

# **Host-plant quality adaptively affects the diapause threshold: evidence from leaf beetles in willow plantations**

**Peter Dalin and Sören Nylin**

Peter Dalin ([Peter.Dalin@slu.se](mailto:Peter.Dalin@slu.se)) Swedish University of Agricultural Sciences, Department of Ecology, P.O. Box 7044, SE-750 07 Uppsala, Sweden. Fax: +46-18-672383.

Sören Nylin ([Soren.Nylin@zoologi.su.se](mailto:Soren.Nylin@zoologi.su.se)) Department of Zoology, Stockholm University, SE-106 91 Stockholm, Sweden. Fax: +46-8-167715.

1 **Abstract**

2 Voltinism (the number of generations produced per year) of herbivorous insects can vary  
3 depending on environmental conditions. The leaf beetle *Phratora vulgatissima* is commonly  
4 univoltine in central Sweden but will sometimes initiate a second generation on coppiced  
5 willows (*Salix viminalis*) grown in plantations for bioenergy purposes. The study investigated  
6 whether increased voltinism by *P. vulgatissima* can be explained by (1) rapid life-cycle  
7 development in plantations allowing two generations, or (2) postponed diapause induction on  
8 willows grown in plantations. In the field, no difference was found in the phenology or  
9 development of first-generation broods between plantations (*S. viminalis*) and natural willow  
10 habitats (*S. cinerea*). On re-sprouting shoots of recently coppiced *S. viminalis*, however, the  
11 induction of diapause occurred 1-2 weeks later than on mature (un-coppiced) plants. A  
12 laboratory experiment indicated no genetic difference in the critical day-length for diapause  
13 induction (CDL) between beetles from plantations and natural habitats. Although development  
14 time was unaffected by host-plant quality, CDL was prolonged by almost an hour when the  
15 beetles were reared on a sub-optimal willow species (*S. phylicifolia*). A subsequent experiment  
16 found that diapause incidence, when the beetles were reared on new leaves from re-sprouting  
17 shoots of previously coppiced *S. viminalis* and *S. cinerea* plants, was significantly less than when  
18 the beetles were reared on mature leaves from un-coppiced plants. The study suggests that *P.*  
19 *vulgatissima* has a plastic diapause threshold influenced by host-plant quality. The use of host-  
20 plant quality as a diapause-inducing stimulus is likely to be adaptive in cases where food  
21 resources are unpredictable, such as when new host-plant tissue is produced after a disturbance.  
22 Coppiced willows may allow two beetle generations because re-sprouting willows continue to  
23 produce new leaves throughout the season.

24

25 **Introduction**

26 The number of generations that insects produce per year (i.e. voltinism) is an important life-  
27 history trait that can strongly affect population growth, fitness and potential rate of adaptability  
28 (Kurota and Shimada 2002, Steinbauer et al. 2004, Yamanaka et al. 2008). Voltinism in  
29 herbivorous insects often varies across latitude and altitude (Tauber et al. 1986, Masaki 1999).  
30 Insects with a wide distribution range may therefore produce one generation per year (univoltine  
31 life-cycle) at northern latitudes where the growing season is relatively short, but produce two  
32 (bivoltine) or even more (multivoltine) generations further south. Such latitudinal clines in  
33 voltinism partly reflect local adaptations to seasonal environments, with a genetic basis, but  
34 plasticity in life-history traits is also a crucial component of the insects' seasonal adaptations  
35 (Nylin and Gotthard 1998). Plasticity in voltinism may allow the insect to immediately adjust the  
36 number of generations in response to prevailing environmental conditions, without need for  
37 selection to operate, and there is indeed evidence that recent climate change has led to extra  
38 generations in response to warmer temperature conditions, through plasticity (Altermatt 2010,  
39 Poyry et al. 2011). For most insects, however, we have too limited knowledge about what  
40 processes influence such life-history plasticity to be able to predict under what circumstances  
41 voltinism may change.

42

43 In temperate climate zones, insect voltinism is determined by the number of generations  
44 produced before the seasonal timing of winter diapause (Tauber et al. 1986, Danks, 2007).  
45 Diapause is a dormant stage characterized by lowered metabolic rates, cold hardening and  
46 cessation of reproductive development in insects that overwinters as adults (Kostal 2006). Many  
47 insects have a facultative diapause, i.e. individuals make a “decision” during development to

48 either enter diapause (thus, to wait with reproduction until the following year) or to exhibit direct  
49 development and to become reproductively active as adults and produce another generation  
50 within the same year (Gotthard 2008). This decision-making is determined by seasonal cues to  
51 which the insects respond for the induction of diapause, with day-length (photoperiod) being the  
52 most important cue (Nelson et al. 2010, Saunders 2010). Hence, in insects with facultative  
53 diapause voltinism is in a sense always a plastic trait, responding primarily to day-length. On the  
54 other hand, the critical day-length (CDL), defined as the day-length when 50% of a population  
55 enters diapause (Tauber et al. 1986, Saunders 2010) is a genetically determined property that  
56 varies adaptively among insect populations (Bradshaw 1976, Solbreck and Sillen-Tullberg 1981;  
57 Masaki 1999, Dalin et al. 2010). This critical day-length sets the timing for when diapause  
58 induction occurs over the course of the year and can therefore severely limit the possibilities for  
59 altered voltinism (Tobin et al. 2008).

60

61 Importantly, however, other factors that - in contrast to day-length - can vary from year to year  
62 as well as seasonally, such as temperature and (in the case of herbivorous insects) host-plant  
63 quality, may also plastically affect the incidence of diapause and hence voltinism (Tauber et al.  
64 1986). These effects can be indirect or direct, and it is seldom clear whether they are adaptations  
65 *per se*, i.e. have been selected for rather than just having incidental positive effects on fitness  
66 (Gotthard and Nylin 1995). Both temperature and host-plant conditions can strongly influence  
67 growth and development during the season and will in the field therefore indirectly influence  
68 voltinism by affecting the timing of when the insect reaches the critical stage for diapause  
69 induction. For example, slow growth on a poor host-plant or in response to low temperature  
70 conditions will delay the critical stage for diapause induction. If the critical stage is reached after

71 day-length has declined below CDL; the insect will choose the developmental pathway leading  
72 to diapause. Furthermore, laboratory studies suggest that temperature and host-plant quality can  
73 modify the insects' photoperiodic responses (Masaki 1999, Ishihara and Ohgushi 2006, Dolezal  
74 and Sehnal 2007, Dalin et al. 2010), and such more direct effects on voltinism are stronger  
75 candidates for being true adaptations to environmental variation (in the sense of Gotthard and  
76 Nylin 1995). Since temperature - for physical and chemical reasons - affects so many processes  
77 in the insect, it may be almost impossible to disentangle adaptive responses to temperature from  
78 spurious indirect effects, but effects of host plant quality provides an interesting opportunity for a  
79 deeper understanding of voltinism plasticity.

80

81 For example, Hunter and McNeil (1997) showed that the generalist lepidopteran *Choristoneura*  
82 *rosaceana* (Lepidoptera: Tortricidae) was more likely to enter diapause when reared on a poor  
83 quality food than when reared on high-quality food under controlled laboratory conditions, and  
84 similar results were found in the polyphagous comma butterfly *Polygonia c-album* (Wedell et al.  
85 1997). These studies suggest that food quality can influence the induction of diapause and  
86 voltinism of herbivorous insects, and such a plastic diapause threshold could prevent the insects  
87 from producing maladaptive generations on host-plants of low or declining quality. A poor host-  
88 plant may indicate that the plant cannot support rapid-enough growth and development for  
89 another generation to develop within the same year and, thus, that it is better to wait with  
90 reproduction until the following year. Even in these laboratory experiments, however, it is still  
91 not clear whether the insects can use the chemical properties of the host-plant as a direct signal  
92 or cue – similar to photoperiod – influencing the induction of diapause, or whether the  
93 potentially adaptive response is rather to growth rate, as determined by host quality, or indeed

94 even simply constitutes a spurious physiological side-effect of the host plant (Wedell et al.  
95 1997).

96

97 The leaf beetle *Phratora vulgatissima* is an important pest in willow plantations grown for  
98 bioenergy in northern Europe (Sage and Tucker 1998, Björkman et al. 2000, Dalin et al. 2009). It  
99 is commonly univoltine in northern Europe and bivoltine in central Europe. The species  
100 sometimes initiate a partial second generation also in northern Europe (Dalin 2011). This has  
101 particularly been observed in short-rotation coppiced willow (*Salix viminalis*) plantations grown  
102 for biomass productions in Sweden (P. Dalin, pers. obs.). The leaf beetle overwinters in the adult  
103 stage and emerges from overwintering sites in the spring. The phenology of adult emergence is  
104 usually well synchronized with willow bud-break in the spring. Adults feed on newly developed  
105 leaves and oviposit on the ventral side of the leaves. Larvae of the first generation continue to  
106 feed on leaves during the summer before they pupate in the soil. The next generation of adult  
107 beetles (first-generation adults) emerges in late July or beginning of August in central Sweden  
108 (Dalin 2011). These adults are normally in reproductive diapause and become the overwintering  
109 generation. However, those individuals that complete development to adulthood before August  
110 may become reproductively active and initiate a second generation (Dalin 2011).

111

112 The purpose of this study was to investigate whether the partial second generation of *P.*  
113 *vulgatissima* in willow plantations could be explained by (1.) advanced phenology and  
114 accelerated development of first-generation broods in willow plantations, or (2) postponed  
115 diapause induction of beetles in willow plantations. The central Swedish population that we  
116 study has previously been shown to have a facultative diapause induced by day-length with a

117 CDL estimated to be 18 hours 10 minutes (Dalin 2011). A second purpose of our study was to  
118 investigate whether CDL may differ between *P. vulgatissima* populations from willow  
119 plantations (*S. viminalis*) and natural willow stands (*S. cinerea*). This was tested by rearing the  
120 insects under controlled conditions in the laboratory. A genetic difference in CDL between host-  
121 populations, with an expected shorter CDL in beetles from plantations, could explain why the  
122 species is more likely to produce a second generation in plantations. We also tested the  
123 hypothesis that *P. vulgatissima* has a plastic diapause threshold that is influenced by host-plant  
124 quality. First, we predicted CDL to be prolonged when the species was reared on a sub-optimal  
125 willow; in this case *S. phyllicifolia* that contains relatively high concentrations of phenolic  
126 glycosides. Second, if the beetles are able to postpone diapause in response to vigorous host-  
127 plant growth on previously coppiced willows, we predicted diapause incidence to be reduced if  
128 the species was reared on newly produced leaves from coppiced willows (*S. viminalis* and *S.*  
129 *cinerea*) than when reared on older leaves from mature and un-coppiced plants.

130

## 131 **Materials and Methods**

### 132 *Life-cycle development and diapause induction of field populations*

133 During the summer 2009, we studied when natural populations of *P. vulgatissima* enter diapause  
134 in the field on *S. viminalis* in plantations and on *S. cinerea* in natural willow habitats. A  
135 postponed diapause induction in plantations could explain why the species is more likely to  
136 initiate a second generation in this habitat. In the following year (2010), we studied the  
137 phenology and life-cycle development of first-generation broods of naturally occurring  
138 populations of *P. vulgatissima* in one willow plantation and one natural willow habitat in the  
139 field. If the beetles are able to complete development of the first generation faster in willow

140 plantations, this could explain why *P. vulgatissima* is more prone to produce a second generation  
141 in this habitat.

142  
143 From mid-July in 2009 (Julian date: 196), when the first-generation adults started to emerge in  
144 the field, we collected adult beetles once every week to estimate the proportion of females in  
145 diapause over time. The study was done at two willow plantations (*S. viminalis*) and two natural  
146 willow habitats (*S. cinerea*) located within 20 km from the Ultuna campus of the Swedish  
147 University of Agricultural Sciences in Uppsala (59°49'N, 17°40'E). The first willow plantation  
148 consisted of newly coppiced *S. viminalis* plants (first-year shoots) growing in an experimental  
149 bioenergy plantations near the campus (Weih and Nordh 2005). The second plantation consisted  
150 of more mature *S. viminalis* plants that had been left to grow for five years since the last coppice.  
151 The two natural habitats consisted of mainly mature *S. cinerea* plants growing in a mixed conifer  
152 forest, but also a few coppiced plants with re-sprouting shoots that had been cut back by a  
153 harvester machine during the previous year to prevent the trees from interfering with traffic on a  
154 nearby road. The four sites were chosen because they were easy to access and harbored similar  
155 and moderate densities of *P. vulgatissima*. Female beetles were collected from plants by the hand  
156 and beetles were brought to the laboratory and dissected under a microscope to confirm  
157 reproductive status (Dalin 2011). Collections were made on July 15, July 22, July 29, August 7,  
158 and the last collection was made on August 14 (Julian date: 226) when all (100%) females were  
159 found to be in diapause at all four study sites. The proportion of beetles in diapause was plotted  
160 over time. Due to poor emergence of adult beetles at one of the natural sites, data from the two  
161 natural habitats were pooled together in figure 1.

162



163 From mid April to October (Julian dates 102-285) in 2010, we monitored the phenology and life-  
164 cycle development of *P. vulgatissima* in one willow plantation (*S. viminalis*) and one natural  
165 willow habitat (*S. cinerea*) near Uppsala (59°53'N, 17°38'N). The *S. viminalis* plantation  
166 consisted of two-year old shoots (coppiced during the winter 2008/2009) whereas the natural  
167 habitat consisted on mature (un-coppiced) *S. cinerea* plants. The two sites were visited at least  
168 once, but most often twice, per week to estimate the number of adults, eggs and larvae of *P.*  
169 *vulgatissima* on the plants in the two habitats. The number of individuals in different  
170 developmental stages was counted during five-minute observation periods. One five-minute  
171 period was devoted to search for adult beetles on the dorsal side of leaves. