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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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12 **Abstract**

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

34

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

36

37 **Introduction**

38 Many organisms use the photoperiod as the main environmental cue for phenological
39 synchronization, since it is a reliable indicator of time of year (Nelson et al. 2010) and it
40 allows organisms to predict coming seasonal shifts accurately. This has led to various
41 photoperiodic adaptations in physiological, behavioural and life history traits (Nelson et al.
42 2010). Species undergoing latitudinal range expansion face a change in the photoperiod,
43 which can lead to a mismatch between the timing of seasonal adjustments in physiological
44 and life history traits with seasonal environmental changes (Bradshaw and Holzapfel 2007).
45 This mismatch can lead to lowered survival, for example, due to unsynchronized preparation
46 for overwintering or timing of migration (Rankin and Burchsted 1992). Consequently,
47 latitudinal range expansion may fail unless evolutionary changes in critical photoperiod take
48 place (Gaston 2003; Bradshaw and Holzapfel 2007; Urbanski et al. 2012) and species are able
49 to adjust their behavioural and physiological traits with the abiotic and biotic requirements of
50 the new environment (Elton 1958).

51 Range expansion to and persistence in novel environments may be possible also
52 without any genetic changes (i.e. adaptive evolution) if invading individuals possess
53 phenotypic plasticity in their abilities to avoid stressful conditions (Sakai et al. 2001; West-
54 Eberhard 2003; Duckworth 2009, Sunday et al. 2014). Phenotypic plasticity can manifest
55 itself on many organismal levels, including in physiological and behavioural traits (West-
56 Eberhard 2003). Through behavioural plasticity maladapted individuals might persist, thus
57 allowing time for physiological adaptation to occur (Sakai et al. 2001; Phillips and Suarez
58 2012) (Fig. 1). The potential roles of behavioural plasticity in facilitating the establishment of
59 species, especially invasive species, have been increasingly appreciated (Sagata and Lester
60 2009; Wright et al. 2010; Gabel et al. 2011; Hanshew and Garcia 2012; Phillips and Suarez
61 2012). Therefore, in order to better predict species invasions into novel environments with

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

66 One such important stress avoidance mechanism by which temperate insects survive
67 harsh winter conditions and which can show between-population differences as a result of
68 plastic or genetic change is diapause (Tauber et al. 1986). Correct timing and physiological
69 synchronization with photoperiod is often vital for diapause preparation since overwintering
70 mortality usually is very high in unprepared individuals (Danks 1987; Hahn and Denlinger
71 2007, see Fig. 1 path 1). Diapause is usually triggered by a shortening of the photoperiod
72 (preceding autumn and winter), but can be modulated by for instance, low temperatures or the
73 quality or abundance of food (Danilevskij 1965; Tauber et al. 1986; Danks 1987). Diapause
74 itself is usually characterized by reproductive arrest, suppressed metabolism and high
75 tolerance to many environmental stressors (Tauber et al. 1986; Kostal 2006). In many species,
76 preparation for diapause is also accompanied by changes in behaviour (Beck and Hanec 1960;
77 Beekman et al. 1998) including, active foraging for building energy stores, and seeking out
78 overwintering locations (Tauber et al. 1986). Individuals generally choose overwintering
79 microhabitats where they are buffered against exposure to adverse climatic conditions such as
80 extreme temperatures, large temperature fluctuations and desiccation (Danks 1987). This
81 opens the possibility that individuals can survive winters in novel environments without
82 physiological adaptation (Fig. 1, path 2).

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

87 America (Casagrande 1987; Alyokhin 2009), and currently occupies an area covering 16
88 million km² in North America, Europe and Asia (EPPO 2006). The beetles that invaded
89 Europe, Bordeaux, France, in the 1920s have been suggested to come from lower latitudes in
90 North America (Johnson 1967). There was a clear genetic bottleneck during the initial
91 introduction and establishment in Europe, resulting in reduced genetic variation (Grapputo et
92 al. 2005; Piironen et al. 2013). Despite this reduction, the subsequent range expansion of *L.*
93 *decemlineata* has been very rapid (Piironen et al. 2013) and the annual expansion distance
94 has been estimated to be between 80 and 130 km (Johnson 1967; EPPO 2006). Reasons for
95 the successful invasion include the beetles' versatile life-cycle and capability to overwinter in
96 diapause (Casagrande 1987; Hsiao 1985; de Kort 1990; Mahdjoub and Menu 2008; Alyokhin
97 2009) where cold-tolerance is significantly increased (Boiteau and Coleman 1996; Costanzo
98 et al. 1998).

99 In *L. decemlineata*, diapause initiation occurs at around 15 days after adult eclosion
100 under short photoperiods (Lefevre and de Kort 1989). During the pre-diapause period
101 storage lipid content increases dramatically (Lehmann et al. 2012) and metabolic rate shows a
102 bell-shaped pattern, which peaks at 3-5 days post eclosion (May 1989; Piironen et al. 2010).
103 In addition to physiological changes, pre-diapause behaviour is characterized by adults
104 becoming negatively phototactic and by burrowing into the soil (Alyokhin 2009). Burrowing
105 into the soil is commonly associated with pre-diapause not only in *L. decemlineata* (de Kort
106 1990; Piironen et al. 2011; Izzo et al. 2013) but also in other coleopterans (Rasa 1999; Kuang
107 et al. 2011) and in insects in general (Roubik and Michener 1980; Beekman et al. 1998;
108 Solbreck and Windefalk 2012). Adult *L. decemlineata* beetles can also burrow into the soil
109 even though reproductively active (Hsiao 1985; Tauber et al. 1988; Alyokhin 2009).
110 Burrowing is probably a means to avoid exposure to stressful climatic conditions and harmful
111 biotic interactions (Tauber et al. 1986; Danks 1987). Since burrowing can be decoupled from

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

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

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

137 economic utilization of limited energy reserves during overwintering are vital for successful
138 overwintering, accumulation of lipids and suppression of resting metabolic rate (RMR) are
139 commonly used as physiological measures of preparation for diapause (Coleman and Boiteau
140 1996; Guppy and Withers 1999; Kostal 2006; Hahn and Denlinger 2007; Lehmann et al.
141 2012). 2) In case burrowing behaviour is plastic, we predict that beetles burrow also under
142 non-diapause inducing photoperiods (Fig. 1, path 2). If burrowing on its own has been
143 sufficient for beetles to survive winters faced, we predict a weak association between
144 burrowing and high lipid content as well as low metabolic rate in beetles burrowed under non-
145 diapause inducing conditions (Fig. 1, path 2). 3) In parallel with pre-diapause behaviour, we
146 study whether populations differ in resting metabolic rate (RMR) trajectories when reared
147 under diapause inducing and non-inducing conditions. We predict that the steepness of the
148 RMR trajectory slope increases with latitude in beetles preparing for diapause, which could
149 relate to more effective use of limited energy reserves (May 1989; Guppy and Withers 1999.

150

151 **Materials and methods**

152 *Study animals*

153 Three *L. decemlineata* populations were used in this study. They were the descendants of
154 beetles collected from potato fields near Petroskoi in northern Russia (61°49'N, 34°10'E) (N
155 = 917) in the summer of 2006, from potato fields near Belchow in Poland (52°01'N, 20°34'E)
156 (N = 400) in 2010 and from potato fields near Padua in Italy (45°48'N, 12°07'E) (N = 249) in
157 the summer of 2010. The Petroskoi population represents the northernmost current range limit
158 (EPPO 2006). The parental generations were reared in the laboratory under a long day
159 photoperiod (18 h light, 23°C, 60% relative humidity) on potato (*Solanum tuberosum* of the
160 *van Gogh* variety). The next generation beetles were then overwintered at 5°C in climate
161 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*

187 *tuberosum* of the *van Gogh* variety). To investigate burrowing behaviour in response to
188 photoperiod, 7-day-old beetles were moved from the petri dishes into plastic jars (120 ml)
189 containing peat soil (40 ml). The jars had clear lids to allow full penetration of light and holes
190 for air exchange. The soil was kept moistened and adults were fed daily with fresh potato
191 leaves. Beetles were fed *ad libitum* to avoid burrowing as a response to lack of food (Hsiao
192 1985; Alyokhin 2009). Burrowing into the soil (burrowed into the soil or still on the soil
193 surface) was recorded when beetles were 10 days old.

