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233

Tiina S. Salminen

Timing Is Everything

Photoperiodicity Shaping Life-History Traits and Gene Expression

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ABSTRACT

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The effects of photoperiodic cues on the timing and shaping of life-history traits in northern latitude species are vast. In my doctoral thesis, I have studied the factors affecting the ability of *Drosophila virilis* group flies (mainly *D. montana*) to survive in northern latitudes with high seasonal and daily variation in environmental conditions. This was done by studying the role of photoperiod and temperature cues in evoking changes in reproductive diapause and other life-history traits important in adaptation to a seasonally varying environment, as well as by studying the expression patterns of genes affecting these traits. The main findings were that photoperiodic cues can induce changes in life-history traits at both pre (egg-to-eclosion development time and juvenile body mass) and post (reproductive diapause) eclosion stages. Measuring the day length and adjustment of life-history traits can be restarted after eclosion, which enables the flies to react quickly and optimally to changing photoperiods at different developmental stages. The sensitive period for induction of reproductive diapause was found to be temperature-dependent and connected to the enhanced rate of ovarian development in higher temperatures. When the females were able to detect both photo- and thermoperiods mimicking seasonal variation of their natural habitat, the seasonal switch to diapause happened within a remarkably short time window. The correct timing of this switch, as well as the environmental conditions that the females are experiencing during young adulthood, was found to have an effect also later in life as the effects of diapause was still seen e.g. in cold tolerance levels, after diapause was terminated as well as in the gene expression patterns.

Keywords: DNA-microarray; Drosophila; gene expression; life-history trait; reproductive diapause; vitellogenesis.

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CONTENTS

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-V. I am the first author in papers I, II and IV and carried out most of the study planning, experimental work, statistical analysis and writing. In study III, where I am the second author, I participated in the study planning, designing the DNA-microarray probes and writing the manuscript and I also prepared most of the samples used in the experiment. In study V, where I am the second author, I participated in the study planning, constructing the study lines and determining the female ovarian stages and I also performed the egg-to-eclosion development time experiment and participated in writing.

I Salminen T.S., Vesala L. & Hoikkala A. 2011. Detecting seasonal time: photoperiodic regulation of life-history traits in a northern fly species, *Drosophila montana.* Manuscript.

II Salminen T.S. & Hoikkala A. 2011. Effects of temperature on the induction of photoperiodic reproductive diapause in *Drosophila montana* malt fly. Manuscript.

III Kankare M., Salminen T.S., Laiho A., Vesala L. & Hoikkala A. 2010. Changes in gene expression linked with adult reproductive diapause in a northern malt fly species: a candidate gene microarray study. BMC Ecology 10(3): 1-9.

IV Salminen T.S., Vesala L., Laiho A., Merisalo M., Kankare M. & Hoikkala A. 2011. Plastic phenotypic and expressional changes regulated by seasonal cues enable insects to survive over the harsh winter period at high latitudes. Manuscript.

V Kankare M., Salminen T.S., Lampinen H. & Hoikkala A. 2011. Sequence variation in the *couch potato* gene and its effects on life-history traits in a northern malt fly, *Drosophila montana.* Submitted manuscript.

ABBREVIATIONS

CCRT	chill coma recovery time
CDL	critical day length
DNA	deoxyribonucleic acid
FC	fold change
L:D	light: dark cycle
qPCR	quantitative real time PCR
RDN	required day number
RNA	ribonucleic acid
SP	sensitive period
ZG	zeitgeber

1 INTRODUCTION

1.1 Photoperiodism

There is an optimal time for everything in nature: to develop, breed and hibernate, to eat, move and sleep, and to migrate and accumulate energy supplies. How do individuals know how to optimally time these events during the day or during different seasons? There are two major rhythms in the biosphere that can be followed through photoperiodic cues: the daily cycle caused by Earth's rotation around its own axis and the annual cycle of the seasons caused by Earth's rotation around the sun. In the northern latitudes, photoperiods, or hours of light during a day, are changing regularly during the year, increasing towards the summer and decreasing towards the autumn. Photoperiodism is an ability of organisms to assess and use the day length as an anticipatory cue to time seasonal events in their life. This ability cannot be overlooked when studying the mechanisms underlying the life-histories of any species living at temperate and polar latitudes. For example, hundreds of insect species are known to use photoperiodism to regulate their seasonal activities (Saunders 1982, Masaki 1983, Nishizuka et al. 1988). Fitness of the plants and animals living in a seasonal environment involves an ability to express an appropriate phenotype during different seasons in order to exploit these opportunities and, at the same time, avoid being exposed to lethal conditions (Bradshaw & Holzapfel 2007). Hence, fitness in a seasonal environment is all about timing.

Recent rapid climate change has resulted in warmer winters and earlier springs as well as the later onset of winters and longer growing seasons. Species which have adapted to time their life-history traits according to the photoperiodic cues may in the future suffer from a mismatch between photoperiod and temperature, as the warming climate does not alter day length at any locality on Earth. This causes challenges especially for immobile species, such as plants. Natural populations of several species from different taxonomic groups have already begun to respond to this mismatch by shifting their

distribution and timing of growth and reproduction (Parmesan & Yohe 2003). Unfortunately, the responses of many populations are likely to be inadequate to counter the speed and magnitude of climate change (Hoffman & Sgro 2011).

1.2 Timing and regulation of life-history traits

The ability of species to cope with seasonal changes in environmental conditions is greatly enhanced by phenotypic plasticity in various kinds of life-history traits. For example, changes in photoperiodic cues and temperature can induce changes in traits such as behaviour, coloration, development time, body mass, temperature tolerance and cuticular structure, allowing production of the most optimal phenotype according to seasonal requirements. The principal cue for the timing of major seasonal events in northern insect species is photoperiod (Bradshaw & Holzapfel 2007). Photoperiodic regulation of seasonal responses in life-history traits is common in many taxonomic groups. For example, in insects the photoperiodic control of seasonal events has been found in more than 500 species (Nishizuka et al. 1988). As mentioned above, some life-history traits can be fine-tuned according to the prevalent environmental cues, whereas others are regulated in a switch-like manner between two or more clearly distinguishable phenotypes. Both the photoperiod and the temperature can induce these kinds of switches alone, but they can also function in cooperation. Photoperiod is known to act as a go/no-go signal for diapause (see chapter 1.2) induction in several insect species (Bradshaw & Holzapfel 2010). The most drastic example of the switch-like effects of temperature is perhaps the temperature induced sex-determination in reptiles (Bull 1980).

Although seasonal changes in cold tolerance (cold acclimation) are usually thought to be regulated mainly by temperature, also photoperiod can have a marked effect on it (Hodková & Hodek 2004, Vesala et al. unpublished). Photoperiodic signals can help individuals to prepare physiologically for the forthcoming winter at the correct time through mechanisms that involve e.g. production of cryoprotectants, upregulation of heat shock proteins and possibly accumulation of antimicrobial peptides. However, in cold tolerance, as in several other life-history traits, simultaneous changes in thermo- and photoperiod enable organisms to produce optimal phenotypes during different seasons.

1.3 Circadian and circannual responses

All photoperiodic responses share a common character: the highly accurate measurement of a day or night length. This forecasting/response system relies on the function of two major molecular clock mechanisms, the circadian clock and the photoperiodic calendar (e.g. Košťál 2011). Circadian clock (*circa* =

about, *diem* = day) is an internal timekeeping system that allows species to predict changes in a day and produce a continuum of phenotypes fine-tuned to prevalent photoperiodic conditions. The endogenous circadian system functions to organize both behaviour and physiology, allowing organisms to anticipate environmental changes, for example in light, temperature, and food and mate availability (Allada & Chung 2010). The circadian clock is aligned to the environment through *Zeitgebers* ("time givers"), the most notably being the daily light/dark cycle. Light is the best understood *Zeitgeber*, but other factors, such as daily changes in temperature and social behavior (Levine et al. 2002) can act as inputs to the circadian clock. Circadian rhythms are entrained by sun-driven changes in light and temperature and although the phase of the clock can be sensitive to these changes, the circadian period is remarkably stable over a wide temperature range, a phenomenon termed temperature compensation (Allada & Chung 2010).

The photoperiodic calendar consists of light receptors (input pathways), the photoperiodic clock and counter, and output pathways (Košťál 2011). It acts as a go/no-go seasonal switch on the basis of accumulated information, committing organisms to seasonal activities or states such as migration, dormancy or reproductive diapause (Bradshaw & Holzapfel 2010). In this system, the clock scores day (or night) length as short or long, while the counter stores information on the number of daily cycles received. Recent studies have suggested that the two systems could work in cooperation or that some of the circadian clock genes could play a role in both clock systems (Saunders 2009, Bradshaw & Holzapfel 2010, Košťál 2011).

1.4 Diapause

1.4.1 The importance of diapause and why study it

Diapause is a widely recognized life-history trait and an adaptation in insects experiencing drastic seasonal changes in their environment (Beck 1980, Saunders 2002). Diapause is mostly under photoperiodic control in species that encounter clearly detectable seasonal changes in photoperiod. It should be noted that terms like dormancy, quiescence and diapause are often confused. Dormancy refers to any state of suppressed development and quiescence is an immediate physiological threshold response where suppressed and normal functions can alternate depending on an environmental factor. The term diapause, on the other hand, refers to a profound endogenously controlled interruption of development and it usually starts well before the actual adverse condition (Danks 1987).

Insects' ability to enter a state of developmental arrest gives them an opportunity to exploit seasonally fluctuating resources and also to distribute to areas where they encounter adverse conditions, for example to higher latitudes or altitudes. Diapausing insects usually show enhanced tolerance towards low

and high temperatures, and in addition, to desiccation and starvation. However, enhanced stress tolerance is achieved only if diapause has started at an appropriate time during insect development and also during the right seasonal time. This timing of diapause is usually controlled by species specific genetic factors (obligatory diapause) as well as seasonal cues (facultative diapause) such as day length and temperature (see chapter 1.2.3). Features connected to diapause prepare the diapause-destined insects for a period of developmental arrest, and all of these features can be expected to be associated with metabolic and gene expression patterns not observed in non-diapause-destined individuals (Denlinger 2002).

Insect diapause has been studied in a variety of species, by many different research groups and diapause related studies were started already many decades ago, peaking during the decades of the 1960s and 1970s. However, new information can still be found, as new techniques from the field of molecular biology, biochemistry and genetics are advancing our knowledge about diapause. As Denlinger (2008) noted, information gained from diapause studies is not only important in understanding seasonal biology, but the information can be used in developing pest management strategies, manipulating domesticated species used in pollination and silk production and helping to gain knowledge about how to cryopreserve insect stocks. Information obtained from diapause studies can also be applicable to studies contributing to improve human health and provide insight into questions on aging, obesity and disease transmission (Denlinger 2008).

1.4.2 Metabolic challenges during insect diapause

Since winter diapause usually lasts more than a half of the year, and since individuals are not usually able to acquire energy supplies during this time, survival without eating is a tremendous challenge for diapausing individuals. The energy reserves that the individuals are able to accumulate can have an effect on whether they enter diapause and also when diapause is terminated, as well as on their fitness during the post-diapause period (Hahn & Denlinger 2007). According to Hahn & Denlinger (2011), insects use two strategies to cope with the metabolic challenges during diapause: accumulation of reserves and metabolic depression. Diapause is a metabolically dynamic state, involving shifts between different energy sources during the diapause period and also showing dramatic pulses of metabolic activity that spike with a frequency of several days (Hahn & Denlinger 2011). Diapausing insects with insufficient energy reserves either die during the diapause period or fail to continue their post-diapause development (Hahn & Denlinger 2007). Other options for individuals with insufficient energy reserves exist as well. They can try to avert diapause, as in the case of the blow fly *Calliphora vicina,* which enters diapause in the larval stage (Saunders 1997). They can aim to breed during the same season, or they can be forced to terminate diapause prematurely as their energy reserves are depleted (as noted in *C. vicina*; Saunders 1997). Or, if the insects retain their ability to feed, they can try to compensate the energy deficiency by

feeding during diapause, as in the case of the damselfly *Lestes eurinus* (de Block et al. 2007).

