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1 **Northward range expansion requires synchronization of both**
2 **overwintering behaviour and physiology with photoperiod in the invasive**
3 **Colorado potato beetle (*Leptinotarsa decemlineata*)**

4
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Abstract

Photoperiodic phenological adaptations are prevalent in many organisms living in seasonal environments. As both photoperiod and growth season length changes with latitude, species undergoing latitudinal range expansion often need to synchronize their life-cycle with a changing photoperiod and growth season length. Since adaptive synchronization often involves a large number of time-consuming genetic changes, behavioural plasticity might be a faster way to adjust to novel conditions. We compared behavioural and physiological traits in overwintering (diapause) preparation in three latitudinally different European Colorado potato beetle (*Leptinotarsa decemlineata*) populations reared under two photoperiods. Our aim was to study whether behavioural plasticity could play a role in rapid range expansion into seasonal environments. Our results show that while burrowing into the soil occurred in the southernmost studied population also under a non-diapause inducing long photoperiod, the storage lipid content of these beetles was very low compared to the northern populations. Similar behavioural plasticity was however not found in the northern populations. Furthermore, the strongest suppression of energy metabolism was seen in pre-diapause beetles from the northernmost population. These results could indicate accelerated diapause preparation and possibly energetic adjustments due to temporal constraints imposed by a shorter, northern, growth season. Our results indicate that behavioural plasticity in burrowing may have facilitated initial range expansion of *L. decemlineata* in Europe. However, long term persistence at high latitudes has required synchronization of burrowing behaviour with physiological traits. The results underline that eco-physiological life-history traits of insects, such as diapause, should be included in studies on range expansion.

Key words: Coleoptera, diapause, latitude, metabolic rate, behavioural plasticity

Introduction

Many organisms use the photoperiod as the main environmental cue for phenological synchronization, since it is a reliable indicator of time of year (Nelson et al. 2010) and it allows organisms to predict coming seasonal shifts accurately. This has led to various photoperiodic adaptations in physiological, behavioural and life history traits (Nelson et al. 2010). Species undergoing latitudinal range expansion face a change in the photoperiod, which can lead to a mismatch between the timing of seasonal adjustments in physiological and life history traits with seasonal environmental changes (Bradshaw and Holzapfel 2007). This mismatch can lead to lowered survival, for example, due to unsynchronized preparation for overwintering or timing of migration (Rankin and Burchsted 1992). Consequently, latitudinal range expansion may fail unless evolutionary changes in critical photoperiod take place (Gaston 2003; Bradshaw and Holzapfel 2007; Urbanski et al. 2012) and species are able to adjust their behavioural and physiological traits with the abiotic and biotic requirements of the new environment (Elton 1958).

Range expansion to and persistence in novel environments may be possible also without any genetic changes (i.e. adaptive evolution) if invading individuals possess phenotypic plasticity in their abilities to avoid stressful conditions (Sakai et al. 2001; West-Eberhard 2003; Duckworth 2009, Sunday et al. 2014). Phenotypic plasticity can manifest itself on many organismal levels, including in physiological and behavioural traits (West-Eberhard 2003). Through behavioural plasticity maladapted individuals might persist, thus allowing time for physiological adaptation to occur (Sakai et al. 2001; Phillips and Suarez 2012) (Fig. 1). The potential roles of behavioural plasticity in facilitating the establishment of species, especially invasive species, have been increasingly appreciated (Sagata and Lester 2009; Wright et al. 2010; Gabel et al. 2011; Hanshew and Garcia 2012; Phillips and Suarez 2012). Therefore, in order to better predict species invasions into novel environments with

seasonally occurring periods of stress (Fig. 1) and to be able to better assess the capacity of species to respond to climate change (Bradshaw and Holzapfel 2001; Lee 2002; Saikkonen et al. 2012; Sunday et al. 2014), it is important to consider the relative contribution of behavioural plasticity vs. adaptation for stress avoidance.

One such important stress avoidance mechanism by which temperate insects survive harsh winter conditions and which can show between-population differences as a result of plastic or genetic change is diapause (Tauber et al. 1986). Correct timing and physiological synchronization with photoperiod is often vital for diapause preparation since overwintering mortality usually is very high in unprepared individuals (Danks 1987; Hahn and Denlinger 2007, see Fig. 1 path 1). Diapause is usually triggered by a shortening of the photoperiod (preceding autumn and winter), but can be modulated by for instance, low temperatures or the quality or abundance of food (Danilevskij 1965; Tauber et al. 1986; Danks 1987). Diapause itself is usually characterized by reproductive arrest, suppressed metabolism and high tolerance to many environmental stressors (Tauber et al. 1986; Kostal 2006). In many species, preparation for diapause is also accompanied by changes in behaviour (Beck and Hanec 1960; Beekman et al. 1998) including, active foraging for building energy stores, and seeking out overwintering locations (Tauber et al. 1986). Individuals generally choose overwintering microhabitats where they are buffered against exposure to adverse climatic conditions such as extreme temperatures, large temperature fluctuations and desiccation (Danks 1987). This opens the possibility that individuals can survive winters in novel environments without physiological adaptation (Fig. 1, path 2).

