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To my family

ABSTRACT

Adaptation to seasonal changes in environmental conditions is of crucial importance for species living at high latitudes. This involves not only tolerance to cold temperatures, but also an ability to timely predict the forthcoming cold season from gradual changes in the environment. The forecasting systems of several northern species have been found to rely on changes in photoperiodic cues, which are the most reliable seasonal cue at high latitudes. In my thesis, I examine the genetic basis of variation in one of the strongest adaptations to seasonally varying environments in insects - adult reproductive diapause - and other associated life-history traits in *Drosophila montana*. Study I revealed significant and predictable clinal variation in the critical day length (CDL) for diapause incidence in presence of high gene flow. Study II showed high variation in factors affecting the diapause incidence both within and between the same clinal populations and showed that while variation in females' photoperiodic responses decreases during laboratory maintenance, the mean CDLs of the strains remain the same. Study III was carried out with a "resonance" experiment, revealed no rhythmicity in the females' diapause response, which suggests that the photoperiodic timer of *D. montana* is based on a non-oscillatory hourglass timer or rapidly dampening circadian oscillator. In study IV we performed a QTL analysis by crossing two *D. montana* strains that differed in their diapause incidence, cold tolerance, egg-to-eclosion development time and body weight at a specific day length. This study revealed both unique and combined QTL for the studied traits, the most interesting result being a large-effect QTL for diapause on the X chromosome. While we did not find evidence for the involvement of a circadian clock in the photoperiodic regulation of reproductive diapause in *D. montana* in the above-mentioned studies, some QTL for traits regulated by different time-measuring systems were overlapping (IV).

Keywords: Adaptation; diapause; *Drosophila montana*; life history; seasonality.

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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-IV.

I   Tyukmaeva V.I., Salminen T.S., Kankare M., Knott K.E. & Hoikkala A. 2011. Aadaptation to a seasonally varying environment: a strong latitudinal cline in reproductive diapause combined with high gene flow in *Drosophila montana*. *Ecology and Evolution* 1: 160-168.

II Lankinen P., Tyukmaeva V.I. & Hoikkala A. 2012. Northern *Drosophila montana* flies show steep photoperiodic response curves and high genetic variation in critical day length evoking reproductive diapause. Manuscript.

III Kauranen H., Tyukmaeva V.I. & Hoikkala A. 2012. The flies of a northern Drosophila species with robust diapause lose their rhythmicity and ability to enter diapause in prolonged scotophases. Manuscript.

IV Tyukmaeva V.I., Veltsos P., Slate J., Gregson E., Kauranen H., Kankare M., Ritchie M.G., Butlin R.K. & Hoikkala A. 2012. Genetic basis of population divergence in life-history traits important in adaptation to seasonally varying environment in a northern insect species. Manuscript.

	Planning	Data	Analyses	Writing
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ABBREVIATIONS

CCRT   chill coma recovery time
CDL   critical day length
DNA   deoxyribonucleic acid
LD   light:dark cycle
LL   constant light
NH   Nanda-Hamner protocol
PPRC   photoperiodic response curve
QTL   quantitative trait loci
SNP   single nucleotide polymorphism

FIGURES

1 INTRODUCTION

> *"Adaptation is the heart and soul of evolution"*
> *Niles Eldredge*

1.1 Seasonal adaptation

Evolutionary mechanisms through which species and populations get adapted to their environment present a corner-stone for understanding the origin and maintenance of biodiversity. While organisms attempt to maximize their fitness in the prevailing environmental conditions, limited resources and changing environmental conditions lead to various kinds of constraints and trade-offs between fitness traits making this goal practically unattainable. However, genetic variation in life-history traits, which is maintained e.g. by counteracting selection pressures, helps populations to cope with short- and long-term changes in environmental conditions.

The major source of energy on Earth is solar radiation, which is not distributed evenly on the planet's surface. The fact that the amount of solar radiation also shows high seasonal variation especially in the temperate and polar zones, creates a powerful factor for natural selection – seasonality. Short favorable periods for growth and reproduction in these areas induce especially strong selection pressures for appropriate timing of the changes in life history, such as traits linked with development, progeny production and preparing for the cold period.

Overwintering is a demanding life-stage that implies resistance to various adverse factors associated with cold conditions. A downside of overwintering is a rather high level of natural mortality, largely due to the effects of environmental stresses, depletion of energy resources and, in some cases, predation. The most important life-history traits that have a high impact on individuals' survival include body weight, development time and cold tolerance. As can be expected, these traits often show latitudinal clines caused by various kinds of environmental factors (e.g. Magiafoglou et al. 2002, Hackman et al. 1970,

Demont et al. 2008). There are, for example, two opposite views explaining latitudinal variation in body size. The first one, Bergmann's rule, says that an increase in body size with latitude is caused by a decrease in mean temperature. Whereas the second one, converse Bergmann's rule, says that body size decreases with the length of the growing season. These rules are confounded by trade-offs between body size and other life-history traits like developmental time, which changes e.g. as a function of the number of generations per year (see e.g. Blackenhorn & Demont 2004).

Even though the above mentioned traits are important in overwintering, in many species the key feature for survival over this period is a stage of dormancy, which often also has an impact on several other fitness traits. In my thesis I mainly concentrate on studying reproductive diapause, which is a widely used dormancy trait in insects living in seasonal environments.

1.2 Insects' "escape" strategies

Insect species have evolved various kinds of strategies to avoid unfavorable conditions. One of them, migration, is often referred as an "escape in space", and the other one, dormancy, as an "escape in time". Most species produce one (univoltine species) or several (multivoltine species) generations per growing season, but in some long-lived species the individuals' life cycle can take several years and involve dormancy at several different developmental stages. This has been found to happen e.g. in the dragonfly *Tetragoneuria cynosura*, in which the early and mature nymphs can enter diapause during different winters (Lutz and Jenner 1964).

The range of dormancies is wide, but they can be divided generally into two ecological types: hibernation, which is a response to cold conditions, and aestivation, which is a response to high temperatures and arid conditions. Another way to classify dormancies is to divide them into quiescence and diapause. Quiescence, also known as "non-adaptive torpor", is induced directly by adverse conditions and is terminated immediately after the inducing factors are removed. A good example of this kind of dormancy is dehydratation of *Polypedilum vanderplanki* larvae, which continue development as soon as drought is over (Hinton 1951). Diapause, which is one of the main overwintering dormancies of northern insects, differs from quiescence mainly in that it can be induced by environmental cues well before the cold season and that it can last for long periods even when the harsh environmental conditions are over (Tauber 1986).