194

195 *Resting metabolic rate*

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

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

230

231 *Total lipid content*

232 To investigate the size of energy reserves, abdominal total lipid content was measured from
233 10-day-old beetles. After the measurement of RMR, beetles were weighed, anesthetized on
234 ice, the elytrae removed and the whole abdomen cut away and stored at -80°C until analysis.
235 Total lipid content of the abdomen was measured with Chloroform:Methanol (Chl:Meth)
236 extraction (Folch et al. 1957) as described previously (Lehmann et al. 2012). Abdomen were

237 dried for 72 h at 55 °C, after which lipids were extracted by placing the abdomen in 10 ml of
238 2:1 Chl:Meth for 72 h. Lipid content was then assessed gravimetrically after drying the
239 samples for 72 h at 55 °C. Around 85% of abdominal total lipids extracted in this way are
240 neutral lipids, which mainly are storage triacylglycerids (Lehmann et al. 2012). During the
241 first 10 days of adult life, *L. decemlineata* does not accumulate glycerol (P. Lehmann,
242 unpublished), which could interfere with the Chl:Meth extraction method (Williams et al.
243 2011). We used absolute lipid content (mg) as well as relative lipid content (%) for the
244 analyses (see below). Absolute lipid content was calculated by subtracting lean mass (post-
245 extraction dry mass) from dry mass, while total relative lipid content (w/w) was calculated by
246 dividing absolute lipid content by dry mass.

247

248 *Statistical analyses*

249 The potential difference between the years (2010 and 2013) was studied by adding year as a
250 random (block) factor in all tested models. Since year was non-significant in all the analyses,
251 the data for both years were pooled for Petroskoi and Padua. All statistical tests were
252 performed with the IBM SPSS statistics 20.0 (IBM SPSS Inc., Chicago, IL, USA) statistical
253 software package. To study whether burrowing differed between photoperiods or among
254 populations and among populations within photoperiods, we used a binary logistic
255 generalized linear model (GZLM) with population (Petroskoi, Belchow and Padua) and
256 photoperiod (short day and long day) as factors. The interaction between photoperiod and
257 population was removed from the final model since it was non-significant (Sokal and Rohlf
258 2003). To study the association between population, rearing photoperiod and burrowing
259 behaviour on CO₂ production of 10-day-old beetles (RMR, ml CO₂ h⁻¹), we used a general
260 linear model (GLM). RMR was log-transformed to improve normality and linearity.
261 Burrowing behaviour (burrowed, surface) was included as a fixed factor in addition to

262 photoperiod, population and sex. Activity during the RMR measurement and body mass were
263 added as covariates. The non-significant main factor sex, as well as interactions which were
264 non-significant, was removed from the final model (Sokal and Rohlf 2003). If the interaction
265 terms were significant, the main level effects were post-hoc tested with univariate F-tests and
266 pair-wise tests done on group estimated marginal means with Bonferroni multiple-comparison
267 (BMC) corrections. The results were consistent when the above model was built using mass-
268 specific RMR ($\text{ml CO}_2 \text{ g}^{-1} \text{ h}^{-1}$). To study the association between population and burrowing
269 behaviour on lipid content, the abdominal total lipid content (mg) of 10-day-old beetles were
270 analysed with a factorial GLM with sex, population and burrowing behaviour entered as fixed
271 factors and abdomen lean mass (post extraction dry mass) as a covariate. The data were split
272 by photoperiod to improve the power of the test, due to uneven group sizes. The main factor
273 sex was removed from the final model (Sokal and Rohlf 2003). If the interaction was
274 significant, the main level effects were post-hoc tested with univariate tests as described
275 above. We furthermore investigated the strength of association between energy sequestering
276 and utilization as the relationship between log-transformed RMR ($\text{ml CO}_2 \text{ h}^{-1}$) and total
277 relative lipid content (% of abdominal dry mass). This was done using a GZLM with an
278 identity link function where log-RMR was the dependent variable and lipid content as well as
279 body mass were added as covariates. The data was split by population and photoperiod.
280 Finally, to investigate the association between age, population and rearing photoperiod on
281 RMR, the log-transformed CO_2 ($\text{ml CO}_2 \text{ h}^{-1}$) was analysed with a factorial GLM. Age (5 and
282 10 days), sex, population and photoperiod were added as fixed factors and activity as well as
283 body mass was added as covariates. The main factor sex was removed from the final model
284 (Sokal and Rohlf 2003). If the interaction was significant, the main level effects were post-
285 hoc tested with univariate tests as described above.

286

287 **Results**

288 *Burrowing behaviour*

289 While there was no difference in burrowing propensity between the short and long
290 photoperiod in any of the populations (Wald $\chi^2 = 0.006$, $df = 1$, $P = 0.936$) (Fig. 2), a
291 difference was detected when burrowing was compared among the populations (Wald $\chi^2 =$
292 10.300 , $df = 2$, $P = 0.006$). A significantly lower proportion of the beetles from the Paduan
293 population were burrowed than in the Petroskoi (Bonferroni Multiple Comparison, BMC: $P =$
294 0.004) and Belchow (BMC: $P = 0.045$) populations, which in turn did not differ from each
295 other (BMC: $P = 1.000$).

296

297 *Burrowing and RMR*

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

305

306 *Burrowing and total lipid content*

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

312 the surface from all populations had similar amounts of lipids (BMC: $P > 0.679$), the
313 burrowed beetles from Padua had significantly lower lipid content than those from Petroskoi
314 and Belchow (BMC: $P < 0.001$ and $P = 0.006$ respectively) and the latter populations did not
315 differ significantly from each other (BMC: $P = 1.000$). The burrowed beetles from Petroskoi
316 and Belchow had significantly higher lipid content than those on the surface (Univariate test:
317 $F_{1, 126} = 19.129$, $P < 0.001$ and Univariate test: $F_{1, 126} = 19.963$, $P < 0.001$ respectively) while
318 the burrowed beetles from Padua had as low lipid content as those on the surface (Univariate
319 test: $F_{1, 126} = 0.354$, $P = 0.553$).

320

321 *Relationship between total lipid content and RMR*

322 A negative relationship between RMR and lipid content was seen in beetles from all
323 populations when reared under SD conditions (Fig. 4). The relationship was significant for the
324 beetles from Padua and Petroskoi (Wald $\chi^2 = 7.330$, $df = 1$, $P = 0.007$ and Wald $\chi^2 = 31.676$,
325 $df = 1$, $P < 0.001$, respectively), while a marginally significant trend was seen for the beetles
326 from Belchow (Wald $\chi^2 = 3.508$, $df = 1$, $P = 0.061$). When reared in the LD photoperiod, the
327 beetles from Petroskoi showed the strongest negative relationship between RMR and lipid
328 content, which however was non-significant (Wald $\chi^2 = 3.113$, $df = 1$, $P = 0.078$). Also in
329 beetles from Padua or Belchow was the relationship negative but non-significant (Wald $\chi^2 =$
330 0.169 , $df = 1$, $P = 0.681$ or Wald $\chi^2 = 0.999$, $df = 1$, $P = 0.318$, respectively).

331

332 *RMR trajectory with age*

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

337 $F_{1, 502} = 15.379, P < 0.001$). By day 10, the difference in RMR between photoperiods had
338 disappeared (Univariate test: $F_{1, 502} = 1.483, P = 0.224$) but there were differences among
339 populations (Univariate test: $F_{2, 502} = 7.660, P = 0.001$). The beetles from Petroskoi had
340 significantly lower RMR than the beetles from Belchov or Padua (BMC): $P < 0.001$ and $P =$
341 0.017 , respectively) which, in turn, did not differ significantly from each other (BMC: $P =$
342 0.840).

343

344 **Discussion**

345 Successful expansion to a novel environment often requires that an animal either is able to
346 match its life-history with the biotic and abiotic requirements of the novel environment (Fig.
347 1, path 1; Elton 1958; Sakai et al. 2001; Lee 2002) or is capable of avoiding adverse
348 conditions through behaviour (Fig. 1, path 2; Sakai et al. 2001; Duckworth 2009; Wright et al.
349 2010; Phillips and Suarez 2012; Sunday et al. 2014). In the present study we investigated
350 whether the rapid latitudinal range expansion of *L. decemlineata* into novel, high latitude
351 environments in Europe has been associated with behavioural plasticity in overwintering
352 behaviour.

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

362 overwintering in diapause and consequently diapause has become prevalent especially at high
363 latitudes (Danilevskij 1965; Hsiao 1985). Similar results are seen in the mosquito *Aedes*
364 *albopictus* where invasion and initial range expansion in North America took place without
365 significant phenological (photoperiodic) adaptation (Urbanski et al. 2012). Populations have
366 instead secondarily become adapted to local seasonal conditions (Urbanski et al. 2012). There
367 is a growing number of studies on both plants and animals which suggest that phenotypic
368 plasticity can play a major role in buffering initial responses to novel environmental
369 conditions, be this during range expansion or in response to climate change (Sexton et al.
370 2002, Merilä and Hendry 2014; Sunday et al. 2014).

