*	FBgn0004573
14-3-3-epsilon	31196	*14-3-3ε*	FBgn0020238
Ace	17907	*Acetylcholine esterase*	FBgn0000024
Acon	9244	*Aconitase*	FBgn0010100
*Act_42A**	12051	*Actin 42A*	FBgn0000043
Adar	12598	*Adenosine deaminase acting on RNA*	FBgn0026086
Adf1	15845	*Adh transcription factor 1*	FBgn0000054
Adh	3481	*Alcohol dehydrogenase*	FBgn0000055
amd	10501	*a methyl dopa-resistant*	FBgn0000075
Amph	8604	*Amphiphysin*	FBgn0027356
Ankyrin	1651	*Ankyrin*	FBgn0011747
ari-1	5659	*ariadne*	FBgn0017418
Arr1	5711	*Arrestin 1*	FBgn0000120
Arr2	5962	*Arrestin 2*	FBgn0000121
ato	7508	*atonal*	FBgn0010433
Atp-alpha	5670	*Na pump a subunit*	FBgn0002921
b	7811	*black*	FBgn0000153
B4	9239	*B4*	FBgn0023407
bsk	5680	*basket*	FBgn0000229
Btk29A	8049	*Btk family kinase at 29A*	FBgn0003502
btv	15148	*beethoven*	FBgn0023096
cac	1522	*cacophony*	FBgn0005563
Caki	6703	*Calcium/calmodulin-dependent protein kinase*	FBgn0013759
Cam	8472	*Calmodulin*	FBgn0000253
CaMKII	18069	*Calsium/calmodulin-depend.prot.kin.II*	FBgn0004624
Cat	6871	*Catalase*	FBgn0000261
Cdc37	12019	*Cdc37*	FBgn0011573
Cdk9	5197	*Cyclin-dependent kinase 9*	FBgn0019949
CdsA	7962	*CDP diglyceride synthetase*	FBgn0010350
CG3061	3061	*CG3061*	FBgn0038195
CG3814	3814	*CG3814*	FBgn0025692
CG4049	4049	*CG4049*	FBgn0034976
CG4164	4164	*CG4164*	FBgn0031256
CG5001	5001	*Heat shock prot.cognate DnaJ, N-term.*	FBgn0031322
CG6785	6785	*CG6785*	FBgn0032399
CG7182	7182	*Heat shock protein 70 (Hsp70)*	FBgn0035878
CG7650	7650	*CG7650*	FBgn0036519
CG8630	8630	*CG8630*	FBgn0038130
CG9743	9743	*CG9743*	FBgn0039756
CG9747	9747	*CG9747*	FBgn0039754
CG9934	9934	*CG9934*	FBgn0032467
CG10990	10990	*CG10990*	FBgn0030520
CG11251	11251	*CG11251*	FBgn0036346
CG12030	12030	*CG12030*	FBgn0035147
CG12020	12020	*Heat shock prot.cognate DnaJ, C-term.*	FBgn0035273
CG14650	14650	*Heat shock prot.cognate DnaJ, N-term.*	FBgn0037252
CG15531	15531	*CG15531*	FBgn0039755

CG17928	17928	*CG17928*	FBgn0032603
CG34366	34366	*CG34366*	FBgn0085395
CG42318	42318	*CG42318*	FBgn0260941
CkIIalfa	17520	*Casein kinase II aplha subunit*	FBgn0000258
Clk	7391	*Clock*	FBgn0023076
CoI	34067	*Mitochondrial Cytochrome c oxidase I*	FBgn0013674
cpo	31243	*couch potato*	FBgn0000363
CrebB17A	6103	*Cyclic-AMP resp.el.bind.prot. B17A*	FBgn0014467
cry	3772	*cryptochrome*	FBgn0025680
Csp	3695	*Cysteine string protein*	FBgn0004179
cwo	17100	*clockwork orange*	FBgn0259938
cyc	8727	*cycle*	FBgn0023094
Cyt-b5-r	13279	*Cytochrome b5-related*	FBgn0000406
dare	12390	*defective in the avoidance of repellents*	FBgn0015582
Dat	3318	*Dopamine N acetyltransferase*	FBgn0019643
dco	2048	*discs overgrown/double time*	FBgn0002413
Ddc	10697	*Dopa decarboxylase*	FBgn0000422
desat1	5887	*desat1*	FBgn0086687
esat2	5925	*desat2*	FBgn0043043
disco	9908	*disconnected*	FBgn0000459
DnaJ-1	10578	*DnaJ-like-1*	FBgn001565
dnc	32498	*dunce*	FBgn0000479
Dr	1897	*Drop*	FBgn0000492
drd	33968	*drop dead, beltless*	FBgn0260006
drl	17348	*derailed*	FBgn0015380
Droj2	8863	*DnaJ-like-2*	FBgn0038145
drpr	2086	*draper*	FBgn0027594
dsf	9019	*dissatisfaction*	FBgn0015381
Dsor1	15793	*Downstream of raf1*	FBgn0010269
dy	9355	*dusky*	FBgn0004511
e	3331	*ebony*	FBgn0000527
eag	10952	*ether a go-go*	FBgn0000535
*Ef1alfa48D**	8280	*Elongation factor 1 alpha 48D*	FBgn0000556
*eIF-4a**	9075	*Eukaryotic initiation factor 4a*	FBgn0001942
Eip71CD	7266	*Ecdysone-induced protein 28/29kD*	FBgn0000565
ey	1464	*eyeless*	FBgn0005558
FKBP59	4535	*FK506-binding protein FKBP59*	FBgn0029174
Fmr1	6203	*Fmr1*	FBgn0028734
for	10033	*foraging*	FBgn0000721
fru	14307	*fruitless*	FBgn0004652
Fst	9434	*Frost*	FBgn0037724
Gad1	14994	*Glutamic acid decarboxylase 1*	FBgn0004516
*Gapdh1**	12055	*Glyceraldehyde 3 phosp.dehydrogen. 1*	FBgn000109
*Gapdh2**	8893	*Glyceraldehyde 3 phosp.dehydrogen. 2*	FBgn0001092
Gbeta76C	8770	*G protein beta subunit 76C*	FBgn0004623
gl	7672	*glass*	FBgn0004618
Gp93	5520	*Glycoprotein 93*	FBgn0039562
*Gr66A**	7189	*Gustatory receptor 66a*	FBgn0035870
G-salpha60A	2835	*G protein sa 60A*	FBgn0001123
hang	32575	*hangover*	FBgn0026575
Histone_H3.3A	5825	*Histone H3.3A*	FBgn0014857
hiw	32592	*highwire*	FBgn0030600
homer	11324	*homer*	FBgn0025777

Hop	2720	*Hsp70/Hsp90 organizing protein hom.*	FBgn0024352
Hsc70-2	7756	*Heat shock protein cognate 2*	FBgn0001217
Hsc70-3	4147	*Heat shock protein cognate 3*	FBgn0001218
Hsc70-4	4264	*Heat shock protein cognate 4*	FBgn0001219
Hsc70-5	8542	*Heat shock protein cognate 5*	FBgn0001220
Hsc70Cb	6603	*Hsc70Cb*	FBgn0026418
Hsf	5748	*Heat shock factor*	FBgn0001222
Hsp20	4461	*Hsp20/CG4461*	FBgn0035982
Hsp23	4463	*Heat shock protein 23*	FBgn0001224
Hsp26	4183	*Heat shock protein 26*	FBgn0001225
Hsp60	12101	*Heat shock protein 60*	FBgn0015245
Hsp60B	2830	*Heat shock protein 60 related*	FBgn0011244
Hsp67Bc	4190	*Heat shock gene 67Bc*	FBgn0001229
Hsp68	5463	*Heat shock protein 68*	FBgn0001230
Hsp83	1242	*Heat shock protein 83*	FBgn0001233
Hsr_omega	31400	*Heat shock RNA ω*	FBgn0001234
ilp7	13317	*Insulin-like peptide 7*	FBgn0044046
imd	5576	*immune deficiency*	FBgn0013983
inaC	6518	*inactivation no afterpotential C*	FBgn0004784
inaD	3504	*inactivation no afterpotential D*	FBgn0001263
inaF	2457	*inactivation no afterpotential F*	FBgn0260812
InR	18402	*Insulin-like receptor*	FBgn0013984
ix	13201	*intersex*	FBgn0001276
jet	8873	*jetlag*	FBgn0031652
Jon25Bi	8867	*Jonah 25Bi*	FBgn0020906
Jon66Cii	7170	*Jonah 66Cii*	FBgn0035887
ken	5575	*ken and barbie*	FBgn0011236
knk	6217	*knickkopf*	FBgn0001321
l(2)efl	4533	*lethal (2) essential for life*	FBgn0011296
LanA	10236	*Laminin A*	FBgn0002526
lark	8597	*lark*	FBgn0011640
Madm	1098	*MLF1-adaptor molecule*	FBgn0027497
MAPk-Ak2	3086	*MAP kinase activated protein-kinase-2*	FBgn0013987
Mekk1	7717	*Mekk1*	FBgn0024329
Mhc	17927	*Myosin heavy chain*	FBgn0086783
mle	11680	*maleless*	FBgn0002774
mnb	42273	*minibrain*	FBgn0259168
Mpk2	5475	*Mpk2*	FBgn0015765
na	1517	*narrow abdomen*	FBgn0002917
nan	5842	*nanchung*	FBgn0036414
neb	10718	*nebbish*	FBgn0004374
Nf1	8318	*Neurofibromin 1*	FBgn0015269
ninaC	5125	*neither inactivation nor afterpotential C*	FBgn0002938
ninaD	31783	*neither inactivation nor afterpotential D*	FBgn0002939
ninaE	4550	*neither inactivation nor afterpotential E*	FBgn0002940
nompA	13207	*no mechanoreceptor potential A*	FBgn0016047
nonA	4211	*no on or off transient A*	FBgn0004227
norpA	3620	*no receptor potential A*	FBgn0262738
numb	3779	*numb*	FBgn0002973
ogre	3039	*optic ganglion reduced*	FBgn0004646
Ork1	1615	*Open rectifier K^+ channel 1*	FBgn0017561
ort	7411	*ora transientless*	FBgn0003011
p38b	7393	*p38b*	FBgn002484