1.3 Diapause

Diapause is a neurohormonally mediated state of low metabolic activity, which involves cessation of development and/or reproduction. An important feature of diapause is that it is triggered by "token stimuli", i.e. environmental cues that do not represent unfavorable conditions themselves but are seasonally correlated with them (Tauber et al. 1986). Diapause is associated with many processes, such as fat body hypertrophy, cessation of metamorphosis or development, acquisition of cold hardiness or resistance to desiccation, storage of distinctive metabolites in the haemolymph, or reduced metabolism (Hahn & Denlinger 2007). Facultative diapause needs to be triggered by external cues, while obligatory diapause takes place at a certain life stage without any external stimuli. Depending on the duration and type of diapause termination, the diapause may also be classified as "intense" or "shallow".

Diapause typically occurs at a certain development stage, which varies between species. For example in silkworm *Bombix mori* (Kogure 1933) and the green vetch aphid *Megoura viciae* (Lees 1959) embryonic diapause occurs at an egg stage, while in the European corn borer *Ostrinia nubilalis* (Beck & Hanec 1960) and the parasitic wasp *Nasonia vitripennis* (Saunders 1965a) diapause occurs at larval stage. Pupal diapause is common in many Lepidoptera species, including *Acronycta rumicis* (Danilevskii 1965). Furthermore, in several insect species the females undergo diapause at an adult stage and postpone their sexual maturation and reproduction to the next favorable season. This kind of adult reproductive diapause is wide-spread e.g. among the species of genus *Drosophila* (Lumme 1978). Also Colorado potato beetles *Leptinotarsa decemlineata* (De Wilde & De Boer 1961) and the heteropterian bug *Pyrrhocornis apterus* (Hodek 1968) enter diapause at the adult stage.

Diapause is a dynamic state and it generally involves three separable phases (Tauber 1986, Kostal 2006). The initiation phase is characterized by a cessation of direct development (morphogenesis) followed by regulated metabolic suppression and building of energy resources. At the same time insects start to search for a suitable microhabitat for overwintering. In the maintenance phase the endogenous developmental arrest continuous and metabolic rate stays relatively low and constant. At termination phase specific changes in environmental conditions decrease the diapause intensity to its minimum, and by the end of this phase the development of the insect starts to continue.

Diapause has a significant effect on insects' life history profiles including longevity, fecundity and stress resistance (Tauber 1986). This phenomenon has important applications also in the fields of pest management (e.g. Hausmann et al. 2004) and insect domestication (e.g. the silkworm *Bombix mori*; Denlinger 2008). In addition, the knowledge accumulated in diapause studies can be used as a model when studying aging, obesity, disease transmission and cryoprotection (Denlinger 2008). All in all, diapause represents the strongest adaptation to seasonally changing environments, and its induction creates one of

the most important trade-offs in seasonal insects – a trade-off between reproduction and survival.

1.4 Correct timing of development and reproduction is regulated by environmental cues

Living in a seasonal environment is all about timing: it is crucial to maximize the organism's exploration of the favorable season and minimize its exposure to adverse conditions. Both males and females should to be able to prepare for the forthcoming changes in environmental conditions well in advance, store resources and stay in a developmental stage that is best for their overwinter survival. In the temperate zone, day length presents the most reliable cue for the timing of different life history events, as these cues are the same today as they were 10,000 years ago, but also other cues, such as temperature, play a role in time measurement (Saunders 2002). Importantly, the fact that the day length changes at different latitudes at a different pace enables organisms to anticipate seasonal changes in their local environment and adjust changes in their life-history traits accordingly.

Photoperiodic time measurement systems rely on the function of two major molecular clock mechanisms, the circadian clock and the photoperiodic calendar (Bradshaw & Holzapfel 2007). Circadian clocks are regulated by transcriptional-translational feedback loops involving a number of genes, and many of their features are conserved across taxa (e.g. Allada & Chung 2010). These clocks regulate a variety of metabolic and hormonal processes and they have been found to evoke rhythmic changes in seasonally varying life-history traits of organisms, such as developmental timing (Kyriacou et al. 1990, Paranjpe et al. 2005) or cold tolerance (Espinoza et al. 2008). The photoperiodic calendar, on the other hand, consists of light receptors, a photoperiodic clock that scores day/night length as short or long, counters that store information on the number of daily cycles received, and output pathways. This calendar acts as a seasonal switch on the basis of accumulated information and commits insects e.g. to enter reproductive diapause once a threshold, the critical day length (CDL), is surpassed (Bradshaw & Holzapfel 2010). CDL is often used as an operational population characteristic for reproductive diapause, and it refers to the day length at which half of the females of a population will enter diapause. There is no direct evidence for a causal role of circadian clocks in the photoperiodic calendar, but recent studies have suggested that the two systems could work in cooperation, or that some of the circadian clock genes (e.g. *per* and *timeless*) could play an independent role in both clock systems (Saunders 2009, Bradshaw & Holzapfel 2010, Koštál 2011). There are a number of theories and models to explain the function of the two time-measuring mechanisms, reaching from completely separated or overlapping functions to partial cooperation between the mechanisms (for review, see Vaz Nunes & Saunders 1999).

In obligatory reproductive diapause individuals do not need to make a "decision" on whether to enter dormancy or to invest resources in reproduction, but in facultative diapause the correct timing of diapause has important consequences both for survival and progeny production. Individuals that enter reproductive diapause too early face two kinds of risks: they may deplete their energy reservoirs already during the warm period and/or they may not produce any progeny if they die before the next favorable season (Hahn & Denlinger 2007). Conversely, if individuals that emerge in late summer mature sexually and produce progeny instead of entering diapause, they and/or their progeny may not survive over the winter (Musolin & Numata 2003). According to Taylor (1980) the optimal switching time to diapause occurs well in advance, leaving a certain amount of time between the diapause incidence and the onset of winter. The length of this period can be calculated as a sum of the life cycle stages that an organism needs to complete before the harsh season. In wild populations, individuals have been found to enter reproductive diapause even when there would be time to produce one more generation before the winter (Lumme et al. 1978).

1.5 Genetic variation in life-history traits important in overwintering

There is currently a variety of methods available for tracing genetic variation in life-history traits, as well as for localizing and identifying genes that affect these traits. Classic quantitative genetic methods, like selection experiments, various kinds of crossing schemes and the use of isofemale strains, give important information on the amount of additive genetic variation in the studied traits, and Quantitative Trait Loci (QTL) studies help to localize genes affecting these traits on specific chromosomal regions. Finally, gene expression studies (transcriptomics, microarray, qPCR and RNA-interference) offer an alternative way to identify candidate genes for the studied traits and also to measure the effects of these genes on trait values more directly.