371 Our results have implications for the use of burrowing as an indicator of diapause.
372 Since burrowed beetles from Padua show high overwintering mortality when reared under a
373 long day photoperiod, but not when reared under a short day photoperiod (Lehmann et al.
374 2012), burrowing behaviour on its own is not a sufficient, general, biomarker of diapause
375 initiation. Instead, it seems that beetles can burrow for other reasons than diapause (Hsiao
376 1985). In fact, several studies suggest that *L. decemlineata* does not overwinter in diapause in
377 its native range (northern Mexico) (Hsiao 1985; Alyokhin 2009; Izzo et al. 2013), and is
378 known to burrow into the soil for other reasons, such as avoidance of parasites, drought, high
379 temperatures or lack of food (Hsiao 1985; Tauber et al. 1986; Danks 1987). The capacity of
380 animals to use behaviour to choose optimal microclimates and avoid stress, and the
381 implications behavioural phenotypic plasticity has in mitigating deleterious effects associated
382 with stress due to for instance climate change, range expansion or anthropogenic factors has
383 been increasingly appreciated (Wright et al. 2010; Phillips and Suarez 2012; Sunday et al.
384 2014).

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

387 linked to diapause-related physiological traits. In the beetles from Padua, burrowing was
388 accompanied with high lipid content only when reared under short day conditions (Fig. 3b).
389 Since lipid accumulation is an inherent part of diapause preparation (Hahn and Denlinger
390 2007; Lehmann et al. 2012), the results suggest that the burrowed Paduan beetles reared under
391 long day conditions had not prepared for diapause. Indeed the Paduan population suffer from
392 high winter mortality when reared under a long day (18 hours light) photoperiod (Lehmann et
393 al. 2012). Unlike the Paduan beetles, the beetles from Petroskoi and Belchow seem to prepare
394 for diapause also under a long photoperiod, as indicated by their high lipid content in both
395 photoperiods (Lehmann et al. 2012; see also Lehmann et al. 2014). Taken together these
396 results suggest that physiological adaptation has been important for successful establishment
397 at higher latitudes (Fig. 1, path 1; Piiroinen et al. 2011; Lehmann et al. 2012). Because
398 adaptive changes take time, the shift from behavioural-plasticity-driven to photoperiodic-
399 adaptation-driven range expansion could partly explain why the latitudinal range expansion of
400 *L. decemlineata* in Europe has slowed down during the last 30 years (Hsiao 1985; EPPO
401 2006). Other reasons can include temporal constraints due to a short summer (but see Boman
402 et al. 2008) or thermal constraints during a longer and harsher winter (Valosaari et al. 2008).
403 Furthermore, changes in anthropogenic factors (such as pesticide usage) should not be
404 overlooked when assessing range expansion of an agricultural pest (Alyokhin 2009; Piiroinen
405 et al. 2013).

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

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

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

437 whether they are undergoing reproductive maturation or pre-diapause development. However,
438 the northernmost population had lower RMR at age 10 days than the other populations (Fig.
439 5) even when the proportion of burrowed beetles is taken into account (data not shown). Since
440 the populations did not differ at the age of 5 days, this result suggests that RMR in beetles
441 from Petroskoi has a steeper negative age-dependent slope than southern beetles. These
442 results could suggest that the northernmost beetles prepare for diapause faster than the other
443 populations, which could relate to temporal constraints imposed by a shorter growth season
444 (and time to prepare for diapause) at high latitudes. Alternatively, since energy consumption
445 decreases with metabolic rate, a lower absolute RMR could be linked to stronger conservation
446 of limited energy reserves during the winter (Guppy and Withers 1999; Hahn and Denlinger
447 2007), which becomes progressively longer at higher latitude. Clines in RMR are known in
448 many ectothermic species with large ranges, and therefore RMR has been suggested to be
449 linked to adaptation to season length and range expansion propensity (Chown and Gaston
450 1999). Both negative (Clarke 1993) and positive (Block and Young 1978) within-species
451 correlations between latitude and RMR have been documented (Chown and Gaston 1999).
452 While we cannot separate whether the differences between the populations in RMR in the
453 present study relate to slope or absolute value, the results suggest that RMR varies with
454 latitude in *L. decemlineata* and that this variation could have contributed to the expansion
455 process, as suggested also in other species (Block and Young 1978; Clarke 1993; Chown and
456 Gaston 1999; Guppy and Withers 1999).

457

458 *Conclusions*

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

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

480

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490

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655 **Tables**

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

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

661

662 Table 2 GLM (ANCOVAs) testing differences in RMR of adult *L. decemlineata* from three
 663 populations reared under two photoperiods in a) 10-day-old beetles either burrowed or on soil
 664 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		

665

666 Table 3 GLMs (ANCOVAs) of total abdominal lipid content (mg) of 10-day-old adult *L.*
 667 *decemlineata* 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

670 **Figure captions**

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

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

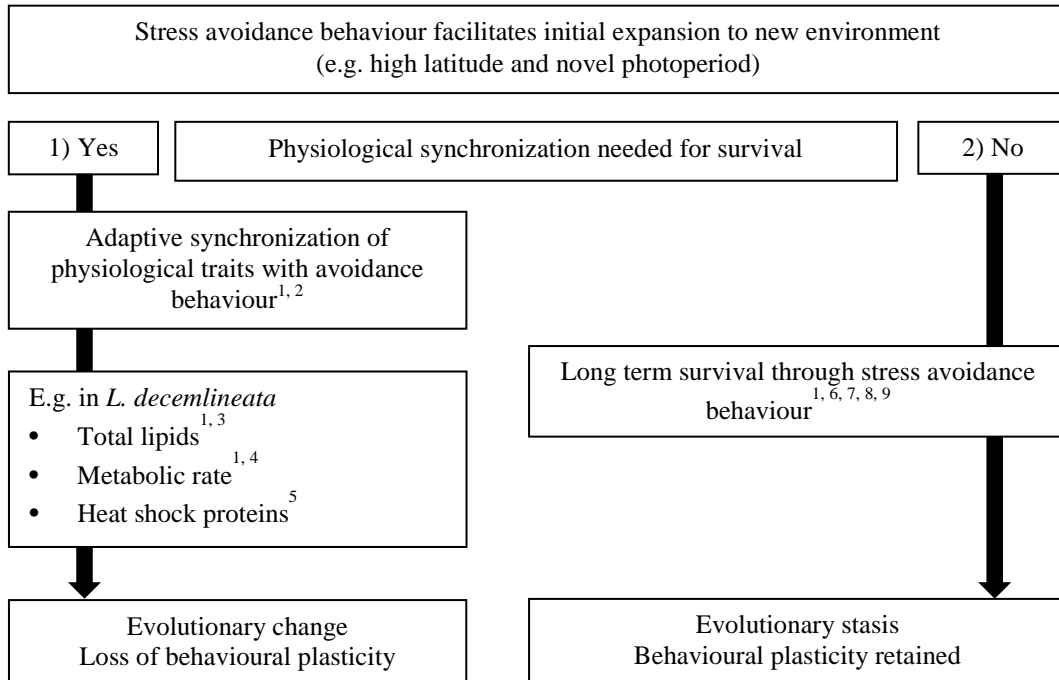
680 **Fig. 3** Difference in (a/b) mass-specific resting metabolic rate and (c/d) total abdominal lipid
681 content of *L. decemlineata* beetles on the soil surface and burrowed into the soil when reared
682 under a (a/c) short or (b/d) long photoperiod. Although mass-specific values are plotted,
683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