To study whether rapid range expansion into seasonal environments is driven by adaptation or behavioural plasticity, we used the very successful pest invader, the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) which has undergone rapid latitudinal range expansion. *L. decemlineata* is native to Mexico and the south-western United States of

America (Casagrande 1987; Alyokhin 2009), and currently occupies an area covering 16 million km² in North America, Europe and Asia (EPPO 2006). The beetles that invaded Europe, Bordeaux, France, in the 1920s have been suggested to come from lower latitudes in North America (Johnson 1967). There was a clear genetic bottleneck during the initial introduction and establishment in Europe, resulting in reduced genetic variation (Grapputo et al. 2005; Piironen et al. 2013). Despite this reduction, the subsequent range expansion of *L. decemlineata* has been very rapid (Piironen et al. 2013) and the annual expansion distance has been estimated to be between 80 and 130 km (Johnson 1967; EPPO 2006). Reasons for the successful invasion include the beetles' versatile life-cycle and capability to overwinter in diapause (Casagrande 1987; Hsiao 1985; de Kort 1990; Mahdjoub and Menu 2008; Alyokhin 2009) where cold-tolerance is significantly increased (Boiteau and Coleman 1996; Costanzo et al. 1998).

In *L. decemlineata*, diapause initiation occurs at around 15 days after adult eclosion under short photoperiods (Lefevre and de Kort 1989). During the pre-diapause period storage lipid content increases dramatically (Lehmann et al. 2012) and metabolic rate shows a bell-shaped pattern, which peaks at 3-5 days post eclosion (May 1989; Piironen et al. 2010). In addition to physiological changes, pre-diapause behaviour is characterized by adults becoming negatively phototactic and by burrowing into the soil (Alyokhin 2009). Burrowing into the soil is commonly associated with pre-diapause not only in *L. decemlineata* (de Kort 1990; Piironen et al. 2011; Izzo et al. 2013) but also in other coleopterans (Rasa 1999; Kuang et al. 2011) and in insects in general (Roubik and Michener 1980; Beekman et al. 1998; Solbreck and Windefalk 2012). Adult *L. decemlineata* beetles can also burrow into the soil even though reproductively active (Hsiao 1985; Tauber et al. 1988; Alyokhin 2009). Burrowing is probably a means to avoid exposure to stressful climatic conditions and harmful biotic interactions (Tauber et al. 1986; Danks 1987). Since burrowing can be decoupled from

diapause and still be advantageous, plasticity in burrowing behaviour may allow individuals to survive winters at higher latitudes without associated photoperiodically induced physiological changes, which often require local adaptation (Fig. 1). Behavioural plasticity in burrowing could therefore be among the reasons of the invasion success and rapid range expansion of *L. decemlineata* (Casagrande 1987; de Kort 1990; Alyokhin 2009).

Our aim in this study is to investigate whether behavioural plasticity could have played a role in the rapid range expansion of *L. decemlineata* in Europe. This can be indirectly studied by comparing the burrowing behaviour of individuals reared under two photoperiods from three populations of different latitudinal origin: northern (Petroskoi, Russia), middle (Belchow, Poland) and southern (Padua, Italy) Europe. We take advantage of prior knowledge on differences in their photoperiodic responses: the northernmost population uses a longer photoperiod as cue for diapause initiation than the southernmost population (Piironen et al. 2011; Lehmann et al. 2012; 2014). If range expansion is mainly driven by photoperiodic adaptation, we expect populations to burrow for diapause only when reared under photoperiods which match their local late summer conditions (Piironen et al. 2011) and that diapause-related physiological traits are strongly correlated with burrowing behaviour (Fig. 1, path 1). However, if behavioural plasticity is maintained in all populations, we expect that burrowing behaviour is observed also when beetles are reared under non-diapause inducing conditions and not necessarily correlated with diapause-related physiological traits (increased lipid content and decreased metabolic rate) (Fig. 1, path 2).

We frame our study with three specific hypotheses. 1) In case range expansion has been accompanied by photoperiodic phenological synchronization, we predict that beetles from all populations burrow only under diapause-inducing photoperiods (Fig. 1, path 1). Furthermore, we predict a strong association between burrowing and high lipid content as well as low metabolic rate in all burrowed beetles (Fig. 1, path 1). As energy sequestering and

economic utilization of limited energy reserves during overwintering are vital for successful overwintering, accumulation of lipids and suppression of resting metabolic rate (RMR) are commonly used as physiological measures of preparation for diapause (Coleman and Boiteau 1996; Guppy and Withers 1999; Kostal 2006; Hahn and Denlinger 2007; Lehmann et al. 2012). 2) In case burrowing behaviour is plastic, we predict that beetles burrow also under non-diapause inducing photoperiods (Fig. 1, path 2). If burrowing on its own has been sufficient for beetles to survive winters faced, we predict a weak association between burrowing and high lipid content as well as low metabolic rate in beetles burrowed under non-diapause inducing conditions (Fig. 1, path 2). 3) In parallel with pre-diapause behaviour, we study whether populations differ in resting metabolic rate (RMR) trajectories when reared under diapause inducing and non-inducing conditions. We predict that the steepness of the RMR trajectory slope increases with latitude in beetles preparing for diapause, which could relate to more effective use of limited energy reserves (May 1989; Guppy and Withers 1999).