QTL analyses have revealed significant QTL for insects' life-history traits e.g. in *Wyeomyia smithii* (Mathias et al. 2007) and the *Culex pipiens* complex (Mori et al. 2007). In both of above-mentioned studies some QTL for different life-history traits mapped to the same chromosomal regions, which suggests that the traits may be controlled in part by the same genes with pleiotropic effects and/or by multiple tightly linked loci. Also studies on various *Drosophila* species have revealed chromosomal areas containing genes that affect one or more life-history traits. QTL studies on *D. melanogaster* have for example revealed significant QTL for cold tolerance on several chromosomes (Morgan & Mackay 2006, Norry et al. 2007, Norry et al. 2008, Svetec et al. 2011), and Williams and Sokolovski (1993) and Schmidt et al. (2008) have detected candidate genes for diapause on the 3rd chromosome of this species. Furthermore, Lumme and Keränen (1978) have

found that the genes affecting the inter-specific difference between *D. virilis* and *D. lummei* in diapause induction are located on the X chromosome, and Lumme (1981) showed that in *D. littoralis* the critical day length for diapause is largely regulated by a single autosomal unit located on the right arm of the combined 3rd and 4th chromosome. However, the road from the identification of QTL to the identification of genes affecting the studied traits is long and cumbersome and not necessarily successful.

1.6   *D. montana* as a study object for seasonal adaptation

Northern *Drosophila* species are good study objects for tracing genetic changes that have taken place during the adaptation to seasonally varying environments. For example, in Fennoscandia 23 *Drosophila* species have been found to live above 65°N latitude and the females of all these species, except *D. alpina*, spend the winter in adult reproductive diapause (Lumme et al. 1978). One of the species that is extremely well adapted to live at high latitudes and altitudes is *D. montana*, a species of the *D. virilis* group (Baker 1975, Vesala et al. 2012). This species has a robust photoperiodic reproductive diapause (Lumme 1978, I) and it shows high cold tolerance that increases towards the winter (Vesala and Hoikkala 2011, Vesala et al. 2012). On the other hand, developmental time and juvenile body weight of this species have been found to be regulated by photoperiodic cues prior to adult eclosion (Salminen et al. in press), i.e. before the switch to diapause occurs, which makes trait comparisons very interesting. Finally, *D. montana* flies completely lack the morning locomotor activity peak typical for more southern *Drosophila* species, and they are able to maintain their free-running activity rhythm better in constant light than in constant darkness, contrary e.g. to *D. melanogaster* (Kauranen et al. in press). These characters are likely to play an important role in adaptation to seasonality.

1.7   Aims of the thesis

The main aim of my thesis was to examine the genetic basis of variation in one of the strongest adaptations to seasonally varying environments in insects, photoperiodic adult reproductive diapause, as well as other associated life-history traits important in adaptation to seasonally varying environment.

Studies I and II examined variation in the steepness of females' photoperiodic response curves (PPRC) and the critical day (CDL) inducing reproductive diapause in *D. montana* populations along a latitudinal cline in Finland. In the first of these studies we defined clinal variation in CDL and traced the amount of gene flow between the cline populations. In the second study we traced variation within and between cline populations in PPRC and CDL using a larger number of isofemale strains per population. Together these

two studies enabled me to find out whether wild *D. montana* populations possess genetic variation in the above-mentioned traits within and between cline populations, whether this variation is maintained in the presence of gene flow, and whether PPRC and CDL are liable to change during laboratory maintenance.

In study III, we carried out a "resonance" (Nanda-Hamner) experiment to find out whether the induction of the photoperiodic reproductive diapause of *D. montana* females requires the involvement of a circadian oscillator or whether it is based on an hourglass-type time measurement system. The question of a possible connection between the two time-measuring systems is still open after decades of research on several insect species, including *D. melanogaster.*

Finally, we performed a QTL analysis (study IV) between two *D. montana* strains that differed in their diapause incidence, cold tolerance, egg-to-eclosion development time and juvenile body weight in a specific day length. The main questions of this study were: Can we detect significant QTL, and in which chromosomal areas are these QTL located? Do any of the QTL affect more than one of the traits and are there unique QTL for any of the traits? And do the detected QTL regions implicate genes known to play a part in the studied traits and/or time-measuring systems regulating them? The fact that all the studied traits are photoperiodically regulated, either by the photoperiodic calendar or circadian clock, and either before or after eclosion, were of extra interest in this study.

2 MATERIALS AND METHODS

2.1 Study species and populations

All experiments were carried out with *D. montana* isofemale strains consisting of progeny of wild-caught fertilized females. The studies were performed on females, whose reproductive state is relatively easy to determine (see below).

In study I we used females of 24 isofemale strains originated from six *D. montana* populations along a 760 kilometer long latitudinal cline (60º-67ºN) in Finland (Fig. 1). All strains were collected in 2008 and 2009 and maintained in the laboratory for about six generations before being used in the experiment. Study II involved females of 102 isofemale strains from the above-mentioned cline populations as well as from a sea-shore population in Kemi. The strains used in this study had been collected from some populations during different years, which gave us a chance to find out how laboratory maintenance affects the studied traits. In study III we used females of isofemale strains from the northern and southern ends of the latitudinal cline (Pelkosenniemi and Lahti), established in 2009. QTL crosses in study IV were performed using isofemale strains from Oulanka, Finland (03F77) and Vancouver, Canada (can3F20) as parental strains (see Fig. 1). Geographical coordinates and codes of all strains, as well as the distances between cline populations, are given in the original papers.
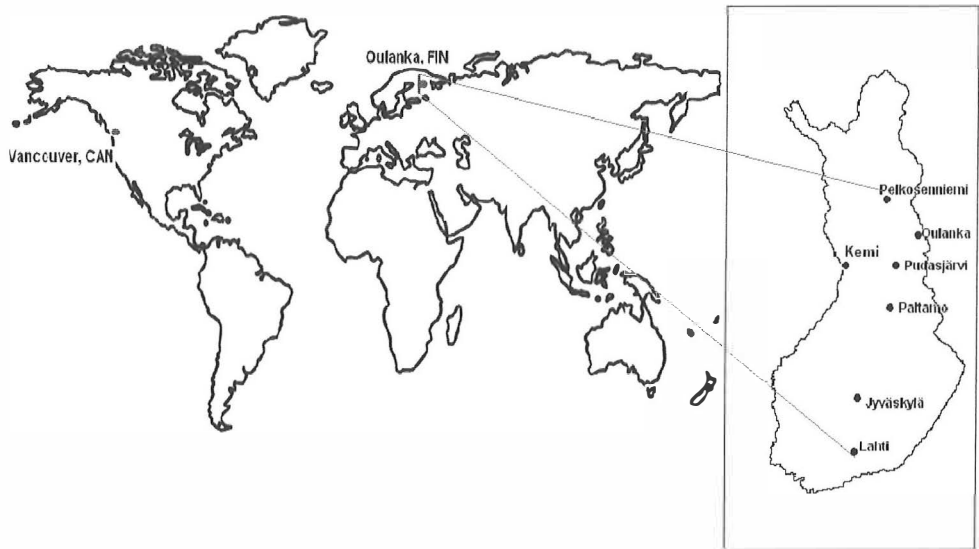
FIGURE 1 Geographical origin of *D. montana* populations used in the experiments.

2.2 Phenotypic measurements

2.2.1 Diapause phenotype

Effects of photoperiod on females' reproductive pathway (direct maturation vs. reproductive diapause) were traced by transferring sets of females into climate chambers with different LD cycles within one day after their emergence (studies I and III) or already as pupae (study II). In the first-mentioned studies freshly emerged flies were sexed under light CO_2 anesthesia, after which the females were placed in vials containing yeast-sucrose-agar medium (Rosato & Kyriacou 2006) and maintained in the climate chambers in 16°C until they reached the age of maturity (about 21 days). In study II the flies were transferred into different photoperiods already as pupae, and the emerging males and females were kept together in bottles containing malt medium (Lakovaara 1969) until they were 21-23 days old. In study IV the females of the QTL crosses were maintained at only one LD cycle (18:6), but otherwise the study protocol was the same as in studies I and III. Studies I and IV were performed by keeping the flies in light-insulated boxes with different LD cycles inside the climate chambers (MEMMERT ICP800), and studies II and III by keeping them in light-insulated boxes with different LD cycles in a temperature controlled room. In both cases the temperature was kept at 16°±1°C.

The reproductive status of the females was determined at the age of 21 - 22 days by dissecting their ovaries under a light microscope. If the ovaries were small and transparent and/or they contained only a little yolk and showed minimal structurization, the females were considered to be "diapausing". If the

ovaries were almost or completely developed and the ovarioles' filaments were well formed and full of yolk, the females were classified as "reproducing" ("non-diapausing") Fig. 1 in study IV.

2.2.2 Cold tolerance

The cold tolerance of the females of the parental strains and the F2 progenies of QTL crosses in study IV was determined using a chill coma recovery time (CCRT) test. The tests were performed with 21 day-old females that had been maintained in a climate chamber at 16:8 LD and 16°C since their emergence. The females were given a cold treatment by transferring them in agar-vials into an incubator (Termaks, KB 8400F) at -7°C and keeping them there for 16 hours. After this time the motionless females were brought into room temperature (21 ± 1°C) and placed individually on dishes with separate compartments and a lid. The CCRT was recorded as the time (in seconds) that a fly needed to be able to stand on its legs after being transferred into room temperature.

2.2.3 Developmental time and juvenile body weight

The parental and progeny females of the QTL crosses were phenotyped also according to their developmental time and body weight (study IV). To gain information on the developmental time of the flies from egg to eclosion, freshly mated females were allowed to lay eggs in malt vials for four hours per day during 14 days (the vials were changed daily to keep larval density low and to measure the development time of the progeny flies repeatedly and hence more precisely). The vials with parental females were maintained in a climate chamber at 16°C and with constant light, while the vials with eggs were transferred immediately after each egg-laying session into 16:8 LD at the same temperature. When the developing progeny reached the pupal stage, the vials were checked once a day to sex the newly eclosed flies and to record the development time of the females. At the same time the body weight of the freshly eclosed females was weighed with a Mettler Toledo scale (XS105 DualRange, 0.01mg).

2.3 Study protocol for the resonance experiment

Possible involvement of the circadian clock in diapause induction was studied by performing a "resonance" experiment, also known as Nanda-Hamner (NH) protocol (Nanda & Hamner 1958), with two strains from Pelkosenniemi and Lahti. The females were collected within 24 hours after emergence and placed in yeast-sucrose-agar vials into an experimental room with automatically controlled temperature (16±1°C) and air conditioning. Vials were divided into 13 wooden experimental chambers (69-264 females per isofemale strain per chamber) illuminated with white LED lights with a light intensity of ~ 700 lx during the photophase. All the chambers had light periods of 12 hours followed by unique

dark periods ranging from 12 hours to 60 hours. We also transferred 108-194 females per strain into constant darkness (light-insulated box) in the same room to trace the effect of the absence of the light signal on the females' photoperiodic response. After 21 days the reproductive stage of the females was determined as described above to calculate the females' diapause propensity in each LD cycle.

2.4 Genetic methods

2.4.1 Microsatellite and SNP markers

Flies' genomic DNA was extracted using the Qiagen DNeasy Tissue Kit (QIAGEN, Germany) according to the manufacturer's protocol in studies I and IV.

Genetic divergence of *D. montana* clinal populations was estimated in study I with 12 polymorphic microsatellite markers using ABI 3100 genetic analyzer and a variety of population genetics software: GENEPOP 4.0.10 (Raymond and Rousset 1995), MICRO-CHECKER 2.2.3 (Van Oosterhout et al. 2004), ARLEQUIN, version 3.5.1.2 (Excoffier et al. 2005), D_{EST} differentiation statistics (D_{ST}, Jost 2008), "DEMEtics" package in R (R Core Team 2008, Gerlach et al. 2010) and STRUCTURE2.3.1 (Pritchard et al. 2000). For more details see paper I.

SNP markers used in the QTL crosses in study IV were identified from two 454 transcriptome assemblies from Vancouver and Oulanka samples collected in 2008 and 2009 (Veltsos, in prep). The markers were scored with an Illumina VeraCode GoldenGate assay and BeadXpress platform to identify the 96 most informative SNPs for the parental strains used in the experiment. The selected 96 SNPs were then scored for 768 individuals from the QTL cross and analyzed using GenomeStudio 2010.1 (Genotyping module 1.7.4, Illumina, San Diego, CA). In total, 16 flies of the parental and 728 females of the F2 generations were used in further analysis.