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

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

692 **Figures**

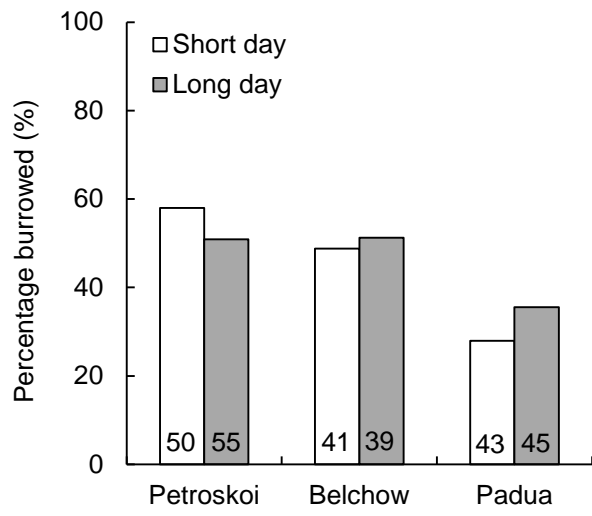
693 Figure 1



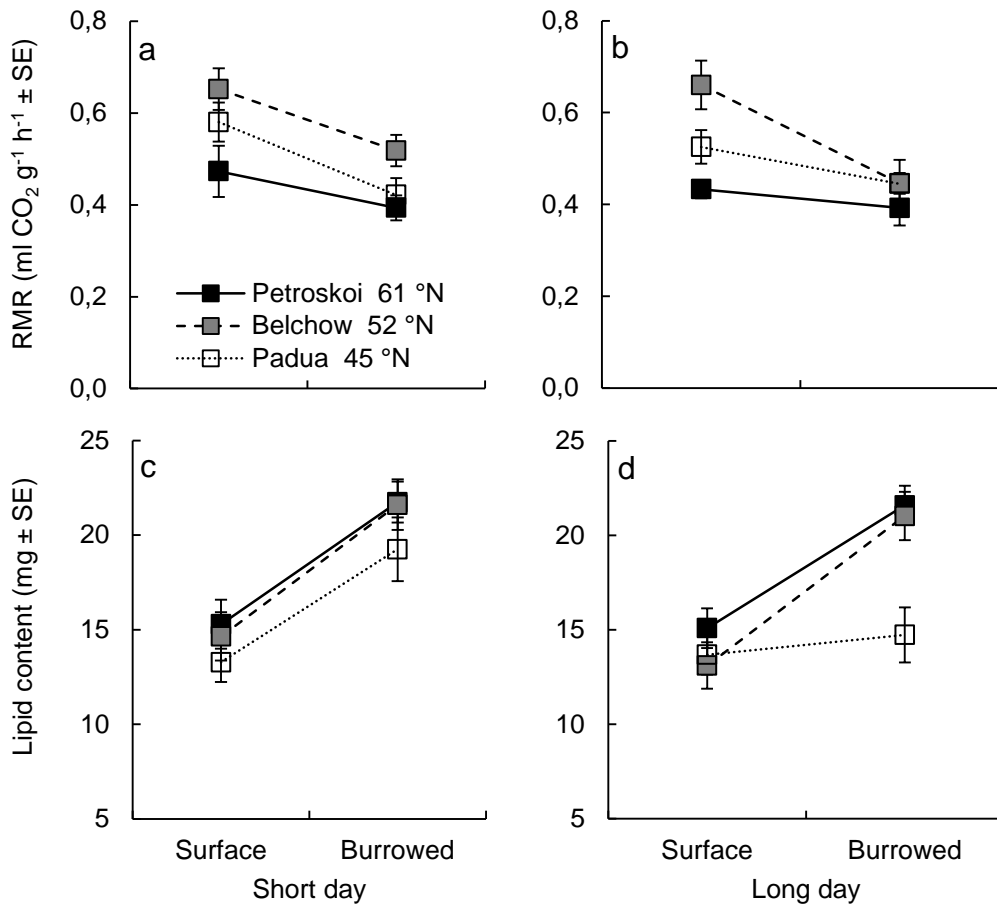
Studies cited in this figure: The present study¹, Williams et al, 2008², Lehmann et al. 2012³, Piiroinen et al. 2011⁴, Lyytinen et al. 2012⁵, Kenneth and Case 1996⁶, Wright et al. 2010⁷, Hanshaw and Garcia 2012⁸, Gabel et al. 2011⁹