The quantity and quality of nutrient stores in insects often varies between non-diapausing and diapausing individuals. However, diapausing individuals are not simply running slower than non-diapausing individuals, but the developmental pathway leading to diapause also has its own metabolic demands (Košťál 2006). Both diapausing and non-diapausing individuals store metabolic reserves in the form of lipids, carbohydrates, and amino acids. To accumulate enough reserves, diapausing individuals must eat more and allocate nutrients away from somatic growth to storage. Hence, individuals must obtain reliable environmental cues of the ongoing season to be able to start to accumulate energy reserves well in advance of the adverse season, in order to prepare for a successful diapause period.

1.4.3 Factors inducing and regulating diapause

The most important environmental cues regulating winter diapause in northern insect species are photoperiod and temperature. These cues may regulate diapause induction directly or through their effects on other factors mentioned below (see more information in studies II and IV). However, there are also other factors controlling the diapause period, such as hormones, maternal effects, nutrition and density. These factors are shortly described here.

Several hormones are known to regulate diapause, but the precise nature of these hormones depends on the species and on the developmental stage in which diapause occurs (Denlinger 1985). In most cases adult diapause can be attributed to the absence of juvenile hormone (JH, secreted in the corpus allatum), since blocking its production results in the cessation of egg maturation (Denlinger 1985), the degeneration of flight muscles, and in cessation of mating activity (Denlinger 2002).

In some species diapause induction can take place already in the previous generation, giving rise to diapausing offspring. This kind of maternal induction of diapause takes place e.g. in the silk moth *Bombyx mori,* in which the photoperiodic cues received by an earlier generation can either induce or avert the diapause response of the next generation (Kogure 1933). Also, the parasitic wasp *Nasonia vitripennis* shows maternal induction of diapause as the developmental pathway of the wasps is already determined by the environmental cues experienced by the mother before the eggs have been deposited within the host flies (Saunders 1966).

Nutrition has been found to affect the induction of diapause also. Diet can modify the degree of the photoperiodic response or even the critical day length (CDL), where 50 % of the females of a population enter diapause (Saunders 2002). For example, in the pink bollworm moth, *Pectinophora gossypiella*, the moths' response to short day length is affected by the quality of their diet (Adkisson 1961). Also density can have an effect on diapause induction, but these effects can be difficult to distinguish from decreased food availability resulting from crowding of insects on their food.

1.4.4 Forms of diapause

Diapause has been found to take place in insects, rotifers, nematodes, earthworms, crustaceans and terrestrial gastropods (see Košťál 2006) and it can take place at different developmental stages. Usually diapause takes place only during one developmental stage within a species. Both immobile and mobile developmental stages may enter diapause, such as embryos (e.g. silkmoth *Bombyx mori*; Denlinger 2002) cocooned larvae and pre-pupae (e.g. moth *Thyrassia penangae*; He et al. 2009), pupae and free-living larvae (e.g. rice stem borer *Scirpophaga incertulas*; Xiao et al. 2010) and adults (monarch butterfly *Danaus plexippus*; Herman 1981, and the flies of several *Drosophila* species; e.g. Lumme 1978, Watabe 1983, Pittendrigh & Takamura 1987, Saunders et al. 1989). In mobile development stages the metabolic suppression induced by diapause is usually less deep than it is in immobile stages (Košťál 2006). Diapause can also be obligatory or facultative. Obligatory diapause is restricted to a certain developmental stage regardless of environmental conditions because it is a part of the species' ontogenetic program. The more common facultative diapause is determined by environmental cues, which either induce or prevent the switch to diapause.

1.4.5 Adult stage reproductive diapause

In photoperiodic adult stage reproductive diapause, oogenesis of females' ovaries is halted at a pre-vitellogenic stage, which is usually induced by short day length. Here, the accumulation of yolk into ovaries is halted and reproduction is postponed to a more favorable season. Hence, if the ovarian development is precedes past the pre-vitellogenic state, the female is no longer capable of entering to reproductive diapause, and it has already allocated some energy to ovaries, which should have been allocated to processes required for overwintering. Females' decision on whether to produce progeny before the autumn or to enter reproductive diapause is crucial for the maintenance of the whole population. Females that enter reproductive diapause too early in the season have low fitness in terms of progeny production. On the other hand, the diapausing females have higher stress resistance, they age more slowly and have better chances to survive over the winter period than non-diapausing females (Tatar et al. 2001). Females that have entered reproductive diapause may also be more cold tolerant (Vesala & Hoikkala 2011). One distinctive feature of reproductive diapause is the allocation of energy from the maturation of ovaries to other functions that help the females survive over the adverse season.

1.4.6 Diapause phases: induction, onset, maintenance and termination

Diapause is usually referred to as a "state", implying that it is a continuous stable condition. However, diapause is a dynamic process consisting of several different phases, all showing distinct features at both phenotypic and gene

expression levels. Insect diapause can be divided into four different eco-physiological phases: induction, onset, maintenance and termination.

As mentioned earlier, the environmental cues that induce diapause can vary between species. These cues are usually received well before the actual adverse season, and these cues will lead to diapause induction only during a fixed and species-specific sensitive period (SP). Photoperiodic induction of reproductive diapause involves two processes: the measurement of qualitative differences between long and short days/nights (photoperiodic clock) and the accumulation of quantitative information on photoperiods (photoperiodic counter) up to an internal threshold at which the induction of diapause is complete (Vaz Nunes & Saunders 1999). Individuals usually need to be exposed to inductive photoperiods for several successive days during the sensitive period before their internal threshold is exceeded and the switch to diapause occurs (Saunders 2002). For example, in *Polygonia c-album* butterflies the diapause pathway is determined already by the time of eclosion (Nylin 1989), while in *D. melanogaster* fruit flies the sensitive period for diapause induction is at the adult stage and it lasts only for 10 hours after eclosion (Saunders & Gilbert 1990). The effects of the photoperiodic counter, which acts as a link between the clock and the photoperiodic response, can be measured quantitatively as the number of short day (or night) cycles (required day number, RDN) needed to induce a diapause response in 50% of individuals in a certain age group (Saunders 1971).

The onset stage of diapause starts when the development of females' ovaries is halted and various metabolic rates are decreasing (Varjas & Saringer 1998). Species that are diapausing at larval or adult stage show a slower decrease in metabolism than species that are diapausing at an immobile stage. Other features that are common to the onset stage of diapause include an increased stress tolerance, migration to overwintering sites, and accumulation of energy reserves (Košťál 2006). The events during the onset phase of the insect's life are extremely important, because a failure to adequately prepare for diapause will reduce the likelihood of survival to the end of diapause.

During the maintenance phase, the metabolic rate is usually held relatively low (Hanski 1988). Among all diapause phases, the maintenance phase has been least studied, even though it is known that in some species the maintenance phase can last for even several years or decades. The duration of diapause is programmed genetically, but also environmental conditions are affecting its duration (Danks 1987, Kalushkov et al. 2001). In some species, diapause is spontaneously terminated without changes in external cues. This is the case e.g. for the tailed zygaenid moth, *Eclysma westwoodii* (Gomi & Takeda 1992) and the corn stalk borer, *Sesamia nonagrioides* (Fantinou et al. 1998). In other cases an external termination cue is required, such as chilling (see Hodek 2002) or changes in the photoperiodic cycles (McLeod & Beck 1963, Masaki 1980). Most authors agree that in species with adult winter diapause and breeding during the spring, the diapause is completed already 4 or 5 months after its induction and changed into quiescence, where morphogenesis is hindered by low ambient temperatures (Hodek 2000). According to this view,

there is usually no particular stimulus terminating diapause, although the diapause state may be modified by environmental signals such as temperature (Hodek 2000). After diapause is terminated, normal development proceeds according to the ontogenic program of the species.

In addition to the phenotypic changes occurring during different diapause phases, also different genes are likely to show high variation in their expression patterns depending on the phase of diapause. Many genes are downregulated during diapause, but a number of genes are uniquely expressed at this time. Furthermore, certain genes are expressed only during the induction or onset phase, whereas others are expressed in the maintenance period and during termination of diapause. All of these changes in gene expression pattern and the variability in the acting genes show how dynamic a process diapause actually is. By dissecting the genetic background of diapause we can find answers to questions related to regulatory mechanisms behind cessation and resumption of development as well as broaden our understanding of stress responses and aging (see study IV).

1.5 Studying the genetic background of diapause

Studies on the genetic basis of adaptively important traits such as different forms of diapause have recently led to the discovery of several interesting genes and genetic pathways acting on the background of these traits. Candidate genes related to diapause can be studied using either bottom-up or top-down approaches. In a bottom-up approach the function of the studied candidate gene is altered or completely knocked down, for example by using RNA-interference techniques, and the effect of this knockdown is then studied at the phenotypic level. In a top-down approach there are already existing differences at the phenotypic level and the genetic background between these phenotypes is compared. During the last decades, a multitude of new methods have arisen which have proved useful for clarifying the mechanistic basis of the diapause processes at a molecular level. With the increasing availability of transcriptomic methods, such as microarray platforms for several species and different taxonomic groups, and next generation sequencing methods, the gene expression differences between diapausing and non-diapausing individuals can be studied quite easily. Insect winter diapause has been suggested to be controlled by several genes that show expression changes during its different phases (Denlinger 2002, Baker et al. 2009). Diapause-specific and non-specific expression patterns have been observed in various studies as well as in various genes, including the genes that are encoding heat shock proteins (Rinehart et al. 2000, Goto and Kimura 2004, Hayward et al. 2005, Tachibana et al. 2005 and Yocum et al. 2005), genes involved in energy metabolism (Blitvich et al. 2001, Lewis et al. 2002, Levin et al. 2003, Uno et al. 2004), genes involved in hormonal regulation (Huybrecth et al. 2004, Xu & Denlinger 2004, Wei et al. 2005) and clock genes (Goto & Denlinger 2002, Pavelka et al. 2003, Spieth et al. 2004,

Dolezel et al. 2005). The genetic background of diapause also has been studied traditionally by mapping genes affecting diapause through various kinds of crosses. For example, in *Drosophila littoralis*, the long critical day length for diapause induction (typical to northern populations) is thought to be caused by incompletely dominant alleles (Lumme & Oikarinen 1977) located on the fused chromosome 3-4 (Lumme 1981).

Candidate genes for the onset or maintenance phases of diapause have been discovered in different species and most of them have later been found to be common in a variety of species and genera, showing that there are some commonalities in the genetic background of diapause. Good examples of these diapause related genes are e.g. the genes involved in circadian rhythmicity, such as *timeless* in *D. melanogaster* (Tauber et al. 2007), *cycle* and *period* in heteropteran bean bug *Riptortus pedestris* (Ikeno et al. 2010), *per* in the linden bug *Pyrrhocoris apterus* (Dolezel et al. 2007); and the insulin pathway genes, such as *Dp110* in *D. melanogaster* (Williams et al. 2006), *daf* in nematodes (Fielenbach & Antebi 2008) and *FOXO* in mosquitoes (Sim & Denlinger 2008). Furthermore, some genes, e.g. *couch potato* (*cpo*) have been suggested to provide a link between the insulin signaling pathway and the downstream hormones involved directly in the regulation of vitellogenesis during females' sexual maturation (Emerson et al. 2009). (*cpo* and more candidate genes for diapause and reproductive diapause are described in more detail in studies III, IV and V).

1.6 Aims of the thesis

The role of photoperiodism in adaptation to seasonally varying environments can best be studied in species that have spread to high latitudes and that have already been the targets of extensive ecological, evolutionary and genetic studies (Fig. 1). The northern *Drosophila virilis* group species fulfil these criteria. While the *D. virilis* species group has originated in continental Asia, some of the species, such as *D. montana*, *D. littoralis* and *D. ezoana,* have spread northwards adapting to different kinds of thermal and photoperiodic environments (Throckmorton 1982). *D. virilis* group species have been used in ecological, evolutionary and genetic studies already for many decades (see Throckmorton 1982). More recent research on the species of this group involves studies on e.g. phylogeography (Spicer & Bell 2002, Mirol et al. 2007), chromosomal evolution (Gubenko & Evgenev 1984, Morales-Hojas et al. 2007), phenotypic divergence (Routtu et al. 2007), heat and cold tolerance (Garbuz et al. 2003, Vesala & Hoikkala, 2011), clinal variation in photoperiodically determined life-history traits (Lankinen 1986, Tyukmaeva et al. 2011) and the genetics of photoperiodic diapause (e.g. Lumme & Oikarinen 1977, Kankare et al. 2010) and male courtship songs (Hoikkala & Lumme 1987).