Materials and methods

Study animals

Three *L. decemlineata* populations were used in this study. They were the descendants of beetles collected from potato fields near Petroskoi in northern Russia (61°49'N, 34°10'E) (N = 917) in the summer of 2006, from potato fields near Belchów in Poland (52°01'N, 20°34'E) (N = 400) in 2010 and from potato fields near Padua in Italy (45°48'N, 12°07'E) (N = 249) in the summer of 2010. The Petroskoi population represents the northernmost current range limit (EPPO 2006). The parental generations were reared in the laboratory under a long day photoperiod (18 h light, 23°C, 60% relative humidity) on potato (*Solanum tuberosum* of the *van Gogh* variety). The next generation beetles were then overwintered at 5°C in climate chambers according to the protocol described previously (Lehmann et al. 2012).

162

163 *Rearing conditions and experimental setup*

164 The experiment was conducted in 2010 and 2013. In 2010, overwintered Petroskoi (24
165 families, fourth laboratory generation) and Paduan beetles (12 families) were mated (1 male
166 to 1 female) within population. The Paduan beetles were first acclimatized to the laboratory
167 conditions (18 h light, 23°C, 60% relative humidity) for two weeks. In 2013, overwintered
168 Petroskoi (26 families, seventh laboratory generation), Belchow (21 families, third laboratory
169 generation) and Padua beetles (32 families, third laboratory generation) were mated (1 male to
170 1 female) within population. Mated pairs were checked for deposited eggs daily. Larvae (10-
171 15 per family) were reared on whole potato plants (which were covered in a plastic housing)
172 in a greenhouse till adulthood at 23°C degrees, 60% relative humidity and 18 hours light.
173 After larvae burrowed into the soil for pupation, pots were checked daily for emerged adults.
174 Adults were sexed and weighed on the day of emergence ($\pm 0.1\text{mg}$, AM100; Mettler).

175 Based on emergence mass, beetles from each family were divided into two photoperiod
176 (long and short day) and age (5 and 10 days) groups so that each family was represented in
177 both photoperiods and ages and the emergence mass was similar among groups (two-way
178 ANOVA: $P > 0.05$ for both main factors and interactions, sexes were analysed separately).
179 The two photoperiods were: long day (LD, 18 h light): 16 hours full light, 6 of darkness with
180 two 1-hour medium-light periods imitating dawn and dusk, and short day (SD, 12 h light): 10
181 hours full light, 12 hours dark with two 1-hour medium-light period imitating dawn and dusk.
182 The day length change during the end of summer (15th of July to 15th of August) is from:
183 18:40 (hours:minutes) to 15:56 in Petroskoi, 16:15 to 14:34 in Belchow and 15:22 to 14:08 in
184 Padua. The temperature was set to 23°C during lights on and 21°C during lights off, with 60%
185 air relative humidity. Beetles were kept individually on petri dishes (diameter of 9 cm)
186 containing a water moistened filter paper and fed daily with fresh potato leaves (*Solanum*

tuberosum of the *van Gogh* variety). To investigate burrowing behaviour in response to photoperiod, 7-day-old beetles were moved from the petri dishes into plastic jars (120 ml) containing peat soil (40 ml). The jars had clear lids to allow full penetration of light and holes for air exchange. The soil was kept moistened and adults were fed daily with fresh potato leaves. Beetles were fed *ad libitum* to avoid burrowing as a response to lack of food (Hsiao 1985; Alyokhin 2009). Burrowing into the soil (burrowed into the soil or still on the soil surface) was recorded when beetles were 10 days old.

Resting metabolic rate

CO₂ production of individual 5- and 10-day-old beetles (Table 1) was used as a measure of resting metabolic rate (RMR). RMR represents the metabolic activity of a post-absorptive animal at rest kept at a neutral temperature (Randall et al. 2002). To avoid CO₂ emissions resulting from the assimilation of nutrients, all beetles were starved for 24 h prior to CO₂ measurements. CO₂ production was measured approximately at the same time during the synthetic photoperiod, between 2 and 4 hours after lights on. This was to avoid any possible circadian rhythms in behavioural or physiological traits interfering with the RMR. Each beetle was measured only at one time point: day 5 or 10. RMR of the 10-day-old beetles was measured after their burrowing behaviour was recorded. CO₂ production was measured with a Li-6252 CO₂ analyser (LiCor, Lincoln, NE, USA) connected to a flow through respirometry system. We performed a weekly calibration of the CO₂ analyser with 470 ppm CO₂ in nitrogen. Ambient air from which water vapour and CO₂ were removed (with Drierite, WA Hammond Drierite, USA and Ascarite II, Acros Organics, USA, respectively) was pumped through the system (SS-2 pump; Sable Systems, Henderson, NV, USA). The flow rate was set to 150 ml min⁻¹ with a mass flow controller (840 Series; Sierra Instruments Inc., California, USA). Eight cylindrical glass respirometry chambers (volume 1.7 ml) (of which one empty

chamber served as the baseline) were connected to a multiplexer (Sable Systems). The chambers were located inside a dark constant temperature cabinet (PTC-1; Sable Systems) in which the temperature was set to 23°C and controlled with a temperature controller (Pelt-5; Sable Systems). Preliminary tests were performed to ensure that the incurrent air temperature that flowed through the respirometry chamber was stabilized with the ambient temperature in the cabinet. The multiplexer was programmed so that the series of seven beetle chambers was measured twice. The recording of each beetle lasted 5 min during which the moving activity of the beetle was recorded with an infra-red light scattering based activity detector (AD-1; Sable Systems). The sampling interval was 1 s. Beetles were weighed (± 0.1 mg, AM100; Mettler) after the measurement. Respirometry data was baseline corrected and converted to ml CO₂ h⁻¹ by the acquisition and analysis software Expedata version 1.1.15 (Sable Systems). The metabolic value was the mean value of the respirometry recordings of two 5 min periods. Between the first and second measurement 60 minutes passed. From the activity data an absolute difference sum was calculated and a linear regression was taken. The steepness of the slope (arbitrary number) describes the level of activity. Preliminary experiments showed that beetles generally remained inactive during the measurements. Nine individuals were active throughout the measurement period and excluded from the later analyses, as their CO₂ emissions likely did not reflect RMR.

Total lipid content

To investigate the size of energy reserves, abdominal total lipid content was measured from 10-day-old beetles. After the measurement of RMR, beetles were weighed, anesthetized on ice, the elytrae removed and the whole abdomen cut away and stored at -80°C until analysis. Total lipid content of the abdomen was measured with Chloroform:Methanol (Chl:Meth) extraction (Folch et al. 1957) as described previously (Lehmann et al. 2012). Abdomen were

dried for 72 h at 55 °C, after which lipids were extracted by placing the abdomen in 10 ml of 2:1 Chl:Meth for 72 h. Lipid content was then assessed gravimetrically after drying the samples for 72 h at 55 °C. Around 85% of abdominal total lipids extracted in this way are neutral lipids, which mainly are storage triacylglycerids (Lehmann et al. 2012). During the first 10 days of adult life, *L. decemlineata* does not accumulate glycerol (P. Lehmann, unpublished), which could interfere with the Chl:Meth extraction method (Williams et al. 2011). We used absolute lipid content (mg) as well as relative lipid content (%) for the analyses (see below). Absolute lipid content was calculated by subtracting lean mass (post-extraction dry mass) from dry mass, while total relative lipid content (w/w) was calculated by dividing absolute lipid content by dry mass.

Statistical analyses

The potential difference between the years (2010 and 2013) was studied by adding year as a random (block) factor in all tested models. Since year was non-significant in all the analyses, the data for both years were pooled for Petroskoi and Padua. All statistical tests were performed with the IBM SPSS statistics 20.0 (IBM SPSS Inc., Chicago, IL, USA) statistical software package. To study whether burrowing differed between photoperiods or among populations and among populations within photoperiods, we used a binary logistic generalized linear model (GZLM) with population (Petroskoi, Belchow and Padua) and photoperiod (short day and long day) as factors. The interaction between photoperiod and population was removed from the final model since it was non-significant (Sokal and Rohlf 2003). To study the association between population, rearing photoperiod and burrowing behaviour on CO₂ production of 10-day-old beetles (RMR, ml CO₂ h⁻¹), we used a general linear model (GLM). RMR was log-transformed to improve normality and linearity. Burrowing behaviour (burrowed, surface) was included as a fixed factor in addition to

photoperiod, population and sex. Activity during the RMR measurement and body mass were added as covariates. The non-significant main factor sex, as well as interactions which were non-significant, was removed from the final model (Sokal and Rohlf 2003). If the interaction terms were significant, the main level effects were post-hoc tested with univariate F-tests and pair-wise tests done on group estimated marginal means with Bonferroni multiple-comparison (BMC) corrections. The results were consistent when the above model was built using mass-specific RMR ($\text{ml CO}_2 \text{ g}^{-1} \text{ h}^{-1}$). To study the association between population and burrowing behaviour on lipid content, the abdominal total lipid content (mg) of 10-day-old beetles were analysed with a factorial GLM with sex, population and burrowing behaviour entered as fixed factors and abdomen lean mass (post extraction dry mass) as a covariate. The data were split by photoperiod to improve the power of the test, due to uneven group sizes. The main factor sex was removed from the final model (Sokal and Rohlf 2003). If the interaction was significant, the main level effects were post-hoc tested with univariate tests as described above. We furthermore investigated the strength of association between energy sequestering and utilization as the relationship between log-transformed RMR ($\text{ml CO}_2 \text{ h}^{-1}$) and total relative lipid content (% of abdominal dry mass). This was done using a GZLM with an identity link function where log-RMR was the dependent variable and lipid content as well as body mass were added as covariates. The data was split by population and photoperiod. Finally, to investigate the association between age, population and rearing photoperiod on RMR, the log-transformed CO_2 ($\text{ml CO}_2 \text{ h}^{-1}$) was analysed with a factorial GLM. Age (5 and 10 days), sex, population and photoperiod were added as fixed factors and activity as well as body mass was added as covariates. The main factor sex was removed from the final model (Sokal and Rohlf 2003). If the interaction was significant, the main level effects were post-hoc tested with univariate tests as described above.

Results

Burrowing behaviour

While there was no difference in burrowing propensity between the short and long photoperiod in any of the populations (Wald $\chi^2 = 0.006$, $df = 1$, $P = 0.936$) (Fig. 2), a difference was detected when burrowing was compared among the populations (Wald $\chi^2 = 10.300$, $df = 2$, $P = 0.006$). A significantly lower proportion of the beetles from the Paduan population were burrowed than in the Petroskoi (Bonferroni Multiple Comparison, BMC: $P = 0.004$) and Belchow (BMC: $P = 0.045$) populations, which in turn did not differ from each other (BMC: $P = 1.000$).

Burrowing and RMR

In 10-day-old beetles, the main effects of burrowing behaviour and population were significant (Table 2a). Beetles which had burrowed into the soil had lower RMR than beetles still on the soil surface (Fig. 3a, 3b). While the beetles from Petroskoi had significantly lower RMR than those from Belchow (BMC: $P = 0.004$), they did not differ from beetles from Padua (BMC: $P = 0.282$). Similarly, no burrowing related difference in RMR of beetles from Padua and Belchow was detected (BMC: $P = 0.252$). Photoperiod did not have a significant effect on RMR.

Burrowing and total lipid content

In the SD photoperiod, the beetles from all populations accumulated similar amounts of lipids which differed depending on burrowing behaviour (Table 3). Beetles which had burrowed into the soil had on average 6.5 mg more lipids than those still on the soil surface (Fig. 3c). In the LD photoperiod, however, the populations differed in total lipid content depending on whether beetles were on the surface or were burrowed into the soil (Fig. 3d). While beetles on

the surface from all populations had similar amounts of lipids (BMC: $P > 0.679$), the burrowed beetles from Padua had significantly lower lipid content than those from Petroskoi and Belchow (BMC: $P < 0.001$ and $P = 0.006$ respectively) and the latter populations did not differ significantly from each other (BMC: $P = 1.000$). The burrowed beetles from Petroskoi and Belchow had significantly higher lipid content than those on the surface (Univariate test: $F_{1,126} = 19.129$, $P < 0.001$ and Univariate test: $F_{1,126} = 19.963$, $P < 0.001$ respectively) while the burrowed beetles from Padua had as low lipid content as those on the surface (Univariate test: $F_{1,126} = 0.354$, $P = 0.553$).

Relationship between total lipid content and RMR

A negative relationship between RMR and lipid content was seen in beetles from all populations when reared under SD conditions (Fig. 4). The relationship was significant for the beetles from Padua and Petroskoi (Wald $\chi^2 = 7.330$, $df = 1$, $P = 0.007$ and Wald $\chi^2 = 31.676$, $df = 1$, $P < 0.001$, respectively), while a marginally significant trend was seen for the beetles from Belchow (Wald $\chi^2 = 3.508$, $df = 1$, $P = 0.061$). When reared in the LD photoperiod, the beetles from Petroskoi showed the strongest negative relationship between RMR and lipid content, which however was non-significant (Wald $\chi^2 = 3.113$, $df = 1$, $P = 0.078$). Also in beetles from Padua or Belchow was the relationship negative but non-significant (Wald $\chi^2 = 0.169$, $df = 1$, $P = 0.681$ or Wald $\chi^2 = 0.999$, $df = 1$, $P = 0.318$, respectively).

RMR trajectory with age

RMR differed with age in a population and photoperiod dependent manner (Table 2b, Fig. 5). At 5 days after emergence, no difference was detected in RMR among the populations in either photoperiod (Univariate test: $F_{2,502} = 1.522$, $P = 0.219$). RMR was higher under the SD than under the LD photoperiod in all populations at 5 days after emergence (Univariate test:

$F_{1, 502} = 15.379$, $P < 0.001$). By day 10, the difference in RMR between photoperiods had disappeared (Univariate test: $F_{1, 502} = 1.483$, $P = 0.224$) but there were differences among populations (Univariate test: $F_{2, 502} = 7.660$, $P = 0.001$). The beetles from Petroskoi had significantly lower RMR than the beetles from Belchov or Padua (BMC): $P < 0.001$ and $P = 0.017$, respectively) which, in turn, did not differ significantly from each other (BMC: $P = 0.840$).

Discussion

Successful expansion to a novel environment often requires that an animal either is able to match its life-history with the biotic and abiotic requirements of the novel environment (Fig. 1, path 1; Elton 1958; Sakai et al. 2001; Lee 2002) or is capable of avoiding adverse conditions through behaviour (Fig. 1, path 2; Sakai et al. 2001; Duckworth 2009; Wright et al. 2010; Phillips and Suarez 2012; Sunday et al. 2014). In the present study we investigated whether the rapid latitudinal range expansion of *L. decemlineata* into novel, high latitude environments in Europe has been associated with behavioural plasticity in overwintering behaviour.

Our study shows that burrowing behaviour was plastic, as burrowing into the soil occurred under both photoperiods in all populations and not only when the photoperiod matched the one in their native area (Fig. 2). This was especially evident in the southern population from Padua which, in case burrowing would be strictly related to photoperiodic adaptation, was expected to burrow into the soil only under the short photoperiod (Lehmann et al. 2012). This behavioural plasticity may have been one major factor that assisted in the initial spread of *L. decemlineata* from native areas in Mexico to unpredictable agricultural habitats in the southern United States of America (Hsiao 1985; Alyokhin 2009). Later, harsh seasonal conditions, experienced when *L. decemlineata* invaded higher latitudes, selected for

overwintering in diapause and consequently diapause has become prevalent especially at high latitudes (Danilevskij 1965; Hsiao 1985). Similar results are seen in the mosquito *Aedes albopictus* where invasion and initial range expansion in North America took place without significant phenological (photoperiodic) adaptation (Urbanski et al. 2012). Populations have instead secondarily become adapted to local seasonal conditions (Urbanski et al. 2012). There is a growing number of studies on both plants and animals which suggest that phenotypic plasticity can play a major role in buffering initial responses to novel environmental conditions, be this during range expansion or in response to climate change (Sexton et al. 2002, Merilä and Hendry 2014; Sunday et al. 2014).

Our results have implications for the use of burrowing as an indicator of diapause. Since burrowed beetles from Padua show high overwintering mortality when reared under a long day photoperiod, but not when reared under a short day photoperiod (Lehmann et al. 2012), burrowing behaviour on its own is not a sufficient, general, biomarker of diapause initiation. Instead, it seems that beetles can burrow for other reasons than diapause (Hsiao 1985). In fact, several studies suggest that *L. decemlineata* does not overwinter in diapause in its native range (northern Mexico) (Hsiao 1985; Alyokhin 2009; Izzo et al. 2013), and is known to burrow into the soil for other reasons, such as avoidance of parasites, drought, high temperatures or lack of food (Hsiao 1985; Tauber et al. 1986; Danks 1987). The capacity of animals to use behaviour to choose optimal microclimates and avoid stress, and the implications behavioural phenotypic plasticity has in mitigating deleterious effects associated with stress due to for instance climate change, range expansion or anthropogenic factors has been increasingly appreciated (Wright et al. 2010; Phillips and Suarez 2012; Sunday et al. 2014).

Even though beetles from all populations burrowed under both photoperiods, indicating behavioural plasticity (Fig. 2), they differed in how burrowing behaviour was

linked to diapause-related physiological traits. In the beetles from Padua, burrowing was accompanied with high lipid content only when reared under short day conditions (Fig. 3b). Since lipid accumulation is an inherent part of diapause preparation (Hahn and Denlinger 2007; Lehmann et al. 2012), the results suggest that the burrowed Paduan beetles reared under long day conditions had not prepared for diapause. Indeed the Paduan population suffer from high winter mortality when reared under a long day (18 hours light) photoperiod (Lehmann et al. 2012). Unlike the Paduan beetles, the beetles from Petroskoi and Belchow seem to prepare for diapause also under a long photoperiod, as indicated by their high lipid content in both photoperiods (Lehmann et al. 2012; see also Lehmann et al. 2014). Taken together these results suggest that physiological adaptation has been important for successful establishment at higher latitudes (Fig. 1, path 1; Piironen et al. 2011; Lehmann et al. 2012). Because adaptive changes take time, the shift from behavioural-plasticity-driven to photoperiodic-adaptation-driven range expansion could partly explain why the latitudinal range expansion of *L. decemlineata* in Europe has slowed down during the last 30 years (Hsiao 1985; EPPO 2006). Other reasons can include temporal constraints due to a short summer (but see Boman et al. 2008) or thermal constraints during a longer and harsher winter (Valosaari et al. 2008). Furthermore, changes in anthropogenic factors (such as pesticide usage) should not be overlooked when assessing range expansion of an agricultural pest (Alyokhin 2009; Piironen et al. 2013).

The other physiological trait, RMR, was strongly linked to burrowing behaviour in all populations (Fig. 3a), irrespective of whether or not beetles had prepared for diapause (based on lipid content). The results suggest that lowered RMR of burrowed beetles is not necessarily related to diapause initiation in *L. decemlineata*, as reported in several other insects (Guppy and Withers 1999). Burrowed individuals might simply get immediate energetic benefits by lowering RMR (Chown and Nicolson 2004). Lower CO₂ production

could also result from a metabolic shift from burning carbohydrates to lipids, not due to an active suppression of metabolic rate itself (Sinclair et al. 2011). Unfortunately we cannot separate these alternatives in the present study since we did not measure O₂ consumption (Lighton 2008). Diapause-related RMR suppression is also often driven by ambient temperature (Guppy and Withers 1999) which was not manipulated in the present study. However diapause initiation at higher latitudes generally occurs under relatively benign conditions in nature (Tauber et al. 1986) and thus the results of the present study likely reflect natural conditions when beetles initiate and burrow for diapause. Therefore the diapause-related suppression of RMR reported in previous studies likely reflects either metabolic responses to changes in ambient temperature, which generally decreases as diapause progresses (Guppy and Withers 1999), or a general shutdown of metabolic functions during later phases of diapause (Boiteau and Coleman 1996; Kostal 2006; Hahn and Denlinger 2007; but see Hodkova and Hodek 2004).