The SNP markers were assigned to the five major chromosomes of *D. montana* according to their predicted location (Schäfer et al. 2009) by cross-referencing with alignment to the *D.virilis* scaffolds in FlyBase. The positions of the markers within these groups were estimated using a version of the CRIMAP software (Green et al. 1990) modified by Xuelu Liu (Animal Genomics and Breeding group, Monsanto Corporation) using CRIGEN, which splits the data into subfamilies to make the linkage mapping faster. The markers showing conflicting linkage were removed from the analysis.

2.4.2 Quantitative trait loci (QTL) analysis

QTL crosses were performed according to a F2 design (Suppl.1 in study IV) between Vancouver and Oulanka isofemale strains. Before choosing the parental strains for these crosses, we crossed females and males of several strains from the two populations with each other and searched for possible differences in their

gene arrangements by examining possible inversion loops in the salivary gland chromosomes of hybrid larvae. The strains finally chosen for the QTL crosses (can3F20 and O3F77) did not show any detectable differences in their gene arrangements. The females of these strains were phenotyped (diapause state, cold tolerance, developmental time, body weight) in 16:8 LD, which corresponds mid-summer (10th July) in Vancouver and the end of summer (22nd August) in Oulanka. In this photoperiod the females of the Vancouver strain were in reproductive state and those of the Oulanka strain were in reproductive diapause, and the differences between strains were significant also in the other studied traits.

To perform the QTL crosses, the flies of the parental strains (P) were sexed within one day after emergence and kept at constant light (LL) and 16°C until they were sexually mature (about 21 days). To obtain sufficient F2 progeny flies, we mated 3 and 5 pairs of Oulanka females with Vancouver males and Vancouver females with Oulanka males, respectively. The flies of the F1 generation were crossed individually with each other (one female and one male with known parents), and the F2 generation were transferred into 16:8 LD as freshly laid eggs. The females of this generation were phenotyped in the same way as we phenotyped the parental strains.

We performed a separate QTL mapping analysis for each of the four phenotypic traits using the R/qtl package version 1.24-9 (Broman et al. 2003) in R software version 2.15.1 (R Core Team). For each trait we first performed Single-QTL and Two-QTL scans and then proceeded to Multiple-QTL mapping using the multiple imputation method. The significance threshold was obtained from 1000 permutations for the scans; the threshold for the X chromosome was calculated separately. The data on diapause phenotype were analyzed as yes/no responses using the binary method option of the package.

Sequences of the markers under significant QTL peaks for each of the studied trait were aligned using the FlyBase Blast tool (http://flybase.org/blast/). Gene annotations with molecular function and involvement in biological processes were collected from *D. melanogaster* using the FlyBase summary information.

3 RESULTS AND DISCUSSION

3.1 Photoperiodic control of insect life cycles

Insects living at high latitudes have to be able to adjust their life cycle to match the seasonal changes in environment, and to do so most species rely on changes in photoperiodic cues. The magnitude and speed of changes in these cues vary between latitudes and the time of year, which induces strong selection pressures for local adaptation. Natural selection favors the best phenotype in a given habitat, but other evolutionary forces, such as gene flow and genetic drift, may dilute the effects of selection. One way to study the balance between selection and gene flow is to trace phenotypic and genetic divergence in parapatric populations forming latitudinal or altitudinal clines (Endler 1977).

We have studied variation in the critical day length (CDL) for diapause induction along a latitudinal *D. montana* cline in Finland (60°N-68°N, about 760km long with distances of 110-315 km between the cline populations). The difference in the average CDLs between the northern and southern ends of the cline was approximately 2 hours (study I) or 1,5 hours (study II). Both of the measures are consistent with Danilevsky's rule, which says that the CDL lengthens 1–1,5 hours per 5 degrees difference in latitude (Danilevsky et al. 1970), and in both studies the mean CDLs were also found to be strongly correlated with latitude. Thus, the high gene flow between cline populations, revealed by a microsatellite analysis in study I, could not prevent local adaptation in the timing of diapause. However, the fact that microsatellite markers did not indicate any population structure does not rule out the possibility that the clinal populations differ from each other at specific chromosomal areas or genes that are under selection.

The CDLs revealed in studies I and II corresponded in all populations to the local day lengths at the end of July and/or beginning of August. Mean CDLs correspond to different time windows at different latitudes, as for example in Pelkosenniemi (~67°N) shortening the day length by one hour takes about one week in July/August, while in Lahti (~61°N) a change of the same magnitude

requires almost two weeks. It should also be noted that the females will enter diapause only if the day length decreases below the critical point during the first week after their eclosion, i.e. during their sensitive period (Salminen et al. in press). Even though the females of all the clinal populations enter diapause at about the same time, the overwintered flies of the southern populations start to mate and lay eggs much earlier (in April to May) than those of the northern ones (June), which enables the first-mentioned populations to produce a second generation during the growing season (Aspi et al. 1993, Lumme 1978). Also, the developmental time, which has been found to be under photoperiodic control in several insect species (Hahn and Denlinger 2007), varies during the growing season and plays an important role in voltinism patterns. Salminen et al. (in press) have shown that *D. montana* flies develop faster and are smaller when maintained before eclosion under short day conditions representing late summer/early autumn than when maintained under long day conditions representing early summer.

In general, the correct timing of diapause induction has important consequences for evolutionary and ecological trade-offs between the females' reproduction and survival. Even though the timing of diapause is regulated mainly by photoperiod, it can be fine-tuned by other environmental factors, specifically temperature. For example, higher temperature may adjust CDL towards shorter day length (e.g. Ichijo 1985, Pittendrigh & Takamura 1987), and in several species, including the apple maggot *Rhagoletis pomonella* (Prokopy 1968) and the cabbage beetle *Colaphellus bowringi* (Xue et al. 2002), the photoperiodic response has been found to be highly dependent on environmental temperature.

3.2   Genetic variation in the timing of diapause

Genetic variation in different properties of diapause, like CDL or diapause incidence, have often been studied by tracing variation in these traits among isofemale strains from wild populations (e.g. Schmidt 2005, Tauber 2007, Magiafoglou 2002). Isofemale strain designs have also been used to calculate the heritability of diapause and other life-history traits (Hoffmann & Parsons 1988), even though this has evoked some criticism (Wahlsten 1979). According to Hoffman et al. (1988), the "isofemale heritabilities" should be calculated only for freshly collected strains (maintained no longer than five generations in laboratory conditions), which have not have undergone bottlenecks. Heritability of diapause has been found to be rather high, e.g. in *Gryllus veletis* (Begin & Roff 2002), and to vary significantly between populations, such as in the striped ground cricket *Allonemobious fasciatus* (Mousseau & Roff 1989). We decided not to calculate the heritability of CDL because in study I we had too few strains and in study II most of the strains had been maintained in the laboratory much longer than five generations.