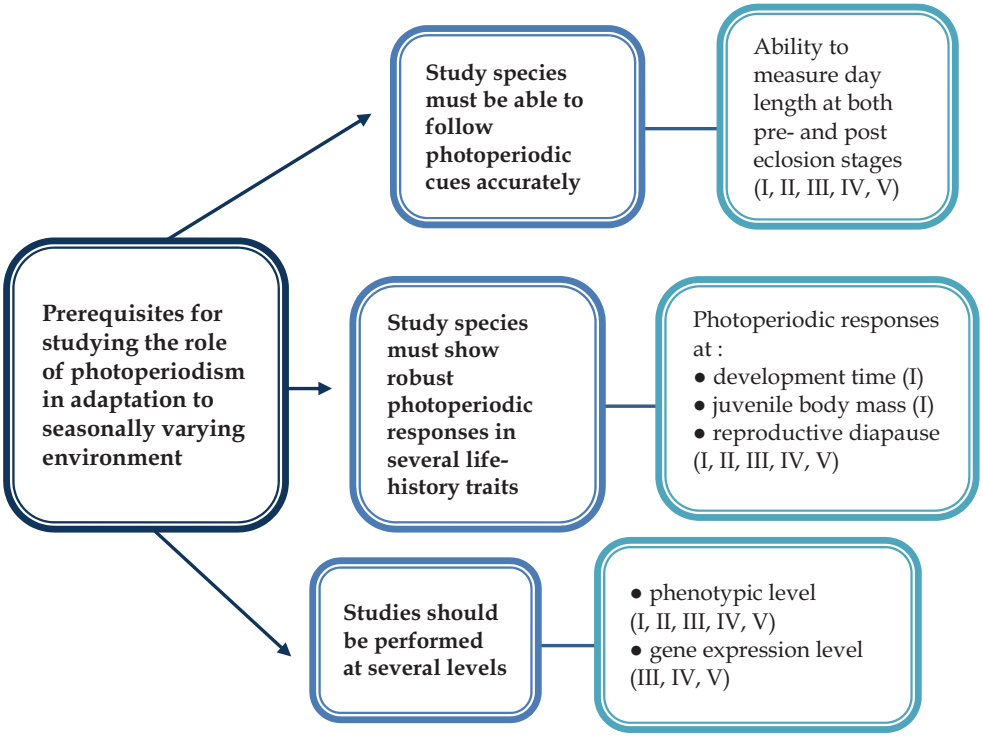

FIGURE 1 Studies on the role of photoperiodism in adaptation to a seasonally varying environment set various kinds of challenges for study organisms and study methods (roman numbers refer to studies performed in this thesis).

The objective of my doctoral thesis was to examine the effects of photoperiod as well as temperature on the timing of life-history traits important in seasonal adaptation, at both the phenotypic and gene expression level. The studies have been done using insect models, mainly *D. montana* flies, to obtain information on the role of environmental cues in shaping life-history traits of insect species living at high latitudes as well as on the genetic background of some of these traits.

The first aim of my doctoral thesis was to trace the effects of different photoperiods on flies' life-history traits determined before (egg-to-eclosion developmental time; juvenile body weight) and after (reproductive diapause) eclosion in *D. montana,* to gain information on how this species uses photoperiodic cues in seasonal anticipation responses (I). I also wanted to determine the timing and duration, as well as the temperature-dependence of the sensitive period, during which diapause induction can take place (II). The induction phase of diapause is an extremely important time-point during an individual's life-cycle, and the decision made during this point has long lasting effects on the individual's life-history traits.

My second aim was to trace gene expression changes involved in diapause induction and maintenance. To be able to do this, we constructed a species-

specific custom designed DNA-microarray for our main study species, *D. montana*. With this array, we searched for candidate genes acting during early stages of reproductive diapause by comparing the expression patterns of young, diapausing and non-diapausing females reared in different photoperiods (III). I also performed another microarray study with a more demanding experimental design. In this study I mimicked the seasonal environmental conditions from the flies' home site in an environmental chamber and studied the effect of gradually changing photoperiod and temperature conditions on both phenotypic traits and on gene expression level (IV). This approach allowed me to trace the effects of gradually changing semi-natural environmental conditions on the seasonal switch to reproductive diapause and to find out whether the overwintering state of the females (overwintering either at vitellogenic state or in reproductive diapause) can have long-term effects on their stress tolerance after overwintering. I also wanted to trace the effect of these gradually changing conditions on gene expression patterns during the onset, maintenance and termination period of reproductive diapause, as well as the overwintering state of the females. And, since one scope of my doctoral thesis is timing, I aimed to find out whether the effects of conditions experienced during early adulthood can be seen later in life in the flies' gene expression profiles.

During study III, we found an interesting link between *couch potato* gene and reproductive diapause. The connection between *cpo* and reproductive diapause was studied further and the DNA-sequence- and amino acid level variation was studied between many Drosophila species as well as in different strains within some of these species (V).

2 MATERIALS AND METHODS

2.1 Study species and populations

Drosophila montana is the main study species in all five studies included in my PhD thesis, but I also used *D. littoralis* and *D. ezoana* in studies IV and V and obtained some DNA sequence information for *D. virilis* and *D. lummei* strains in study V. Most of the studies were performed using *D. montana* flies of isofemale strains obtained from three populations from a 760 kilometre long latitudinal cline of the species in Finland. Studies I and II were performed using flies from the end populations of the cline, as they were assumed to show adaptation to different light regimes: the southernmost population is Lahti (N60°59 E25°40: isofemale strains L209, L309, L609 and L909) and the northernmost population is Pelkosenniemi (N67°00 E27°08: isofemale strains 2PT09, 15PT09, 21PT09 and 24PT09). In studies III and IV, we used flies of another northern *D. montana* population, Oulanka (N66°22 E29°20: isofemale strains 3OL8, 26OL8 and 175OJ8), and in study IV, isofemale strains also from *D. littoralis (*strains: 202OJ8, 219OJ8 and 280OJ8) and *D. ezoana* (67OJ8, 124OJ8 and 143OJ8) from Oulanka. All of the isofemale strains used in the studies consisted of progenies of females collected from the wild populations during the summers 2006, 2008 and 2009 and their codes refer to the stock maintained in the University of Jyväskylä.

Environmental conditions at the three above-mentioned sites differ in the amplitude of daily and seasonal changes in day length and temperature, as well as in the length of the growing season and the duration and severity of the winter. In Lahti the day length varies annually from 5.51 hours to 19.17 hours, in Oulanka from 2.16 hours to 24 hours and in Pelkosenniemi from 1.32 hours to 24 hours. The critical day length (CDL), evoking female diapause response in 50 per cent of the females of the population, changes gradually along the latitudinal cline from Lahti to Pelkosenniemi; the difference in CDL between these southern- and northernmost populations being more than two hours (Tyukmaeva et al. 2011).

The females of northern *D. virilis* group species overwinter in reproductive diapause, where their ovarian development is halted to a pre-vitellogenic state (Lumme 1978, Watabe 1983). Diapause occurs well in advance of the actual adverse winter conditions, and it is terminated during the spring when ovarian development proceeds from the pre-vitellogenic state to vitellogenesis and females are ready to mate and to lay fertilized eggs (Fig. 2). The females of the next generation may produce progeny during the same summer if they emerge before the population specific CDL. Due to differences in the length of the warm period (growth period) between southern and northern Finland, *D. montana* flies in the Lahti population usually have a second generation in summer (a bivoltine population), while in Pelkosenniemi and Oulanka populations this generation is practically missing (univoltine or partially bivoltine populations). Even though it is not possible to make any far-reaching conclusions on differences between uni- and bivoltine populations by performing studies on only two or three populations from a single latitudinal cline, these studies help to identify possible latitudinal adaptations. Since there are differences at the phenotypic level between the flies in the cline, these studies provide a starting point for more detailed genotype analysis in the future.

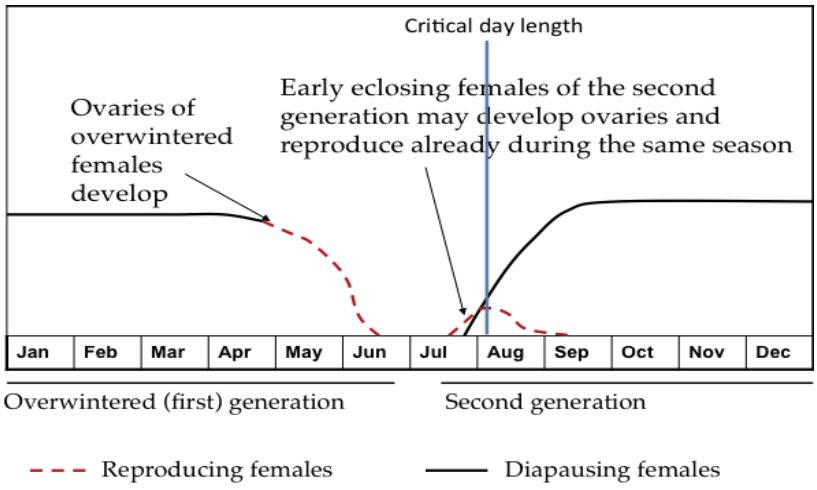

FIGURE 2 Life-cycle pattern of northern Drosophila females.

2.2 Rearing conditions (I, II, III, IV, V)

Isofemale strains used in the studies consisted of progenies of females collected from the wild during summers 2006, 2008 and 2009. They have been maintained since their establishment in diapause preventing conditions in a fly rearing

room with constant light, +19°C, and with a humidity of 60%. 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; following Lakovaara 1969).

The experimental flies were maintained in several different environmental chambers with different light:dark cycles and temperatures, which were kept constant in all studies, except in study IV. In studies I and II the flies were kept in small wooden environmental chambers with controlled light:dark cycles, which were located inside a larger environmental chamber with controlled temperature, or a temperature controlled room. These wooden chambers had bright white interiors and they were lighted by one light bulb (behind plexiglass) or by LED-lights. Chambers were always identical within the experiments. In study IV, the flies were maintained for nearly one year in a large environmental chamber (Fig. 3) with fluctuating day lengths and light intensities as well as day and night temperatures, mimicking the seasonal conditions of the study flies' collection site, Oulanka (see Fig. 1 in study IV).

FIGURE 3 One of the environmental chambers used in the experiments (IV). In cases where there were many vials of flies in the chamber, the positions of the vials were changed regularly to allow the flies to experience as similar conditions as possible. (Photo: Tiina S. Salminen)

2.3 Phenotypic measurements

2.3.1 Egg-to-eclosion development time (I)

Anticipation of seasonal changes in environmental conditions can take place at different developmental stages, both before and after eclosion. The effect of population specific long (early summer), intermediate (late summer), and short (early autumn) day lengths on egg-to-eclosion development times was studied in *D. montana* females and males from four isofemale strains from Lahti and Pelkosenniemi populations. Vials that contained eggs were placed randomly into experimental chambers with different light:dark (L:D) cycles in a temperature controlled room at +16 ± 0,5°C. After pupal stages were detected, the vials were checked every eight hours for the presence of emerged flies. The flies were sexed and the development times of the flies from egg-to-eclosion were recorded.

2.3.2 Juvenile body mass (I)

Photoperiod can also have a direct or indirect effect on body mass. Here, I measured the body weights of females and males from four isofemale strains from Lahti and Pelkosenniemi, reared in population specific long (early summer), intermediate (late summer), and short (early autumn) day lengths. The effect of photoperiod on body mass was studied by weighing (scale: Mettler Toledo XS204 analytical balance with a precision of 0.1 mg) the females and males within 24h after their eclosion to exclude the effect of post-eclosion feeding on the body mass.

2.3.3 Ovarian development (I, II, III, IV, V)

Female ovarian development was studied at the age of 14 (III), 18 (II) or 21 (I, IV, V) days after eclosion. Preliminary study indicated that the ovaries are fully developed already in females at the age of 14 days, when they are kept at >16°C under long day conditions inducing vitellogenesis (T.S. Salminen, unpublished). In some of the studies the ovarian development stage was measured from 18 or 21 day old females, because temperature conditions lower than 16°C or transferring females between two different light:dark cycles, could possibly confuse the photoperiodic counter and require more time for flies to make the decision as to whether to develop vitellogenic ovaries or to enter reproductive diapause. Also, lower temperature might delay the development rate of the ovaries. In this case, some ovaries would appear to be from diapausing females, but in reality they would just be immature due to an inadequate amount of time to develop mature ovaries.