While RMR did not seem to be associated with the decision to enter diapause, it was associated with age, population and photoperiod. Beetles from all populations reared under short day conditions had higher RMR than beetles reared under long day conditions when 5 days old (Fig. 5). Taken together with the lipid data of 10-day-old beetles this result suggest that while beetles from Petroskoi and Belchow prepare for overwintering under both photoperiods, but they might do so faster when the photoperiod is shorter (de Kort 1990; Piironen et al. 2011). Between days 5 and 10, RMR decreased in all populations, and the photoperiodic effect disappeared. The general age-dependent decrease in RMR observed in all populations is probably not related to diapause initiation but is more likely due to developmental changes part of the maturation process (Hack 1997; Guppy and Withers 1999; Roberts and Rosenberg 2006). The 10-day-old adults might, after feeding intensively, lessen feeding and thus have lower gut metabolic activity (Guppy and Withers 1999), regardless of

whether they are undergoing reproductive maturation or pre-diapause development. However, the northernmost population had lower RMR at age 10 days than the other populations (Fig. 5) even when the proportion of burrowed beetles is taken into account (data not shown). Since the populations did not differ at the age of 5 days, this result suggests that RMR in beetles from Petroskoi has a steeper negative age-dependent slope than southern beetles. These results could suggest that the northernmost beetles prepare for diapause faster than the other populations, which could relate to temporal constraints imposed by a shorter growth season (and time to prepare for diapause) at high latitudes. Alternatively, since energy consumption decreases with metabolic rate, a lower absolute RMR could be linked to stronger conservation of limited energy reserves during the winter (Guppy and Withers 1999; Hahn and Denlinger 2007), which becomes progressively longer at higher latitude. Clines in RMR are known in many ectothermic species with large ranges, and therefore RMR has been suggested to be linked to adaptation to season length and range expansion propensity (Chown and Gaston 1999). Both negative (Clarke 1993) and positive (Block and Young 1978) within-species correlations between latitude and RMR have been documented (Chown and Gaston 1999). While we cannot separate whether the differences between the populations in RMR in the present study relate to slope or absolute value, the results suggest that RMR varies with latitude in *L. decemlineata* and that this variation could have contributed to the expansion process, as suggested also in other species (Block and Young 1978; Clarke 1993; Chown and Gaston 1999; Guppy and Withers 1999).

Conclusions

In this study we found behavioural plasticity in overwintering behaviour in European *L. decemlineata*, since Paduan beetles burrowed into the soil when reared under a non-diapause inducing long photoperiod. Even though RMR was lower in burrowed beetles regardless of

photoperiod, lipid content was higher in burrowed beetles only when they were reared under diapause inducing photoperiods. Since low lipid content in burrowed beetles is associated with low winter survival (Lehmann et al. 2012), these data suggest that neither RMR at the age when beetles burrow nor burrowing behaviour necessarily are good indicators of diapause initiation. This study suggests that burrowing behaviour on its own has not been sufficient for successful range expansion into environments with strong seasonality (Fig. 1, path 2) even though it might have assisted in the initial invasion of more benign environments in southern Europe. Instead synchronization of burrowing behaviour and relevant diapause related physiological traits with photoperiod have been needed for survival of *L. decemlineata* during the long and harsh winters in northern Europe (Fig. 1, path 1). Lastly, a steeper slope in age-dependent RMR trajectory of northern *L. decemlineata* suggests that diapause preparation becomes faster as latitude increases (Lehmann et al. 2014) which could be due to temporal constraints. Our results underline that eco-physiological and life-history traits, such as diapause, should be included in studies on range expansion (including applied pest-management modelling). Only then can we make more reliable range expansion predictions, in particular in the face of climate change (Bradshaw and Holzapfel 2001; Gomi 2007; Mahdjoub and Menu 2008; Bean et al. 2012; Saikkonen et al. 2012; Urbanski et al. 2012, Jönsson et al. 2013, Sunday et al. 2014).

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Tables

Table 1 The number of *Leptinotarsa decemlineata* adults used in the lipid and metabolic (shown in brackets) study from the two age groups and three populations (sexes are pooled). For the 5-day-old beetles, no lipid quantification was performed and for the 10-day-old beetles the number burrowed beetles is also shown. The mean weight of beetles (mg $\pm$ SE) is also shown.