Studies I and II revealed great variation in CDL both within and between the clinal populations. One factor maintaining variation within populations could be alternating selection pressures. As environmental conditions naturally differ between years, it can be beneficial for the females during some years to develop ovaries and produce progeny still in late summer, while during other years it may be more beneficial to enter diapause already in July. Diapause can also involve different kinds of trade-offs and correlations with other life-history traits in wild populations (e.g. Kroon et al. 1998, Schmidt & Conde 2006). One factor maintaining variation in CDL in bivoltine populations is selection favouring different types of life-history traits in summer vs. overwintering generations. Detected variation in CDL may also be partly due to migration of flies between the clinal populations, as *D. montana* flies can migrate up to 1 km/day (Aspi et al. 1993). While study I showed no restrictions to gene flow between the cline populations, it did show isolation by distance, reflecting the low dispersal rate of flies between the ends of the cline.

In study II we also traced possible changes in the means and variances of the PPRC (photoperiodic response curve) and CDL of the study strains during laboratory maintenance. This study showed that the mean CDL of the strains does not depend on the number of years that the strain has been maintained in the laboratory, which is consistent with previous findings by Oikarinen and Lumme (1979) in *D. littoralis*. On the other hand, the "steepness" of photoperiodic response curves (PPRCs) increased during the laboratory culture, which suggests that variation in the photoperiodic responses of the study strains to specific day length decreased during the laboratory maintenance, probably due to inbreeding.

As mentioned above, variation in the timing of diapause within populations can enhance survival of populations over the years that differ in the severity of the winter and in the length of the growing period, and may also enable them to respond to long-term changes in environmental conditions. For example, in pitcher-plant mosquitoes *Wyeomyia smithii*, populations have been found to show a decrease in CDL over the years as a response to the lengthening of the growing season caused by climate change (Bradshaw & Holzapfel 2001).

3.3 Involvement of the circadian clock in the photoperiodic regulation of diapause

The role of the circadian clock in regulating seasonal events is still under intense debate and many models explaining its involvement in the photoperiodic calendar have been suggested during the past decades (e.g. Kostal 2011). In photoperiodic time measurement models, which assume the involvement of the circadian clock in the induction of photoperiodic responses, the rise and decline of insects' photosensitivity is presumed to cycle internally between sensitive and insensitive phases during prolonged dark periods after a single light period.

Studies performed using e.g. the Nanda-Hamner (NH) protocol have yielded both positive and negative results (i.e. supporting or refuting this kind of cycling) depending on the species and environmental conditions. For example in *Pterostichus nigrata* the diapause incidence has been found to be affected by the circadian clock in temperatures 15-20°C (Thiele 1977), whereas in *Adoxophyes orana* similar studies have produced negative results (Bonnemaison 1977). Several species, such as *Pieris brassicae* (Veerman et al. 1988), have also been found to show or not show circadian cycling in their photoperiodic responses depending on the temperature.

In our study the percentage of diapausing *D. montana* females decreased gradually to very low percentages along with the increase in the length of the dark period, showing no peaks and troughs in any cycle length. If the photoperiodic timer of *D. montana* would be driven by the circadian clock, the females should have recognized LD cycles with a periodicity close to 24 hours as short day lengths and entered diapause (i.e. in those treatments with total length of the cycle at 24, 48 and 72 hours). The absence of this expected "resonance" pattern can be explained by the hourglass model control of the photoperiodic calendar. However, the lack of rhythmic diapause response and the decline in *D. montana* females' diapause percentage in LD cycles with very long nights and continuous darkness could also be accounted for by the damped circadian oscillator version of the external coincidence model (Bünning 1969, Lewis & Saunders 1987, Saunders & Lewis 1987a, b). The internal coincidence model can be rejected since the flies of this species show only the evening activity peak in entrained conditions and cannot measure the time between morning and evening peaks (Kauranen et al. 2012).

Bradshaw et al. (2003a) have suggested that the long summer days at high latitudes have enhanced selection for a reduced response to light by the circadian clock or directly to the critical day (or night) length for diapause induction. Bradshaw & Holzapfel (2001) have also argued that in various arthropod species the "coupling strength" of the circadian clock and the photoperiodic calendar has declined in northern populations, so that an hourglass timer may have taken over the function of the photoperiodic calendar. Interestingly, in some species, such as the ground beetle *Pterostichus nigrita* (Thiele 1977) and the European corn borer *Ostrinia nubilalis* (Skopik & Takeda 1987), NH experiments have produced negative results only for the most northern populations. In the present study we found no differences in the diapause responses between the strains originating from northern and southern Finland, even though the critical day for diapause induction differs between the females from these localities by about two hours (Study I). This could be due to the fact that both populations are from rather high latitudes.

As Saunders (2010) states, the diversity of photoperiodic mechanisms among arthropods suggests considerable divergence or even separate evolutionary origin of the time measuring systems, which may also be reflected in the results of NH and other resonance experiments. The NH protocol has also been criticized for its inability to discriminate between an hourglass-like and circadian oscillation based timers, since a rhythmic response in the NH protocol is no proof

for the presence of oscillators (Bradshaw et al. 1998, Bradshaw et al. 2003a) and a negative response cannot rule out the possibility of the heavily damping oscillator (Veerman 2001).

3.4 Genetic basis of population divergence in life-history traits important in overwintering (QTL mapping)

Insect species with a wide geographic distribution offer a good opportunity to study the genetic basis of photoperiodically regulated life-history traits important in local adaptation. We performed a QTL mapping analysis (study IV) to trace the genetic basis of population differences in several photoperiodically regulated traits (diapause, cold tolerance, developmental time, body weight). The study was performed at the photoperiod of 16:8 LD, which corresponds to early summer in Vancouver and late summer in Oulanka, and at which the females of the parental strains of the QTL cross showed high divergence in all the studied traits.