Ovarian development stage was determined by dissecting the females under a microscope and gently pulling the ovaries out with tweezers (Fig. 4). The ovaries were classified into two or three stages in different studies. In

studies where only two categories were used (I, III, IV, V), the developing/intermediate ovaries were combined in the same category with the developed ovaries (see below).

The three stage classification was as follows:
a) mall and transparent ovaries with no visible egg chamber formation or yolk accumulation were classified as pre-vitellogenic ovaries typical of young females with immature ovaries (II, IV) or older females in reproductive diapause (I, II, III, IV, V)
b) intermediate size ovaries, with some egg chamber segments visible and some yolk accumulated into them were classified as developing (intermediate) ovaries belonging to females that had not yet completely reached sexual maturity (II, IV). These females were not diapausing, as they had proceeded past the pre-vitellogenic stage
c) large ovaries, with visible eggs were classified as developed or vitellogenic ovaries typical of mature non-diapausing females (I, II, III, IV, V).

FIGURE 4 *Drosophila montana* females with pre-vitellogenic (uppermost, diapausing), intermediate (middle one; developing) and vitellogenic (lowermost; developed) ovaries. (Photo: Tiina S. Salminen & Mikko Merisalo).

2.3.4 Sensitive period for reproductive diapause (I, II)

To find out whether the sensitive developmental stage for the onset of reproductive diapause and/or vitellogenesis is before or after elosion and whether the photoperiodic counter can be reset after eclosion (I), I performed transfer experiments between short pre-eclosion and long post-eclosion day lengths and vice versa. The results obtained from these experiments were

compared to those obtained for females that had been reared under the same day length at pre- and post-eclosion level. The females' ovarian development stage was determined by dissecting the ovaries of the females at the age of 21 days and categorizing the ovaries as diapausing or vitellogenic, as explained above.

To determine more accurately the timing and duration of the sensitive period for female diapause induction after eclosion and to trace the effects of temperature on the sensitive period, I performed more transfer experiments between long and short day lengths and vice versa in both 16ºC and 19ºC (II). I also checked the development rate of the females' ovaries during the first eight days after eclosion when the females were kept in vitellogenesis inducing long day length in the above-mentioned temperatures (II). All of these studies were done using the females of strains from Lahti and Pelkosenniemi populations.

2.3.5 Effect of overwintering state on post-winter cold tolerance (IV)

Stress tolerance of *D. montana*, *D. littoralis* and *D. ezoana* females that overwintered either in a vitellogenic state or in reproductive diapause in an environmental chamber mimicking the seasonal conditions of their collection site, Oulanka (Finland; 66ºN), was measured using cold as a stress factor (IV). Cold tolerance was measured during the semi-natural spring conditions when the females were 250-days-old using the chill coma recovery time (CCRT) method (David et al. 2008). Females that overwintered in reproductive diapause were transferred into the chamber within one day of their eclosion at the diapause inducing photoperiod of 16:8LD (late August). Females that overwintered in a vitellogenic state were kept for one week in constant light and 19°C to induce ovarian development before transferring them into a climate chamber with a photoperiod of 15:9LD. Hence, at the time of the chill coma recovery time (CCRT) –tests, these two types of females were equally old (250 days) and had been living in the same environmental conditions (day length 15:9LD), with the only exception being the first week after eclosion. Also, all the females had vitellogenic ovaries during CCRT measurements, as the females that had overwintered in reproductive diapause had already terminated their diapause in the spring-like photoperiodic conditions. Chill coma was induced by keeping the females for 16 hours at -7°C (Vesala & Hoikkala 2011). After the cold treatment, the recovery times of the females were measured at room temperature by recording the time it took for the flies to stand up.

2.4 Genetic methods

2.4.1 RNA extractions and cDNA synthesis (III, IV, V)

Total RNA was extracted using a Qiagen RNA extraction kit with RNase-Free DNase treatment according to the manufacturer's protocols (Qiagen). Pools of thirteen and eleven flies were used for studies III and V, respectively. For the microarray part of study IV, Ambion RNAqueous 96 well total RNA Isolation Kit with DNase treatment (Qiagen) was applied for RNA extraction using the whole bodies of two females. For the qPCR part of study IV, RNA was extracted from whole bodies of five female flies using a modified Tri-reagent (Ambion) protocol followed by an extra clean-up step with RNeasy columns (RNeasy mini kit, Qiagen) and with a DNAse (Qiagen) treatment. Extracted RNA from the microarray samples in study IV was purified with a MinElute kit (Qiagen), while in studies III and V, a Qiagen RNAeasy purification kit was used. Concentration and the purity of all the RNA samples were measured with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and integrity was measured with Agilent's Bioanalyzer (only for study IV). Finally, cDNA was generated either with Sigma's Enhanced Avian HS RT-PCR kit (V) or with iScript Reverse Transcription Supermix for qPCR (Bio-Rad Laboratories) (III and IV) using diluted RNA samples of equal concentration and following the manufacturer's instructions.

2.4.2 Constructing custom designed species-specific DNA-microarray (III)

The first version of the species-specific candidate gene microarray constructed for *D. montana* included 101 genes related to diapause, vitellogenesis, phototransduction, courtship behavior, temperature response, locomotion activity, aging, and the circadian clock. The genes were selected from the *D. melanogaster* genome using FlyBase gene ontology searches on biological response. The most conserved regions of the genes were indentified by aligning the exon regions of the *D. melanogaster* gene to their orthologs in *D. virilis,* a sister species of *D. montana*, using Blat search in the UCSC Genome Bioinformatics database. The gene sequences of *D. montana* were amplified with PCR and sequenced using both DNA and cDNA as templates to observe possible intron regions. The online program e-Array 5.3 (Agilent) was used to design one to four 60 bp oligonucleotide probes per gene matching exon regions. Finally, the probes were synthesized in situ with liquid chemistry and arrayed on an "Agilent 60-mer Multi-Pack Gene Expression Microarray" platform with one-colour system.

For the second version of the microarray (used in study IV) we included new probes for 118 genes related to the same traits as mentioned above using the same protocol to obtain gene sequences from *D. montana* and to design the microarray probes. This updated version of the microarray contained probes for

219 genes with 2-5 probes per gene (see Supplementary material in study IV for the complete list of genes in the array).

2.4.3 Quantitative real time PCR (III, IV, V)

Quantitative real time PCR (qPCR) was used for validation of the microarray results (III, IV) as well as to study the effect of a deletion in the *couch potato* gene on its expression profile (V). Three (III & V) to five (IV) biological replicates and three technical replicates for each of the samples were assayed in qPCR analysis using a selected set of experimental and control genes. Amplification efficiency values of all the primer pairs were inspected using 2-fold serial dilutions of pooled cDNA (from all the used treatments) with three technical replicates and 6-9 dilution points. 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: denaturation at 95°C for 3 min, followed by 10 seconds at 95°C, annealing at specific T_A for 10 seconds and extension at 72°C for 30 seconds, repeated 40 times and followed by melting curve analysis (65°C-95°C) for checking the purity of the qPCR reaction. Gene expression values were calculated with a normalized expression method (ΔΔ(Ct)) using CFX96 Manager Software 2.0 (Bio-Rad Laboratories) with real efficiency values. Statistical significance of the results was computed with the program REST 2009 (http://www.gene-quantification.de/rest-2009.html) using 10000 iterations and bootstrapping method.

2.4.4 DNA extractions and sequence variation at nucleotide and aminoacid levels in *couch potato* (*cpo*) gene (V)

DNA was extracted from the whole bodies of individual flies with a salt extraction method (Gloor & Engels, 1992). To obtain DNA sequences from the *cpo* gene, we first identified the most conserved regions of this gene by aligning *D. melanogaster cpo* exons to their orthologs in *D. virilis* and *D. mojavensis*, using Blat search in the UCSC Genome Bioinformatics database (http://genome.ucsc.edu/). Based on these alignments, we designed several primer pairs with the program Primer3 (http://frodo.wi.mit.edu/primer3/) and/or manually. *D. montana cpo* sequences were then amplified with PCR and sequenced using BigDye Terminator 3.1 Sequencing Kit reagents and visualized with a 3130xl Genetic Analyzer (Applied Biosystems), using both DNA and cDNA as templates. The sequence data were analyzed with Sequencing Analysis v5.2 (Applied Biosystems), DNABaser v2 (Cubic Design) and/or BioEdit V 7.0.9 (http://www.mbio.ncsu. edu/bioedit /page2.html) programs. Finally, ExPaSy Translate tool (http://web.expasy.org/translate/) was applied for translation of nucleotide sequences to amino acid sequences.

3 RESULTS AND DISCUSSION

3.1 Seasonal timing of life-history traits (I)

Photoperiod is known to affect several life-history traits in insects, including development time and body mass (Tauber et al. 1986), and also to act as a go/no-go switch or seasonal signal that commits an animal e.g. to migration, dormancy, development or reproduction (Bradshaw & Holzapfel 2010). Hence, the ability of a species to forecast future environmental conditions by taking into account photoperiodic cues is crucial for species that encounter annually repeatable adverse conditions. In the first part of my doctoral thesis, I studied photoperiodic anticipation of seasons by tracing the effects of long (early summer), intermediate (late summer), and short (early autumn), photoperiods on three life-history traits important in adaptation to seasonally variable environments: egg-to-eclosion development time, juvenile body mass, and the sensitive developmental stage for the onset of reproductive diapause. The studies were performed on flies from two northern *Drosophila montana* malt fly populations differing in voltinism patterns (Lahti, N60°59 E25°40, and Pelkosenniemi, N67°00 E27°08). The most interesting findings were consistent between the populations: the flies developed faster when they were maintained before their eclosion in short day conditions representing early autumn than when they were reared in long day conditions representing early summer. This kind of photoperiodic anticipation response has been found also in other species, such the speckled wood butterfly, *Pararge aegeria*, in which larvae experiencing short day length grow faster than those experiencing long days (Nylin et al. 1989). This seasonal anticipation response could be due to the effort of an individual to eclose as fast (development time) and as soon (seasonal timing) as possible during the late summer or early autumn in order to achieve the correct developmental stage required for overwintering, and to have enough time to accumulate sufficient resources before the winter. Insects need energy reserves e.g. to survive over the diapause period and to go through post-diapause development, which includes metabolically expensive functions,

such as metamorphosis. Diapausing insects with low energy reserves have a higher mortality during diapause, as reported in various arthropods (Hahn & Denlinger 2007, Hahn & Denlinger 2011).

In insects, also body size has been shown to be affected by photoperiod (Hahn & Denlinger 2007). In our study, the flies from both Lahti and Pelkosenniemi populations had a lower body mass (measured soon after eclosion) when reared in short day length than when reared in long or intermediate day lengths. However, it is impossible to say whether this was due to the direct effect of short day length on body mass or if it represents a trade-off between development speed and body mass.

In the northern hemisphere, females of most insect species that overwinter in adult stage reproductive diapause, develop vitellogenic ovaries and reproduce only if experiencing summer conditions, while females that are exposed to late summer or early autumn conditions after their emergence enter reproductive diapause. The sensitive period for receiving environmental cues prior to either developing vitellogenic ovaries or halting the development of the ovaries to pre-vitellogneic state, can happen both before and after eclosion, depending on the species. In *D. montana* the sensitive period for the induction of adult stage reproductive diapause was found to take place after eclosion. There was no direct connection between the females' egg-to-eclosion development time and their reproductive state at adulthood, which suggests that these two traits are determined by photoperiodic cues through different time measurement systems. Finally, it was concluded that the flies' development time from egg-to-eclosion is determined by prevalent environmental conditions, e.g. the day length measured through the circadian clock. The sensitive stage for diapause induction, on the other hand, was found to occur after eclosion, leading to specific phenotypes only after a delay, representing an on/off trait characteristic to responses determined by the photoperiodic calendar. These adaptations enable *D. montana* flies to respond to changing photoperiods at different developmental stages and on a short time scale, enabling them to use photoperiodic cues to match their life-history traits according to seasons.