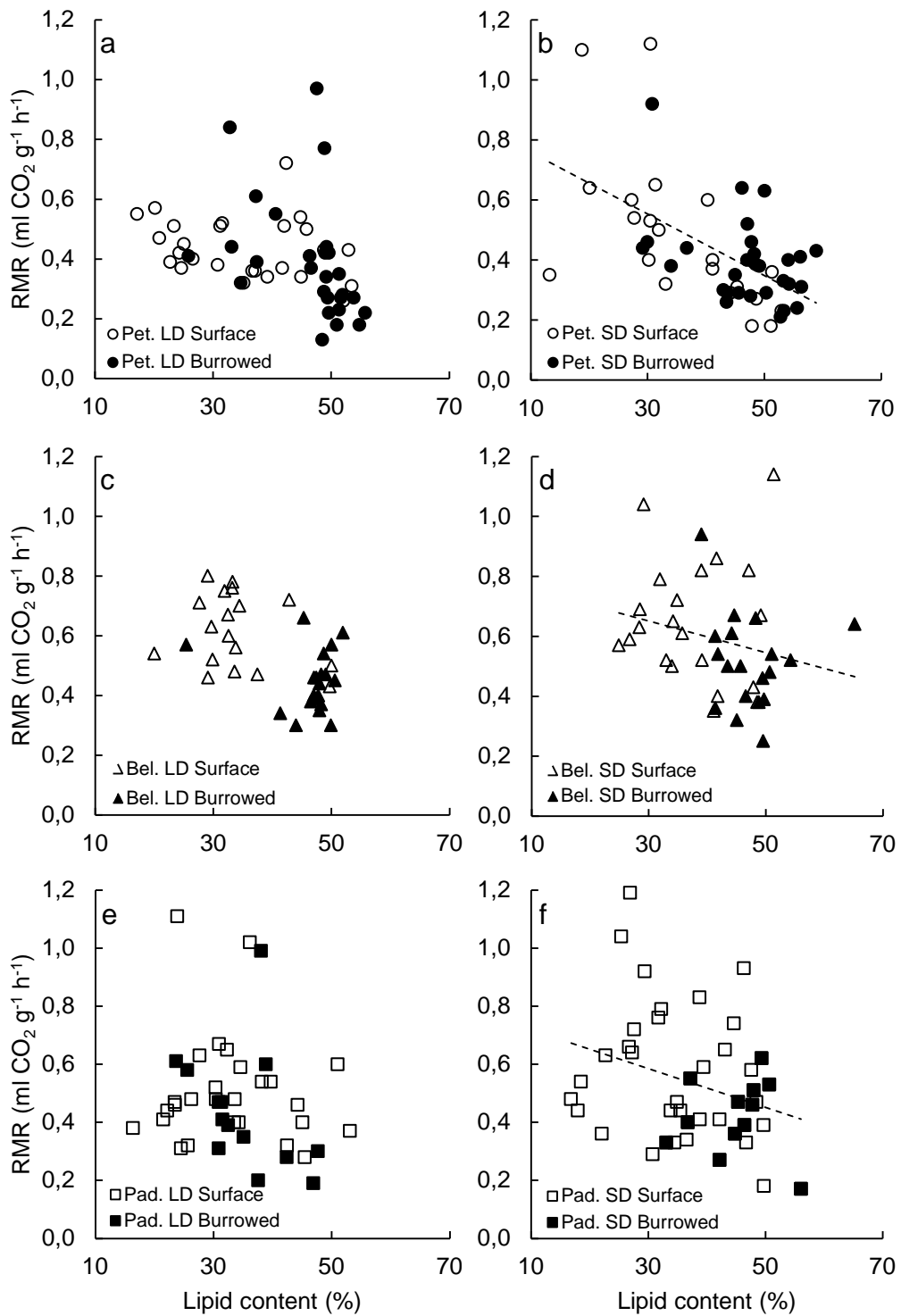
694

695 Figure 2

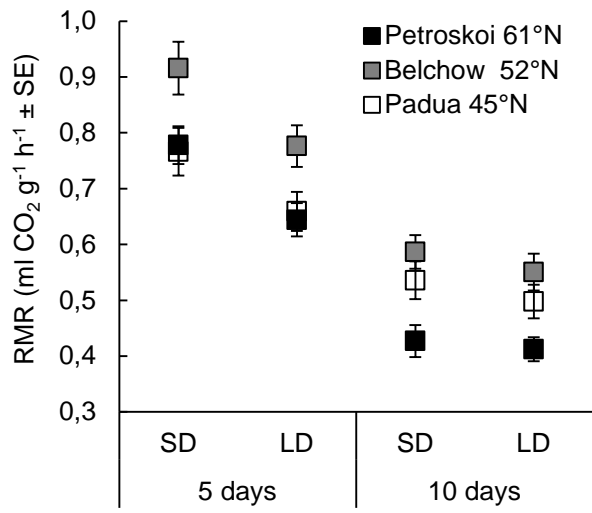


696





701 Figure 5



702