P5cr	6009	*Pyrroline 5-carboyxlate reductase*	FBgn0015781
para	9907	*paralytic*	FBgn0260993
PEK	2087	*Pancreatic eIF-2aplha kinase*	FBgn0037327
per	2647	*period*	FBgn0003068
Pgm	5165	*Phosphogluconate mutase*	FBgn0003076
Pi3K92E	4141	*Pi3K92E*	FBgn0015279
Pka-C1	4379	*cAMP-dependent protein kinase 1*	FBgn0000273
Pka-R2	15862	*cAMP-dependent protein kinase R2*	FBgn0022382
ple	10118	*pale*	FBgn0005626
Pp1-87B	5650	*Protein phosphatase 1 at 87B*	FBgn0004103
Prp5	6227	*CG6227*	FBgn0030631
pwn	11101	*pawn*	FBgn0003174
qtc	14039	*quick-to-court*	FBgn0028572
rdgB	11111	*retinal degeneration B*	FBgn0003218
regucalcin	1803	*regucalcin*	FBgn003036
Rev1	12189	*Rev1*	FBgn003510
Rh3	10888	*Rhodopsin 3*	FBgn0003249
Rh4	9668	*Rhodopsin 4*	FBgn0003250
Rh5	5279	*Rhodopsin 5*	FBgn0014019
Rh6	5192	*Rhodopsin 6*	FBgn0019940
Rop	15811	*Ras opposite*	FBgn0004574
*RpL11**	7726	*Ribosomal protein L11*	FBgn0013325
*RpL19**	2746	*Ribosomal protein L19*	FBgn0002607
*RpL27A**	15442	*Ribosomal protein L27A*	FBgn0261606
*RpL40**	2960	*Ribosomal protein L40*	FBgn0003941
rut	9533	*rutabaga*	FBgn0003301
Sas	5232	*Sialic acid phosphate synthase*	FBgn0038045
sbb	5580	*scribbler*	FBgn0010575
scb	8095	*scab*	FBgn0003328
Sdc	10497	*Syndecan*	FBgn0010415
serT	4545	*Serotonin transporter*	FBgn0010414
sgg	2621	*shaggy*	FBgn0003371
Sh	12348	*Shaker*	FBgn0003380
shakB	34358	*shaking B*	FBgn0085387
shi	18102	*shibire*	FBgn0003392
sisA	1641	*sisterless A*	FBgn0003411
slgA	1417	*sluggish A*	FBgn0003423
slmb	3412	*supernumerary limbs*	FBgn0023423
slo	10693	*slowpoke*	FBgn0003429
Slob	6772	*Slowpoke binding protein*	FBgn0024290
sls	1915	*sallimus*	FBgn0086906
SNF4Agamma	17299	*SNF4/AMP-activ. prot. kinase gamma subunit*	FBgn0025803
so	11121	*sine oculis*	FBgn0003460
Sod	11793	*Superoxide dismutase*	FBgn0003462
spin	8428	*spinster*	FBgn0086676
stc	3647	*shuttle craft*	FBgn0001978
stv	32130	*starvin*	FBgn0086708
syt	3139	*synaptotagmin*	FBgn0004242
Thor	8846	*Thor*	FBgn0261560
tilB	14620	*touch insensitive larva B*	FBgn0014395
tim	3234	*timeless*	FBgn0014396
tipE	1232	*temperature-induced paralytic E*	FBgn0003710
tko	7925	*technical knockout*	FBgn0003714

Tpi	2171	*Triose phosphate isomerase*	FBgn0086355
tra	16724	*transformer*	FBgn0003741
Treh	9364	*Trehalase*	FBgn0003748
Trf	7562	*TBP-related factor*	FBgn0010287
Trh	9122	*Tryptophan hydroxylase*	FBgn0035187
trp	7875	*transient receptor potential*	FBgn0003861
trpgamma	5996	*trpγ*	FBgn003259
trpl	18345	*trp-like*	FBgn0005614
tutl	15427	*turtle*	FBgn0010473
tws	6235	*twins*	FBgn0004889
Ubi-p63e	11624	*Ubiquitin-63E*	FBgn0003943
vri	14029	*vrille*	FBgn0016076
y	3757	*yellow*	FBgn0004034

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