3.2 Temperature can fine-tune the onset of photoperiodic reproductive diapause (II, IV)

Correct timing of reproduction and reproductive diapause is of a great importance for northern insect species and its effects on insect survival and reproduction have been investigated in several species (Tauber et al. 1986, Danks 1987, Denlinger 1991, Gomi et al. 2008). In general, the females that enter reproductive diapause too early during the growing season suffer from a reduced reproductive potential and/or mortality, while the females that reproduce too late may not survive over the cold period and they may also

produce progeny that fail to reach adulthood and/or to collect necessary energy reserves before the cold season. Photoperiodic induction of reproductive diapause involves two processes: the measurement of qualitative differences between long and short days/nights (photoperiodic clock) and the accumulation of quantitative information on photoperiods (photoperiodic counter) up to an internal threshold at which the induction of diapause is complete (Saunders 1981, Vaz Nunes & Saunders 1999). The function of the photoperiodic time measurement system and the induction of diapause can also be affected by temperature in a number of ways. Changes in temperature can, for example, alter the CDL (Saunders & Gilbert 1990), and daily temperature cycles, or thermoperiods, may simulate the effects of light-dark cycles acting as a *Zeitgeber* in their own right.

In study II, the sensitive period (SP) for the induction of reproductive diapause was studied in *D. montana* females by conducting a series of transfer experiments between constant short and long day lengths during eight days after eclosion in 16°C and 19°C. The sensitive period for the induction of reproductive diapause was found to start right after eclosion and last until the females' ovaries had surpassed the pre-vitellogenic stage. The three degrees difference in the temperature was found to enhance ovarian development rate and shorten the period during which females are still capable to enter reproductive diapause. Close to 100% of females of all study strains entered reproductive diapause at 16˚C and in the more northern study strains also at 19˚C when experiencing short day conditions. The sensitive period, where the switch to reproductive diapause takes place, appeared to be temperature dependent and lasted for at least eight days in 16˚C and four to five days in 19˚C. This dependency was due to increased ovarian development rate at higher temperatures. On the other hand, the induction of diapause response in 50% of the females of study strains required (required day number, RDN) that the females had been exposed to diapause-inducing conditions for about three days during the sensitive period, which could explain the lower proportion of diapausing females in higher temperature conditions, as vitellogenesis is faster in higher temperatures and the females have less time to track photoperiods. Temperature has been found to affect diapause propensity of females in several Drosophila species, the percentage of diapausing females being usually higher in lower temperatures (Watabe 1983).

In study IV, I traced the effect of gradually changing photo- and thermoperiods on the seasonal timing of reproductive diapause in three *Drosophila virilis* group species. The daily and seasonal conditions in the climate chamber were set to mimic the annual conditions at the flies' home site, Oulanka (N66°22 E29°20). In this experiment the seasonal switch to reproductive diapause happened during a remarkably short time window. The percentages of diapausing females from different isofemale strains increased from 0-20% to 48-87% between photoperiods of 20:4LD and 19:5LD, which corresponds to a seven day time window in northern Finland. This shift occurred over a shorter time scale than that observed for the Oulanka

population when studies were done at constant day lengths and temperatures (Tuykmaeva et al. 2010). This suggests that the timing of diapause becomes more accurate if the insects can sense annual changes on the basis of more than one environmental cue.

3.3 Effects of flies' overwintering state on their cold tolerance during spring (IV)

D. montana, *D.littoralis* and *D. ezoana* females from the above-mentioned climate chamber experiment (study IV) were used to trace the effects of the females' overwintering state on their cold tolerance in spring time. The winter temperature in the chamber was kept slightly warmer (+4^{o}C) than what occurs in the wild under the snow cover, so that both vitellogenic and diapausing females could survive over the winter conditions. During the artificial spring, when the diapausing females had terminated their diapause and possessed vitellogenic ovaries, the cold tolerance of 250 day old *D. montana*, *D. littoralis* and *D. ezoana* females was measured using a chill coma recovery time method (CCRT; David et al. 2008). In CCRT -tests insects are exposed to a sufficiently low temperature for a few hours, causing a state where the nerve impulses are lost and they are not able to move (Goller & Esch 1990). The time that it takes for an insect to regain its ability to move after being brought back to room temperature is called the chill coma recovery time (e.g. David et al. 1998). The CCRT method is used widely to measure changes in cold tolerance in Drosophila species, although the treatment varies according to species, since Drosophila, as well as other insect species and populations, show a wide range of variation in their low temperature tolerance.

The chill coma recovery times of *D. montana* and *D. littoralis* females varied significantly between females that had spent the winter in vitellogenic or diapause state, while in *D. ezoana* the overwintering state did not have any effect on the recovery time. In *D. montana* and *D. littoralis* the females that had overwintered in diapause recovered faster than those that had overwintered in a vitellogenic state. This finding was extremely interesting because the females had experienced the same environmental conditions since the age of approximately 7-14 days, and both of the groups had had enough time to acclimate to the cold prior to the winter. This result shows how wide an effect the decision of whether to develop mature ovaries or to enter reproductive diapause can have on the females' life-history traits at an older age.

Comparison of the chill coma recovery times between the species showed that *D. littoralis* females were less cold tolerant than the females of the two other species. Oulanka (the collection site of the females used in this experiment) might be close to its northernmost distribution range. On the other hand, *D. montana* and *D. ezoana* have more northerly spread distributions than *D. littoralis*.

3.4 Searching for candidate genes underlying traits of interest with a species-specific DNA-microarray (III)

Studies on gene expression patterns between individuals that are at different developmental stages or have gone through different treatments can help identify genes underlying plasticity in the studied traits. DNA-microarrays offer a feasible method for studying gene expression patterns. Since there was no species-specific commercial DNA-microarray available for our study species, *D. montana*, we designed a candidate gene microarray for it using available genome information from other *Drosphila* species. The microarray provided a practical and cost-effective platform for detecting genes connected to the studied life-history traits. However, one of the drawbacks was that we could trace changes only in a restricted number of genes for which we had probes in our array.

In the first microarray study (III), we searched for candidate genes for reproductive diapause. This study revealed two genes, *couch potato* (*cpo*) and *regucalcin,* both of which showed upregulation in diapausing females when compared to vitellogenic females. In this study *regucalcin* was referred to as *Dca,* based on the literature available during that time. In *D. melanogaster* and the other species of the *Sophophora* subgenus, *Dca* and *regucalcin* are paralogous genes, but in the species of the *Drosophila* subgenus (including *D. montana*), the gene is present in only one copy, which bears more sequence similarity to *regucalcin* than to *Dca* (Arboleda-Bustos & Segarra 2011). Previously, the *cpo* gene had been connected to diapause in *D. melanogaster* (Schmidt et al. 2008), but the upregulation of *regucalcin* in diapausing females was a new finding. Females used in this first array experiment were only 14 days old, so the detected genes might have a role in the late onset period and early maintenance period of reproductive diapause. However, it should be mentioned that ovarian status was not the only thing differing between these studied females, as they were collected from different day lengths; one inducing diapause and the other one inducing vitellogenesis. Hence, the upregulation of these genes might have been connected to diapause or to different photoperiodic cycles. Both of these genes will be good targets for future studies on the molecular basis of reproductive diapause and photoperiodicity.

3.5 Seasonal gene expression patterns and persistence of gene expression profiles (IV)

In the second microarray study (IV) I traced seasonal gene expression patterns in *D. montana* females at different phases of non-diapause- and diapause life-cycles. Here, the samples were collected from eight seasonal time points from the climate chamber, where daily and annual changes had been set to mimic the

conditions in the flies' collecting site in northern Finland (Oulanka; N66°22 E29°20). Samples of non-diapausing females consisted of 7, 50 and 150 days old females that had been transferred soon after eclosion in the climate chamber to early summer conditions known to induce vitellogenesis. Samples of females from the diapause life-cycle consisted of 7, 50, 150, 220 and 250 days old females that had been transferred in the chamber to diapause inducing late summer conditions. This study revealed a set of genes that were upregulated either during non-diapausing or diapausing females at different age groups and/or diapause phases (see study IV). Comparison of the gene expression patterns between non-diapausing and diapausing females of different age revealed a remarkable number of genes that showed more than two-fold differences in expression levels. Throughout the comparisons between nond-diapausing and diapausing females, e.g. *couch potato* was noticed to show high expression levels within the diapause life-cycle, decreasing only after diapause was terminated. *regucalcin* was upregulated only during the onset and early maintenance phase of diapause, while a gene called *Thor* showed highest expression during the overwintering diapause phase (for further results see study IV). Altogether this study revealed several interesting candidate genes specific to different phases of diapause, worth studying further.

One of the most interesting comparisons made in this study was a comparison between the females that were overwintering in the climate chamber in either non-diapause or diapause state and the samples of these females were collected from the overwintering period from the exactly the same environmental condition (see study IV). Females of both of these groups were 150 days old and had experienced the same seasonal conditions in the environmental chamber since the age of 7-14 days and since the late summer conditions. The only thing differing between these females was that the non-diapausing females were reared for one week in vitellogenesis inducing conditions before transferring them to diapause inducing late summer conditions approximately at the same time as the diapause-destined females were transferred to the chamber. Comparison of the gene expression patterns between these females revealed that many of the genes that showed up- or downregulation in either non-diapausing or diapausing females before the overwintering period (7 and 50 days old females), showed similar expression profiles also during the overwintering period. Genes that showed persistence in their expression patterns in non-diapausing females included e.g. *Hsp26*, *Hsp23*, *TipE*, *homer*, *His3.3a* and *Btk29A*, as genes that showed persistence in their expression patterns in diapausing females included e.g. the genes *Act42A*, *per*, *Mhc*, *Cat* and *Thor* (for detail, see study IV).

3.6 Sequence variation in the *couch potato* gene and its effects on life-history traits

In the last study (V), we investigated DNA polymorphism in the *couch potato* (*cpo)* gene, which is known to be involved in reproductive diapause in several insect species (Schmidt et al. 2008, Kankare et al. 2010, Ragland et al. 2010, Zhang & Denlinger 2011). This gene was found to be upregulated in diapausing females in both our microarray/qPCR studies (III and IV) during all diapause phases. We sequenced the most important exons of this gene in several *D. montana* populations and in four other *D. virilis* group species, concentrating on exon 5, where Schmidt et al. (2008) discovered two amino acid mutations showing a correlation with female diapause incidence in wild *D. melanogaster* populations. The study revealed both intra- and interspecific variation at DNA sequence level as well as interspecific differences in the length of the 5th exon as well as a site of a stop codon at the end of this exon. The latter finding suggests that the *D. virilis* group species possess a short transcript of *cpo* gene, in which exons 6-15 are spliced off. Mutation in a similar splice variant has been connected to diapause incidence in *D. melanogaster* (Schmidt et al. 2008). We also found a deletion of six amino acids located in this same transcript in one *D. montana* isofemale line. The deletion appeared to be extremely rare in the wild population, but its frequency increased rapidly during laboratory maintenance. We also discovered deletion to decrease the expression level of the transcript and also to slightly slow down the flies' development speed under diapause inducing short day conditions, but it had no direct effect on the incidence of diapause.

4 CONCLUSIONS

Reproductive diapause has been suggested to affect several other life-history traits, such as aging, developmental speed, longevity and various kinds of stress tolerances in northern insect species. It is, however, difficult to distinguish the effects of photoperiodically regulated reproductive diapause from the direct effects of photoperiod on life-history traits. In my doctoral thesis, I have studied the effect of photoperiodic cues on life-history traits as well as on gene expression patterns. I have shown that photoperiodicity plays an important role in seasonal anticipation responses both at pre- and post-eclosion stages in *Drosophila montana* malt flies and that the sensitive period for diapause induction is a crucial time point in the females' life-cycle, as the consequences of whether to develop ovaries or enter to reproductive diapause has a vast effect on the later life-history traits of females.

My first study (I) showed that *D. montana* flies are able to detect and to response to photoperiods during the pre-eclosion development stages, as well as during the adult stage, and that the flies can re-set their day length measuring system after eclosion. The fly development time from egg-to-eclosion varied in different photoperiods, and both the females and the males developed more slowly when reared under long (early summer) than under short (late summer and early autumn) day conditions. Female egg-to-eclosion development time showed no connection with the female reproductive state at adulthood, which is determined after eclosion. Also, juvenile body mass showed significant variation according to the pre-eclosion rearing day length, as the flies of both sexes were heavier when reared in long compared to short day conditions. However, it is difficult to say whether the variation in flies' body mass was due to photoperiodic cues *per se* or whether it was caused by a trade-off between the development time of the flies and their body mass.