The target traits, diapause, cold tolerance, developmental time, and body weight, have been found to be under photoperiodic regulation in several species (e.g. Horwath & Duman 1983, Vesala et al. 2012, Nylin et al. 1989, Hahn & Denlinger 2007) and also to show phenotypic plasticity at different temperatures (Ghalambor 2007, Vesala et al. 2011). Even though Moorhead (1954) has shown that *D. montana* flies from the West coast of North America (represented here by the Vancouver strain) are significantly larger than "Standard" *D. montana*, the light regime has been found to affect also this trait in several insect species (Abrams et al. 1996). In wild populations, optimal insect size can involve a direct trade-off between the fitness advantages of large size (e.g. high fecundity) and the disadvantages of long developmental time (Nylin & Gotthard 1998).

In study IV the diapausing and non-diapausing F2 females of the QTL cross were found to differ significantly in their cold tolerance, developmental time and body weight, and the two last-mentioned traits showed a positive correlation. It should be noted that while all these traits are regulated by photoperiodic cues, their regulation may occur through different time-measuring systems and/or during different developmental stages. Egg-to-eclosion developmental time and body weight are regulated by photoperiodic cues during the larval and pupal stages, i.e. prior to diapause induction, which occurs in *D. montana* during the first week after eclosion (Salminen et al. in press). Diapause is also the only go/no-go (binary) trait that is regulated by a photoperiodic timer (see Bradshaw & Holzapfel 2010). Furthermore, earlier studies on *D. montana* have shown that seasonal changes in the cold tolerance of females (measured with chill coma recovery test) are regulated mainly by day length and temperature (Vesala et al. 2012a, b), even though diapause also has been found to affect it in some strains of the species (Vesala and Hoikkala 2011). Effects of diapause/dormancy on insect

cold tolerance have been found to vary among species and also to depend on the methods used in studying this trait (Hodkova and Hodek 1997, Goto et al. 2001).

The total effects of QTL explained about 27 % of variation in female propensity to enter diapause, 4 % of strain differences in cold tolerance, 21 % in developmental time, and 17 % in body weight. All these traits had unique QTL, but the study revealed also QTL that covered partly the same chromosomal regions at least on the second and fourth chromosomes. The last-mentioned QTL regions are especially interesting as they could involve genes that play a role in the photoperiodic regulation of several traits under the control of circadian clock or photoperiodic timer. However, the QTL regions were quite wide and the linkage of specific SNP markers with them give only tentative support for their involvement in trait regulation. In our study a SNP marker for *Rhodopsin 6* gene (involved in photoperception) was found to be linked with the QTL responsible for developmental time and body weight, and another marker for *regucalcin* with a QTL for developmental time. The last-mentioned gene has earlier been found to play a role in cold acclimation in *D. melanogaster* (Goto 2000) and also to show expression changes linked with diapause in *D. montana* (Kankare et al. 2010). Annotation of more SNP markers used in our QTL mapping could help to localize more candidate genes. Another alternative would be to check whether any of the known circadian clock genes (e.g. Stehlik et al 2008) are located on QTL regions. Final identification of genes taking part in the regulation of studied traits would require fine-scale mapping as well as direct studies of the function of these genes and their effects on the studied traits at different light-dark cycles.

Our study IV revealed one QTL region specific to diapause. This was located on the X chromosome and explained 21% of strain differences in the percentage of females entering diapause at the tested photoperiod. Finding the relevant gene(s) under this QTL would be extremely interesting as they could be involved in the diapause trait itself and/or in the photoperiodic calendar regulating photoperiodic responses like diapause.

4 CONCLUSIONS AND FUTURE DIRECTIONS

An ability to cope with daily and seasonal changes in environmental conditions is crucial for all species living at high latitudes. Genetic variation in traits involved in adaptation plays a key role in this context; it provides material for natural selection and enables the populations to adapt to short and long term changes in environmental conditions. My main aim was to trace genetic variation in the critical day length (CDL) inducing adult reproductive diapause, as well as the genetic basis of this and three other life-history traits important in adaptation to seasonally varying environments in a northern fly species *D. montana*.

Survival through harsh winter conditions involves not only tolerance to cold temperatures, but even more importantly, an ability to predict the forthcoming cold season from gradual changes in environmental cues early enough to get prepared for the winter. In our studies *D. montana* females showed an ability to anticipate the time when winter is approaching well in advance from photoperiodic cues and to enter diapause before the start of cold season. The populations of this species appeared to be well-adapted to the light regimes specific to their geographical locations (I, II) and (IV). They showed a latitudinal cline in CDL for induction of reproductive diapause (I, II), in spite of high gene flow between populations (I), as well as large variation in CDL within populations (I and II). While variation in females' photoperiodic responses decreased during laboratory maintenance, the mean CDLs of the strains remained the same (II).

Forecasting of seasonal changes has been suggested to rely on the function of two major molecular clock mechanisms, the circadian clock and photoperiodic calendar. In study III, we carried out a "resonance" experiment to determine whether the circadian time-measuring system plays a role in the induction of photoperiodic reproductive diapause in *D. montana*. The study revealed no rhythmicity in females' photoperiodic response (diapause induction), which suggests that the photoperiodic timer of *D. montana* is based on an non-oscillatory hourglass timer or rapidly damping circadian oscillator.

Finally, I found that the traits important in overwintering in *D. montana*, such as diapause, cold tolerance, developmental time and body weight, are polygenic

(IV). QTL analysis between two *D. montana* strains that differed in their diapause incidence, cold tolerance, egg-to-eclosion development time and body weight at a specific day length revealed both unique and combined QTL for the studied traits, with the most interesting large-effect QTL for diapause located on the X chromosome. Final identification of genes taking part in the regulation of the studied traits would, however, require more fine-scale mapping as well as direct studies on the function of these genes and their effects on the studied traits in different light-dark cycles.

Our work evoked many questions worth further studies. One powerful method to estimate local variation in the quantity and quality of genetic variation in CDLs would be a selection experiment. Another approach for tracing differences between clinal populations would be to perform genome scans for the most northern and southern populations of the cline. This method might also help identify "genomic islands" in sections of the genome that have diverged between populations due to different selection pressures, i.e. to detect "regions" of local adaptation at the genetic level. One could also trace variation in the structure and splicing of specific candidate genes known to affect diapause and/or traits connected with it along the latitudinal cline.

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YHTEENVETO (RÉSUMÉ IN FINNISH)

Vuodenaikaisvaihteluun sopeutuminen: geneettinen muuntelu talvehtimiseen liittyvissä elinkiertopiirteissä

Kyky ennustaa päivittäisiä ja vuodenaikojen vaihteluun liittyviä muutoksia ja sopeutua näihin muutoksiin on hyvin tärkeä ominaisuus pohjoisella pallonpuoliskolla eläville eliöille. Toinen tärkeä tekijä on sopeutumiseen liittyvien elinkiertopiirteiden geneettinen muuntelu, joka mahdollistaa luonnonvalinnan toimimisen ja lajien sopeutumisen elinympäristössä tapahtuviin lyhyen ja pitkän aikavälin muutoksiin. Tämän väitöskirjatutkimuksen päätavoitteena oli tutkia hyönteisillä esiintyvän aikuisiän lisääntymislepokauden ja muutamien muiden talvehtimiseen liittyvien elinkiertopiirteiden geneettistä taustaa. Lisääntymislepokausi on eräs tärkeimmistä pohjoisen pallonpuoliskon hyönteisillä esiintyvistä talvehtimiseen liittyvistä sopeumista, ja sen määräytymistä tutkitaan muun muassa määrittämällä kriittinen päivänpituus, jossa puolet populaation naaraista siirtyy lepokauteen. Tutkimukset tehtiin D. virilis -ryhmään kuuluvalla Drosophila montana -mahlakärpäsellä, joka on levinnyt koko pohjoiselle pallonpuoliskolle ja sopeutunut hyvin erilaisiin elinympäristöihin. Tutkittavan lajin naaraat viettävät talven aikuisvaiheen lisääntymislepokaudessa, johon ne siirtyvät jos päivänpituus on tietyn rajan alapuolella naaraiden kotelosta kuoriutumisen aikaan.

Ankarista talvioloista selviytymiseen liittyy olennaisena osana kylmänsietokyvyn lisäksi se, miten ja kuinka hyvissä ajoin eliöt pystyvät ennustamaan elinpiirissään tapahtuvia muutoksia ympäristön signaalien perusteella. Tässä väitöskirjatutkimuksessa osoitettiin, että D. montana -lajin naaraat pystyvät ennustamaan kasvukauden päättymisen päivän/yön pituuden perusteella, ja että ne siirtyvät lisääntymislepokauteen jo elokuun alussa. Tutkimuksessa käytettyjen eri leveysasteilta peräisin olevien populaatioiden osoitettiin olevan hyvin sopeutuneita maantieteellisen sijaintinsa mukaiseen valojaksoon (osatyöt I, II ja IV). Nämä populaatiot muodostivat leveysasteiden mukaisen kliinin lisääntymislepokauden laukaisevan kriittisen päivänpituuden suhteen, vaikka niiden välillä oli runsaasti geenivirtaa (I). Kriittisessä päivänpituudessa esiintyi geneettistä muuntelua sekä populaatioiden sisällä että niiden välillä (I ja II). Tutkittujen kärpäslinjojen (luonnosta pyydystettyjen naaraiden jälkeläistöjen) kriittinen päivänpituus pysyi laboratoriokasvatuksen aikana samana, vaikka ko. linjojen sisäinen muuntelu väheni nähtävästi sisäsiitoksen seurauksena (II).

Vuodenaikaisten muutosten ennustamisen on arveltu perustuvan kahteen sisäiseen kellojärjestelmään, vuorokausirytmejä säätelevään kirkaadiseen kelloon ja vuodenaikaisia muutoksia säätelevään valojaksoiseen kalenteriin. Väitöskirjan kolmannessa työssä tehtiin "resonanssikoe", jolla tutkittiin liittyykö kirkaadisen kellon toiminta D. montana -naaraiden lisääntymislepokauteen siirtymiseen (III). Tässä kokeessa vastakuoriutuneita naaraita kasvatettiin 13 erilaisessa valojaksossa ja naaraiden oletettiin siirtyvän lepokauteen niissä valojaksoissa, joissa valoisan ja pimeän jakson summa oli 24 tuntia tai sen kerrannai-

nen. Tutkimuksessa naaraiden valojaksoisessa vasteessa eli lisääntymislepokauteen siirtymisessä ei havaittu rytmisyyttä, joten ko. vasteen voitiin päätellä perustuvan rytmittömään "tiimalasiajastimeen" tai nopeasti vaimenevaan kirkaadiseen oskillaattoriin.

Viimeisessä väitöskirjan osajulkaisussa selvisi, että talvehtimiseen liittyvät elinkiertopiirteet, kuten lisääntymislepokausi, kylmänkestävyys, kehitysaika ja kuoriutumisen jälkeinen paino ovat polygeenisiä eli useamman geenin säätelyn alla olevia ominaisuuksia (IV). Kahden edellä mainittujen elinkiertopiirteiden suhteen poikkeavan kärpäslinjan välillä tehty risteytys (ns. QTL analyysi) paljasti kärpästen kromosomistossa useita alueita, joissa oli yhteen tai useampaan tutkittuun ominaisuuteen vaikuttavia geenejä. Mielenkiintoisin löytö oli X-kromosomissa sijaitseva naaraiden lisääntymislepokauden määräytymiseen vaikuttava alue, joka selitti suuren osan linjojen välisestä muuntelusta ko. ominaisuudessa. Varsinaisten geenien löytyminen edellä mainituilta alueilta vaatii kuitenkin vielä tarkempien kartoitusten tekemistä sekä geenien toiminnan tutkimista erilaisissa päivänpituuksissa.

Tässä väitöskirjatutkimuksessa osoitettiin, että paikallinen valintapaine lepokauden oikeaan ajoitukseen on riittävän voimakas ylläpitämään maantieteellistä kliiniä lisääntymislepokauden laukaisevassa kriittisessä päivänpituudessa jopa geenivirran läsnäollessa. Lisäksi tutkimuksessa löydettiin useita kromosomialueita, jotka liittyvät lisääntymislepokauteen ja muihin talvehtimisen kannalta tärkeisiin elinkiertopiirteisiin. Tutkimus herätti myös paljon uusia kysymyksiä ja jatkotutkimusten aiheita, joissa lisääntymislepokauden laukaisevan kriittisen päivänpituuden geneettistä taustaa voitaisiin tutkia myös genomitasolla, mikä auttaisi edelleen jäljittämään paikallisiin oloihin sopeutumisessa tärkeitä genomialueita. Olisi myös kiinnostavaa tutkia tiettyjen lepokauteen yhdistettyjen kandidaattigeenien rakennetta, silmikoitumista ja toimintaa eri leveysasteilta peräisin olevissa populaatioissa.

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ORIGINAL PAPERS

I

ADAPTATION TO A SEASONALLY VARYING ENVIRONMENT: A STRONG LATITUDINAL CLINE IN REPRODUCTIVE DIAPAUSE COMBINED WITH HIGH GENE FLOW IN *DROSOPHILA MONTANA*

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