	Short Day			Long Day		
	Petroskoi	Belchow	Padua	Petroskoi	Belchow	Padua
	<i>N</i> (<i>N</i> in	<i>N</i> (<i>N</i> in	<i>N</i> (<i>N</i> in	<i>N</i> (<i>N</i> in	<i>N</i> (<i>N</i> in	<i>N</i> (<i>N</i> in
	RMR)	RMR)	RMR)	RMR)	RMR)	RMR)
5 days old	-- (46)	-- (36)	-- (43)	-- (43)	-- (35)	-- (37)
Weight (mg)	141 $\pm$ 3.6	125 $\pm$ 3.3	130 $\pm$ 3.1	140 $\pm$ 3.5	128 $\pm$ 2.9	132 $\pm$ 3.4
10 days old	50 (47)	40 (41)	43 (42)	53 (55)	39 (39)	42 (43)
Weight (mg)	146 $\pm$ 3.0	136 $\pm$ 2.9	134 $\pm$ 3.0	143 $\pm$ 3.1	139 $\pm$ 3.4	130 $\pm$ 2.9
Burrowed	29 (28)	19 (20)	12 (12)	27 (28)	20 (20)	14 (15)

Table 2 GLM (ANCOVAs) testing differences in RMR of adult *L. decemlineata* from three populations reared under two photoperiods in a) 10-day-old beetles either burrowed or on soil surface and b) 5- and 10-day-old beetles.

Effect	df	MS	F	P
a)				
Intercept	1	10.490	483.290	<0.001
Activity (covariate)	1	0.417	19.193	<0.001
Mass (covariate)	1	0.182	8.405	0.004
Population	2	0.116	5.323	0.005
Photoperiod	1	0.029	1.339	0.248
Burrowing behaviour	1	0.358	16.479	<0.001
Error	265	0.022		
b)				
Intercept	1	20.380	1088.209	<0.001
Activity (covariate)	1	1.043	55.690	<0.001
Mass (covariate)	1	0.461	24.640	<0.001
Age	1	3.645	194.617	<0.001
Population	2	0.050	2.695	0.068
Photoperiod	1	0.255	13.638	<0.001
Age*Population	2	0.114	6.086	0.002
Age*Photoperiod	1	0.019	4.096	0.044
Error	502	0.019		

666 Table 3 GLMs (ANCOVAs) of total abdominal lipid content (mg) of 10-day-old adult *L.*
667 *deceplineata* originating from three populations. Photoperiods were analysed in separate
668 models. Abdomen lean (lipid-free) mass was used as covariate.

	Effect	df	MS	F	P
Short day	Intercept	1	0.216	0.006	0.937
	Abdomen mass (covariate)	1	1001.071	29.449	<0.001
	Population	2	55.222	1.624	0.201
	Burrowing behaviour	1	1241.258	36.514	<0.001
	Population*Burrowing behaviour	2	2.297	0.068	0.935
	Error	126	33.994		
Long day	Intercept	1	194.747	6.742	0.011
	Abdomen mass (covariate)	1	1762.929	61.030	<0.001
	Population	2	179.939	6.210	0.003
	Burrowing behaviour	1	804.683	27.857	<0.001
	Population*Burrowing behaviour	2	122.615	4.245	0.016
	Error	127	28.886		

669

Figure captions

Fig. 1 Conceptual path diagram summarizing the potential role of behavioural plasticity during range expansion into novel stressful environments. Path 1 represents behaviour initially buffering against stressors, but ultimately leading to local adaptations through genetic assimilation (Lee, 2002; Pigliucci and Murren 2003; Duckworth 2009). Path 2 represents behaviour buffering against environmental stressors to such a degree that adaptation is hindered or significantly slowed down (Duckworth 2009; Wright et al. 2010).

Fig. 2 The percentage of *L. decemlineata* beetles burrowed into the soil at age of 10 days after eclosion when reared under a short and long photoperiod. Numbers at base of pillars show sample size.

Fig. 3 Difference in (a/b) mass-specific resting metabolic rate and (c/d) total abdominal lipid content of *L. decemlineata* beetles on the soil surface and burrowed into the soil when reared under a (a/c) short or (b/d) long photoperiod. Although mass-specific values are plotted, analyses were conducted with mass as covariate.

Fig. 4 Mass-specific resting metabolic rate as a function of lipid content (% of abdomen dry mass) for 10-day-old *L. decemlineata* originating from Petroskoi (Pet.), Belchow (Bel.) and Padua (Pad.) reared under a short (SD) or long (LD) photoperiod. Lines are drawn when the relationship was significant. See text for statistical details. Although mass-specific values are plotted, analyses were conducted with beetle total mass (including lipids) as covariate.

Fig. 5 Mass-specific resting metabolic rate of 5- and 10-day-old *L. decemlineata* from three populations reared under either a long (LD) or short (SD) photoperiod. Although mass-specific values are plotted, analyses were conducted with mass as covariate.

692 **Figures**

693 Figure 1

694

Studies cited in this figure: The present study¹, Williams et al, 2008², Lehmann et al. 2012³, Piironen et al. 2011⁴, Lyytinen et al. 2012⁵, Kenneth and Case 1996⁶, Wright et al. 2010⁷, Hanshew and Garcia 2012⁸, Gabel et al. 2011⁹

695 Figure 2

696

701 Figure 5

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