Next I determined more thoroughly the timing and duration of the sensitive period (SP) for diapause induction (II), i.e. the time period when the females are in a correct physiological and developmental stage to enter diapause, and I also traced the effect of temperature on the length of this period. For this purpose I performed a series of experiments in 16°C and 19°C, where

newly eclosed females were transferred from diapause averting day length to diapause inducing day length, and vice versa, during several successive days. Close to 100% of females of all study strains entered reproductive diapause at 16˚C and in the more northern study strains also at 19˚C when experiencing short day conditions. The sensitive period, where the switch to reproductive diapause takes place, appeared to be temperature dependent and lasted for at least eight days in 16˚C and four to five days in 19˚C. This dependency was due to increased ovarian development rate at higher temperatures, which could explain the lower proportion of diapausing females in higher temperature conditions, as vitellogenesis is faster in higher temperatures and the females have less time to track photoperiods before their ovaries develop past the pre-vitellogenic state.

The above-mentioned studies were performed in unchanging photoperiods and constant temperatures, which do not reflect natural conditions. In the wild, the northern flies are exposed to drastic daily and seasonal changes in both of these cues. Maintaining the flies in a climate chamber mimicking seasonal changes in lighting and temperature conditions of their natural environment (IV) showed that the seasonal timing of diapause has a crucial impact on females both at the phenotypic and gene expression level, even after diapause has been terminated. The seasonal switch to diapause was found to happen in all three studied northern *D. virilis* group species (*D. montana*, *D. littoralis* and *D. ezoana*) within a very narrow time window (representing 7 days in nature). The cold tolerance of females that had overwintered in a diapause or non-diapause state was checked during semi-natural spring conditions with a chill coma recovery time (CCRT) –test (IV). At the time of testing, the females that had overwintered at diapause state had already terminated their diapause, so all females had mature ovaries when the CCRT tests were performed. The study showed that *D. montana* and *D. littoralis* females that had overwintered in diapause recovered significantly faster from chill coma than the females that had overwintered in a vitellogenetic stage. Hence, the effect of reproductive diapause was evident even after its termination.

In the three last studies in my thesis (III, IV, V), I combined phenotypic studies with studies on changes in gene expression level. This was started by constructing a species-specific DNA-microarray for *D. montana*, since using the arrays designed for other species can give unreliable results due to low sequence homology in the studied genes between the species. In our first array experiment the gene expression patterns of diapausing and non-diapausing females (as well as between newly eclosed young females, see study III) were compared. The custom designed array revealed some interesting candidate genes for photoperiodic responses/reproductive diapause, such as *couch potato* and *regucalcin*. Next, the seasonal gene expression patterns of *D. montana* females were studied. Fly samples were collected from the climate chamber mimicking seasonal changes in photoperiod and temperature conditions of their natural habitat and these samples represented different phases of non-diapausing and diapausing life-cycles. Interestingly, in some of the genes, the

changes in gene expression patterns induced during early adulthood seemed to persist in later ages, even when the conditions were altered drastically. It was also shown that diapause is a dynamic process as it involves not just a downregulation of genes, but that there are a set of genes typical to diapausing females and to different diapause phases in *D. montana* (IV).

Finally, one candidate gene related to reproductive diapause was taken under closer inspection (V), as DNA polymorphism was investigated in the *couch potato* (*cpo)* gene. This gene was upregulated in diapausing females when compared to non-diapausing females in earlier microarray and qPCR studies (III and IV). Selected exons (according to pre-existing literature of *cpo*) of this gene were sequenced in several *D. virilis* group species. The study revealed both intra- and interspecific variation at DNA sequence level and we also found a deletion of six amino acids located in the last section of exon 5 in one *D. montana* isofemale line. This deletion was shown to decrease the expression level of the shorter *cpo* transcript and the *D. montana* individuals possessing this deletion allele were developing slightly more slowly when reared under diapause inducing short day conditions, when compared to the females possessing the wild type allele. However, the deletion did not have a direct effect on the incidence of diapause.

In this doctoral thesis I have shown how widely northern *Drosophila* species have adapted to use photoperiodic cues to time their life-history traits to fit seasonal regimes. Studies concerning photoperiodism are important, as species may in future suffer from a day length – temperature mismatch due to global climate warming.

5 FUTURE DIRECTIONS

Results obtained in my doctoral thesis can be used for many purposes in the future. For example, Lahti and Pelkosenniemi populations of *D. montana* (I, II) appeared to be adapted to use population specific day lengths in diapause induction. It would be extremely interesting to cross individuals from these populations and to check the photoperiodic responses of F1 and F2 generations (for example, egg-to-eclosion development time and critical day length of diapause).

Insect diapause has been studied already for many decades, but by using currently available molecular, biochemical and genetic techniques, we can have a new insight into diapause, which was not possible in the past. One of the goals of my doctoral thesis was to find candidate genes related to the genetic background of photoperiodic responses and reproductive diapause. This was done by studying gene expression patterns with DNA-microarrays and qPCR technique. The candidate genes found during my doctoral thesis will be good targets for future studies on the molecular basis of diapause and photoperiodic responses. Importantly, in the future, gene expression studies concerning the genetic background of reproductive diapause should be focused on flies exposed to the population specific critical day length, in which half of the females of the population enter diapause. By comparing gene expression between diapausing and non-diapausing females obtained from the same day length, the effect of photoperiod can be excluded from that of reproductive diapause. These studies should also be done by using the next generation sequencing methods, since transcriptome profiling would potentially reveal more candidate genes than did our custom designed microarray, as our array contained probes for a selected set of genes.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Ajoituksen tärkeys: valojaksoisuus elinkiertopiirteitä ja geenien toimintaa muokkaavana tekijänä

Elinkiertopiirteiden, kuten yksilönkehityksen, migraation ja lisääntymisen, ajoittaminen vuodenaikoihin nähden optimaaliseen ajankohtaan on luonnossa todella tärkeää. Pohjoisella pallonpuoliskolla kasvukausi on lyhyt ja sitä seuraava pitkä, pimeä ja kylmä talvi kestää jopa puoli vuotta. Talvikauteen on valmistauduttava jo hyvissä ajoin ennen sen alkamista, joten kyky tunnistaa meneillään oleva vuodenaika ja ennustaa ympäristöolosuhteissa tapahtuvat muutokset on erittäin tärkeä pohjoisten lajien sopeutumisen ja säilymisen kannalta. Valojakso, eli valoisan ja pimeän jakson vaihtelu vuorokauden sisällä, toimii pohjoisilla leveysasteilla luotettavimpana vuodenaikojen vaihtelusta kertovana signaalina. Monet pohjoiset hyönteislajit ovatkin sopeutuneet hyödyntämään valojaksoja vuodenajan määrittämisessä ja ajoittamaan ja säätelemään elinkiertopiirteitään näiden signaalien avulla.

Eräs tärkeimmistä pohjoisten hyönteisten talvisopeumista on lepokausi, jonka aikana yksilönkehitys on pysähtynyt, aineenvaihdunta ja ikääntyminen hidastunut ja stressinsietokyky lisääntynyt. Väitöskirjani päätutkimuslaji, pohjoiselle pallonpuoliskolle levittäytynyt *Drosophila montana* –mahlakärpänen, talvehtii pääosin lyhyiden, loppukesää vastaavien valojaksojen säätelemässä lisääntymislepokaudessa. Lisääntymislepokaudessa naaraiden ovarioiden (munarauhasten) kehitys on pysähtynyt esi-vitellogeeniselle tasolle eli ovaarioihin ei ole kertynyt ruskuaista ja ne ovat kooltaan pienet ja läpikuultavat. Tällaisessa lepokaudessa ovaarioiden kehittämiseen tarkoitetut energiavarannot on suunnattu muihin elintoimintoihin talvehtimiskyvyn parantamiseksi.

Olen tutkinut väitöskirjassani pohjoisten mahlakärpästen kykyä rytmittää elinkiertonsa vuodenaikojen mukaan valojaksoissa tapahtuvien muutosten perusteella sekä jäljittänyt ko. rytmeihin liittyvien geenien toiminnassa tapahtuvia muutoksia erilaisissa olosuhteissa. Aloitin valojaksoisten vasteiden tutkimisen seuraamalla valojakson vaikutusta mahlakärpästen kehitysaikaan munavaiheesta aikuisvaiheeseen saakka. Kun *D. montana* -yksilöitä oli pidetty munavaiheesta lähtien alkukesää muistuttavassa pitkässä valojaksossa, niiden kehitys oli hitaampaa ja ne olivat kuoriutuessaan painavampia kuin yksilöt joita oli pidetty loppukesää muistuttavassa lyhyemmässä valojaksossa. Tämä osoittaa, että *D. montana* yksilöt pystyvät mittaamaan valojakson pituutta ja hienosäätämään elinkiertopiirteitään sopimaan sekä meneillä olevaan että tulevaan vuodenaikaan jo ennen kuoriutumistaan. Lyhyessä valojaksossa (syksyllä) kuoriutuvilla yksilöillä on luonnossa vähemmän aikaa valmistautua talvea varten ja jo päivänkin ero kehitysajassa saattaa tuoda yksilölle valintaedun hitaammin kehittyviin nähden. Lisääntymislepokausi määräytyi niiden valojaksojen mukaisesti, joita naaraat kokivat ensimmäisinä päivinä kuoriutumisen jälkeen, eikä se korreloinut edellä mainittujen ennen kuoriutumista mitattujen elinkiertopiirteiden kanssa. Ajanjakson pituus, jolloin naaraiden diapaussi saattoi käynnistyä,

oli lämpötilasta riippuvainen, sillä korkeammassa lämpötilassa naarailla oli vähemmän aikaa mitata päivänpituuksia peräkkäisistä vuorokausista ovarioiden nopeamman kehittymisen vuoksi.

Etsin väitöskirjatyössäni *D. montanalta* lepokauteen liittyviä geenejä seuraamalla geenien toiminnan tasolla tapahtuvia muutoksia (geenien ilmentyminen eli geenieksprессio) diapaussin eri vaiheissa. Tätä tarkoitusta varten *D. montanalle* suunniteltiin useisiin elinkiertopiirteisiin liittyvistä kandidaattigeeneistä koostuva DNA-mikrosiru, käyttäen apuna sukulaislajien genomitietoa. Ensimmäiselle *D. montana* -lajille spesifiselle DNA-mikrosirulle suunniteltiin geenikoettimia 108 geenistä, joiden oli todettu aiemmin liittyneen lepokauteen tai lisääntymislepokauteen, sirkadiseen rytmiin, liikeaktiivisuuteen, stressivasteisiin, kylmänkestävyyteen ja/tai ikääntymiseen esim. *D. melanogasterilla*. Tätä sirua käyttäen *D. montana* -naaraiden geenien toimintaa tutkittiin pitkässä (sukukupsä naaras) ja lyhyessä (lisääntymislepokaudessa oleva naaras) valojaksossa. Sirun todettiin toimivan hyvin ja koeasetelman avulla tunnistimme valojaksoisiin vasteisiin ja lisääntymislepokauteen liittyen kaksi mielenkiintoista geeniä: *couch potato* –ja *regucalcin* (aiemmin mainittu nimellä *Dca*).

Luonnossa sekä valojakso että lämpötila muuntelevat päivittäin, joten mahdollisimman aitojen vasteiden aikaansaamiseksi myös ympäristötekijöiden tulisi vastata tarkasti luonnossa vallitsevia oloja. Tämän takia ohjelmoin olosuhdekammioon Oulangan leveysastetta vastaavat olosuhteet, joissa valojaksoja, päivä- ja yö lämpötiloja sekä valon intensiteettiä muunneltiin vuodenaikojen mukaisesti lähes vuoden ajan. Tutkimuslajeina käytin Oulangalta kerättyjä kolmen *D. virilis* –lajiryhmään kuuluvan lajin, *D. montana*, *D. littoralis* ja *D. ezoana –lajien* naaraita. Työn yhtenä tavoitteena oli selvittää, missä vaiheessa näiden lajien naaraat siirtyvät lisääntymislepokauteen ja kuinka nopeasti siirtyminen tapahtuu luonnonkaltaisissa oloissa. Luonnonmukaisemmat olot saivatkin naaraat siirtymään lisääntymislepokauteen vuodenaikaan nähden aikaisemmin verrattuna tasaisiin valorytmeihin ja lämpötilaan. Siirtymisvaiheen lyhyys osoittaa, että luonnonoloissa naaraat ovat sopeutuneet käyttämään kasvukauden pituutta optimaalisesti hyväkseen ja tietyn päivänpituuden kynnysarvon jälkeen valintapaine siirtyä lisääntymislepokauteen on erittäin voimakas.

Tutkin myös kuinka talvehtiminen joko lisääntymislepokaudessa tai sukukypsänä yksilönä vaikuttaa *D. montana*, *D. littoralis* ja *D. ezoana* -naarailla talvehtimisen jälkeiseen kylmänkestävyyteen. Vaikka kaikki naaraat olivat mittaamishetkellä sukukypsiä (myös lisääntymislepokaudessa talvehtineet naaraat olivat kehittäneet sukukypsät ovaariot), oli talvehtimisen aikaisen ovaarioiden tilan vaikutus nähtävillä sekä *D. montana* että *D. littoralis* –naarailla: lisääntymislepokaudessa talvehtineet naaraat heräsivät nopeammin kylmäkoomasta kuin sukukypsinä talvehtineet naaraat. Fysiologisten mittausten lisäksi tutkin *D. montana* -naaraiden geenien toiminnassa tapahtuvia muutoksia eri ikäryhmissä kesä-, syksy-, talvi- ja kevätolosuhteissa. Sekä sukukypsillä että lisääntymislepokaudessa olevilla naarailla oli havaittavissa geenejä, jotka toimivat selvästi aktiivisemmin vain jommallakummalla ryhmällä ja myös näiden ryhmien sisällä esiintyi tietyille ikäluokille ominaisia geenieksprессioita. Muutamien geenien

ekspressio säilyi korkeana tai matalana lähes koko tutkittavan elinkierron ajan (7 päivän ikäisestä 150 päivän ikäiseen saakka), sen mukaan millaisissa olosuhteissa naaraat olivat olleet pian kuoriutumisensa jälkeen. Geenien toiminnassa havaittiin erittäin suuria eroja myös eri vuodenaikoja vastaavien olosuhteiden välillä, mikä osoittaa geenien toimivan erittäin plastisesti vaihtelevissa olosuhteissa.

Lukuisista väitöskirjatyössä esiin tulleista geeneistä valitsimme jatkoon *couch potato* (*cpo*) –geenin. Tässä geenissä esiintyvää muuntelua tutkittiin nukleotidi- ja aminohappotasolla useilla *Drosophila virilis* –ryhmän lajeilla keskittyen geenin viidenteen eksoniin, jonka on aikaisemmissa tutkimuksissa osoitettu liittyvän kärpästen lisääntymislepokauteen. Lisäksi selvitettiin yhdestä kärpäslinjasta löytyneen kuuden aminohapon mittaisen deleetion (poistuma) vaikutusta *cpo*-geenin ekspressioon, yksilöiden kehitysaikaan ja lisääntymislepokauteen siirtymiseen. Deleetio vähensi geenien ekspression määrää ja hidasti kehitysaikaa lyhyen päivänpituuden yksilöillä, mutta ei vaikuttanut naaraiden kykyyn siirtyä lisääntymislepokauteen.

Osoitan väitöskirjassani valojaksoisten signaalien tärkeyden pohjoisten lajien elinkiertopiirteitä muokkaavana tekijänä, käyttäen mallina pohjoisessa eläviä Drosophila lajeja. Lajit jotka ajoittavat elinkiertonsa vaiheita, kuten lisääntymistä ja talvehtimiseen valmistautumista lähinnä valojaksoisten signaalien kautta, saattavat tulevaisuudessa kohdata vaikeuksia ilmastonmuutoksen seurauksena muuttuneiden lämpötilojen seurauksena. Valojaksoisuuteen perustuvien elinkiertopiirteiden plastisuuden ja sen geneettisen taustan tutkiminen onkin tärkeää lajien sopeutumiskyvyn arvioimiseksi. Koska hyvinkin kaukaisten lajien välillä on havaittu yhtäläisyyksiä vuodenaikaisrytmeihin liittyvien ominaisuuksien geneettisessä taustassa, työn tuloksia voidaan hyödyntää myös muita hyönteislajeja, ja jopa ihmistä, koskevissa tutkimuksissa.

REFERENCES

Adkisson, P.L. 1961. Effect of larval diet on the seasonal occurrence of diapause in the pink bollworm. *J. Econ. Entomol*. 54: 1107-1112.

Allada, R. & Chung, B.Y. 2010. Circadian organization of behavior and physiology in *Drosophila*. *Annu. Rev. Physiol.* 72: 605-624.

Arboleda-Bustos, C.E. & Segarra, C. 2011. The *Dca* gene involved in cold adaptation in *Drosophila melanogaster* arose by duplication of the ancestral *regucalcin* gene. *Mol. Biol. Evol.* 28: 2185-2195.

Baker, D.A. & Russell, S. 2009. Gene expression during *Drosophila melanogaster* egg development before and after reproductive diapause. *BMC Genomics* 10: 242.

Beck, S.D. 1980. Insect Photoperiodism. 2nd edition, Academic Press, New York, 387 pp.

Blitvich, B.J., Rayms-Keller, A., Blair, C.D. & Beaty, B.J. 2001. Identification and sequence determination of mRNAs detected in dormant (diapausing) *Aedes triseriatus* mosquito embryos. *DNA Sequence* 12: 197–202.

Bradshaw, W.E. & Holzapfel, C.M. 2007. Evolution of animal photoperiodism. *Annu. Rev. Ecol. Evol. Syst* 38: 1-25.

Bradshaw, W.E. & Holzapfel, C.M. 2010. What season is it anyway? Circadian tracking vs. photoperiodic anticipation in insects. *J. Biol. Rhythms* 25: 155-165.

Bull, J.J. 1980. Sex determination in reptiles. *Q. Rev. Biol.* 55: 3-21.

Danks, H.V. 1987. *Insect Dormancy: An Ecological Perspective*. *Biol. Surv. Canada*. Ottawa, Canada. pp. 439.

David, J.R., Gibert, P., Pla, E., Petavy, G., Karan, D. & Moreteau, B. 1998. Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *J. Therm. Biol.* 23: 291-299.

de Block, M., McPeek, M.A. & Stoks, R. 2007. Winter compensatory growth under field conditions partly offsets low energy reserves before winter in a damselfly. *Oikos* 116: 1975–82.

Denlinger, D.L. 1985. Hormonal control of diapause. In Kerkut, G.A. & Gilbert, L.I. (Eds), *Comprehensive Insect Physiology, Biochemistry and Pharmacology*. Pergamon Press, Oxford. pp. 353-412.

Denlinger, D.L. 1991. Relationship between cold hardiness and diapause. In Lee Jr. R.E. & Denlinger, D.L. (Eds), *Insect at low temperature*. Chapman & Hall, New York, pp. 174-198.

Denlinger, D.L. 2002. Regulation of diapause. *Annu. Rev. Entomol*. 47: 93-122.

Denlinger, D.L. 2008. Why study diapause? *Entomol. Res.* 38: 1-9.

Dolezel, D., Šauman I., Koštál, V. & Hodkova, M. 2007. Photoperiodic and Food Signals Control Expression Pattern of the Clock Gene, *Period*, in the Linden Bug, *Pyrrhocoris apterus*. *J. Biol. Rhythms* 22: 335-342.

Dolezel, D., Vaněčková, H., Šauman I. & Hodkova, M. 2005. Is *period* gene causally involved in the photoperiodic regulation of reproductive

diapause in the linden bug, *Pyrrhocoris apterus*? *J. Insect Physiol*. 51: 655–659.

Emerson, K.J, Bradshaw, W.E. & Holzapfel, C.M. 2009. Complications of complexity: integrating environmental, genetic and hormonal control of insect diapause. *Trends Genet.* 25: 217-225.

Fantinou, A.A., Tsitsipis, J.A. & Karandinos, M.G. 1998. Diapause termination in *Sesamia nonagrioides* (Lepidoptera: Noctuidae) under laboratory and field conditions. *Environ. Entomol.* 27: 53–58.

Fielenbach, N. & Antebi, A. 2008. *C. elegans* dauer formation and the molecular basis of plasticity. *Genes Dev.* 22: 2149-2165.

Garbuz, D., Evgenev, M.B., Feder, M.E. & Zatsepina, O.G. 2003. Evolution of thermotolerance and the heat-shock response: evidence from inter/intraspecific comparison and interspecific hybridization in the *virilis* species group of *Drosophila*. I. Thermal phenotype. *J. Exp. Biol.* 206: 2399-2408.

Gloor, G. & Engels, W. 1992. Single-fly DNA preps for PCR. *Dros. Inf. Serv.* 71: 148-149.

Goller, F. & Esch, H. 1990. Comparative study of chill coma temperatures and muscle potentials in insect flight muscles. *J. Exp. Biol.* 150: 221-231.

Gomi, T. & Takeda, M. 1992. A quantitative photoperiodic response terminates summer diapause in the tailed zygaenid moth, *Eclysma westwoodii*. *J. Insect Physiol.* 38: 665–670.

Gomi, T., Kimura, T. & Hirose, S. 2008. Seasonal changes in the reproductive and life-history traits of *Chrysomela populi* L. (Coleoptera: Chrysomelidae). *Entomol. Sci.* 11: 31-38.

Goto, S.G. & Denlinger, D.L. 2002. Short-day and long-day expression patterns of genes involved in the flesh fly clock mechanism: *period*, *timeless*, *cycle* and *cryptochrome*. *J. Insect Physiol*. 48: 803–816.

Goto, S.G. & Kimura, M.T. 2004. Heat-shock-responsive genes are not involved in the adult diapause of *Drosophila triauraria*. *Gene* 326: 117-122.

Gubenko, I.S. & Evgenev, M.B. 1984. Cytological and linkage maps of *Drosophila virilis* chromosomes. *Genetica* 65: 127-139.

Hahn, D.A. & Denlinger, D.L. 2007. Meeting the energetic demands of insect diapause: nutrient storage and utilization. *J. Insect Physiol.* 53: 760-773.

Hahn, D.A. & Denlinger, D.L. 2011. Energetics of insect diapause. *Annu. Rev. Entomol*. 56: 103-121.

Hanski, I. 1988. Four kinds of extra long diapause in insects: a review of theory and observations. *Ann. Zool. Fennici* 25: 37–53.

Hayward, S.S.L., Pavlides, S.C., Tammariello, S.P., Rinehart, J.P. & Denlinger, D.L. 2005. Temporal expression patterns of diapause-associated genes in the flesh fly pupae from the onset of diapause through post-diapause quiescence. *J. Insect. Physiol.* 51: 631-640.

He, H-M., Xian, Z-H., Huang, F., Liu, X-P. & Xue, F-S. 2009. Photoperiodism of diapause induction in *Thyrassia penangae* (Lepidoptera: Zygaenidae). *J. Insect Physiol.* 55: 1003-1008.

Herman, W.S. 1981. Studies on the adult reproductive diapause of the monarch butterfly, *Danaus plexippus*. *Biol. Bull*. 160: 89-106.

Hodek, I. 2002. Controversial aspects of diapause development. *Eur. J. Entomol.* 99: 163–173.

Hodková, M. & Hodek, I. 2004. Photoperiod, diapause and cold hardiness. *Eur. J. Entomol.* 101: 445-458.

Hoffmann, A.A & Sgro, C.M. 2011. Climate change and evolutionary adaptation. *Nature* 470: 479-485.

Hoikkala, A. & Lumme, J. 1987. The Genetic Basis of Evolution of the Male Courtship Sounds in the Drosophila virilis Group. *Evolution* 41: 827-845.

Ikeno, T., Tanaka, S.I., Numata, H. & Goto, S.G. 2010. Photoperiodic diapause under the control of circadian clock genes in an insect. *BMC Biol.* 8: 116.

Kalushkov, P., Hodkova, M., Nedved, O. & Hodek, I. 2001. Effect of thermoperiod on diapause intensity in *Pyrrhocoris apterus* (Heteroptera Pyrrhocoridae). *J. Insect Physiol.* 47: 55-61.

Kankare, M., Salminen, T.S., Laiho, A., Vesala, L. & Hoikkala, A. 2010. Changes in gene expression linked with adult reproductive diapause in a northern malt fly species: a candidate gene microarray study. *BMC Ecol.* 10: 3.

Kogure, M. 1933. The influence of light and temperature on certain characters of the silk-worm, *Bombyx mori*. *J. Dept. Agr. Kyushu Univ*. 4: 1-93.

Koštál, V. 2006. Eco-physiological phases of insect diapause. *J. Insect Physiol*. 52: 113-127.

Koštál, V. 2011. Insect photoperiodic calendar and circadian clock: independence, cooperation or unity? *J. Insect Physiol.* 57: 538-556.

Lakovaara, S. 1969. Malt as a culture medium for Drosophila species. *Dros. Inf. Serv.* 44: 128.

Lankinen, P. 1986. Geographical variation in circadian eclosion rhythm and photoperiodic adult diapause in *Drosophila littoralis*. *J. Comp. Physiol. A.* 159: 123-142.

Levin, D.B., Danks, H.V. & Barber, S.A. 2003. Variations in mitochondrial DNA and gene transcription in freezing-tolerant larvae of *Eurosta solidaginis* (Diptera: Tephritidae) and *Gynaephora groenlandica* (Lepidoptera: Lymantriidae). *Insect Mol. Biol.* 12: 281–289.

Levine, J.D., Funes, P., Dowse, H.B. & Hall, J.C. 2002. Resetting the circadian clock by social experience in *Drosophila melanogaster*. *Science* 298: 2010-2012.

Lewis, D.K., Spurgeon, D., Sappington, D.W. & Keeley, L.L. 2002. A hexamerin protein, AgSP-1, is associated with diapause in the boll weevil. *J. Insect Physiol*. 48: 887–901.

Lumme, J. 1981. Localization of the genetic unit controlling the photoperiodic adult diapause in *Drosophila littoralis*. *Hereditas* 94: 241-244.

Lumme, J. 1978. Phenology and photoperiodic diapause in northern populations of Drosophila. In: Dingle, H. (Ed), *Evolution of insect migration and diapause*, Springer Verlag, New York. pp. 145-170.

Lumme, J. & Oikarinen, A. 1977. The genetic basis of the geographically variable photoperiodic diapauses in *Drosophila littoralis*. *Hereditas* 86: 129-142.

McLeod, D.G.R. & Beck, S.D. 1963. Photoperiodic termination of diapause in an insect. *Biol. Bull.* 124: 84-96.

Masaki, S. 1980. Summer diapause. *Annu. Rev. Entomol*. 25: 1–25.

Masaki, S. 1983. Introduction. In: Brown, V.K. & Hodek, I. (Eds.), *Diapause and life cycle strategies in insects*. Dr. W. Junk Publishers, The Hague. pp. 1-5.

Mirol, P.M., Schäfer, M.A., Orsini, L., Routtu, J., Schlötterer, C., Hoikkala, A. & Butlin, R.K. 2007. Contrasting phylogeographic patterns in *Drosophila virilis* and *Drosophila montana*. *Mol. Ecol.* 16: 1085-1097.

Morales-Hojas, R., Päällysaho, S., Vieira, C.P., Hoikkala, A. & Viera, J. 2007. Comparative polytene chromosome maps of *D. montana* and *D. virilis*. *Chromosoma* 116: 21-27.

Nishizuka, M., Azuma, A & Koštál, V. 1988. Diapause response to photoperiod and temperature in Lepisma saccharina Linnaeus (Thysanura: Lepismatidae). *Entomol. Sci.* 1: 7-14.

Nylin, S. 1989. Effects of changing photoperiods in the life cycle regulation of the comma butterfly, *Polygonia c-album* (nymphalidae). *Ecol. Entomol.* 14: 209-218.

Parmesan, C. & Yohe, G. 2003. A globally coherent fingerprint of climate change impacts across natural systems. *Nature* 421: 37-42.

Pavelka, J., Shimada, K. & Koštál, V. 2003. TIMELESS: a link between fly's circadian and photoperiodic clocks? *Eur. J. Entomol.* 100: 255–265.

Pittendrigh, C.S. & Takamura, T. 1987. Temperature dependence and evolutionary adjustment of critical night length in insect photoperiodism. *Proc. Nat. Acad. Sci. USA* 84: 7169-7173.

Ragland, G.J., Denlinger, D.L. & Hahn, D.A. 2010. Mechanisms of suspended animation are revealed by transcript profiling of diapauses in the flesh fly. *Proc. Natl. Acad. Sci. USA* 107: 14909-14914.

Rinehart, J.P., Yocum, G.D. & Denlinger, D.L. 2000. Developmental regulation of inducible hsp70 transcripts, but not the cognate form, during pupal diapause in the flesh fly, *Sarcophaga crassipalpis*. *Insect. Biochem. Mol. Biol.* 30: 515-521.

Routtu, J., Mazzi, D., Van der Linde, K., Mirol, P., Butlin, R.K. & Hoikkala, A. 2007. The extent of variation in male song, wing and genital characters among allopatric *Drosophila montana* populations. *J. Evol. Biol.* 20: 1591-1601.

Saunders, D.S. 1966. Larval diapause of maternal origin-II. The effect of photoperiod and temperature on *Nasonia vitripennis*. *J. Insect. Physiol*. 12: 569-581.

Saunders, D.S. 1971. The temperature-compensated photoperiodic clock "programming" development and pupal diapause in the flesh-fly, *Sarcophaga argyrostoma*. *J. Insect Physiol*. 17: 801-812.

Saunders, D.S. 1981. Insect photoperiodism: the clock and the counter. *Physiol. Entomol*. 6: 99–116.

Saunders, D.S. 1997. Under-sized larvae from short-day adults of the blow fly, *Calliphora vicina*, side-step the diapause program. *Physiol. Entomol*. 22: 249–255.

Saunders, D.S. 2002. Insect clocks, 3rd ed. Elsevier, Amsterdam.

Saunders, D.S. 2009. Circadian rhythms and the evolution of photoperiodic timing in insects. *Physiol. Entomol.* 34: 301-308.

Saunders D.S. & Gilbert L.I. 1990. Regulation of ovarian diapause in *Drosophila melanogaster* by photoperiod and moderately low temperature. *J. Insect Physiol*. 36: 195–200.

Saunders, D.S., Henrich, V.C. & Gilbert, L.I. 1989. Induction of diapause in *Drosophila melanogaster*: photoperiodic regulation and impact of arrhythmic clock mutations on time measurement. *Proc. Natl. Acad. Sci. USA*. 86: 3748-3752.

Schmidt, P.S., Zhu, C.T., Das, J., Batavia, M., Yang, L. & Eanes, M.F. 2008. An amino acid polymorphism in the *couch potato* gene forms the basis for climatic adaptation in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA*. 105: 16207-16211.

Sim, C. & Denlinger, D.L. 2008. Insulin signaling and FOXO regulate the overwintering diapause of the mosquito *Culex pipiens*. *Proc. Natl. Acad. Sci. USA*. 105: 6777–6781.

Spicer, G.S. & Bell, C.D. 2002. Molecular phylogeny of the *Drosophila virilis* species group (Diptera: Drosophilidae) inferred from mitochondrial 12S and 16S ribosomal RNA genes**.** *Ann. Entomol. Soc. Am.* 95: 156-161.

Spieth, H.R., Xue, F. & Strauss, K. 2004. Induction and inhibition of diapause by the same photoperiod: experimental evidence for a ''double circadian oscillator clock''. *J. Biol. Rhythms* 19: 483–492.

Tachibana, S.-I., Numata, H. & Goto, S.G. 2005. Gene expression of heat- shock proteins (Hsp23, Hsp70 and Hsp90) during and after larval diapause in the blow fly *Lucilia sericata*. *J. Insect Physiol.* 51: 641–647.

Tatar, M. & Yin, C.-M. 2001. Slow aging during insect reproductive diapause: why butterflies, grasshoppers and flies are like worms. *Exp. Gerontol.* 36: 723-738.

Tauber M.J., Tauber C.A. & Masaki, S. 1986. *Seasonal adaptations of insects*. Oxford University Press, New York.

Tauber, E., Zordan, M., Sandrelli, F., Pegoraro, M., Osterwalder, N., Breda, C., Daga, A., Selmin, A., Monge,r K., Benna, C., Rosato, E., Kyriacou, C., Costa, R. & Sokolowski, W. 2007. Natural Selection Favors a Newly Derived timeless Allele in *Drosophila melanogaster*. *Science* 316: 1895-1898.

Throckmorton, L.H. 1982. The virilis species group. In: Ashburner, M., Carson, H.L., Thompson, J.N. Jr. (Eds), *The Genetics and Biology of Drosophila*. Vol. 3b Ed. Academic Press, London. pp. 227-296.

Tyukmaeva, V.I., Salminen, T.S., Kankare, M., Knott, K.E. & Hoikkala, A. 2011. Adaptation to a seasonally varying environment: a strong latitudinal cline in reproductive diapause combined with high gene flow in *Drosophila montana. Ecology and Evolution*. doi:10.1002/ece-3.14.

Uno, T., Nakasuji, A., Shimoda, M. & Aizono, Y. 2004. Expression of *cytochrome c oxidase subunit 1* gene in the brain at an early stage in the termination of pupal diapause in the sweet potato hornworm, *Agrius convonvuli. J. Insect Physiol*. 50: 35–42.

Varjas, L. & Saringer, G. 1998. Oxygen consumption as an indicator of diapause intensity in pupae of *Lacanobia oleracea* and *Mamestra brassicae* reared at different inductive photoperiods. *Acta Phytopathologica at Entomologica Hungarica* 33: 147–151.

Vaz Nunes, M. & Saunders, D.S. 1999. Photoperiodic time measurements in insects: A review of clock models. *J. Biol. Rhythms* 14: 84-104.

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

Watabe, H. 1983. Photoperiodic responses in *Drosophila virilis* species group (Diptera, Drosophilidae) from Japan. *Kontyu, Tokyo* 51: 628-634.

Wei, Z.-J., Zhang, Q.-J., Kang, L., Xu, W.-H. & Denlinger, D.L. 2005. Molecular characterization and expression of prothoracicotropic hormone during development and diapause in the cotton bollworm, *Helicoverpa armigera*. *J. Insect Physiol*. 51: 691–700.

Williams, K.D., Busto, M., Suster, M.L., So, A.K., Ben-Shahar, Y., Leevers, S.J. & Sokolowski, M.B. 2006. Natural variation in *Drosophila melanogaster* diapause due to the insulin-regulated PI3-kinase. *Proc. Natl. Acad. Sci. USA*. 103: 15911-15915.

Xiao, H-J., Mou, F-C., Zhu, X-F. & Xue, F-S. 2010. Diapause induction, maintenance and termination in the rice stem borer *Chilo suppressalis* (Walker). *J. Insect Physiol* 56: 1558-1564.

Xu, W-H. & Denlinger, D.L. 2004. Identification of a cDNA encoding DH, PBAN and other FXPRL neuropeptides from the tobacco hornworm, *Manduca sexta*, and expression associated with pupal diapause. *Peptides* 25: 1099–1106.

Yocum, G.D., Kemp, W.P., Bosch, J. & Knoblett, J.N. 2005. Temporal variation in overwintering gene expression and respiration in the solitary bee *Megachile rotundata*. *J. Insect Physiol*. 51: 621–629.

Zhang, Q. & Denlinger, D.L. 2011. Elevated *couch potato* transcripts associated with adult diapause in the mosquito *Culex pipiens*. *J. Insect Physiol*. 57: 620-627.

ORIGINAL PAPERS

I

DETECTING SEASONAL TIME: PHOTOPERIODIC REGULATION OF LIFE-HISTORY TRAITS IN A NORTHERN FLY SPECIES, *DROSOPHILA MONTANA*

by

Tiina S. Salminen, Laura Vesala & Anneli Hoikkala

Submitted manuscript

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II

EFFECTS OF TEMPERATURE ON THE INDUCTION OF PHOTOPERIODIC REPRODUCTIVE DIAPAUSE IN *DROSOPHILA MONTANA* MALT FLY

by

Tiina S. Salminen & Anneli Hoikkala

Manuscript

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III

CHANGES IN GENE EXPRESSION LINKED WITH ADULT REPRODUCTIVE DIAPAUSE IN A NORTHERN MALT FLY SPECIES: A CANDIDATE GENE MICROARRAY STUDY

by

Maaria Kankare, Tiina S. Salminen, Asta Laiho, Laura Vesala & Anneli Hoikkala 2010

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IV

PLASTIC PHENOTYPIC AND EXPRESSIONAL CHANGES REGULATED BY SEASONAL CUES ENABLE INSECTS TO SURVIVE OVER THE HARSH WINTER PERIOD AT HIGH LATITUDES

by

Tiina S. Salminen, Laura Vesala, Asta Laiho, Mikko Merisalo, Maaria Kankare & Anneli Hoikkala

Manuscript

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V

SEQUENCE VARIATION IN THE *COUCH POTATO* GENE AND ITS EFFECTS ON LIFE-HISTORY TRAITS IN A NORTHERN MALT FLY, *DROSOPHILA MONTANA*

by

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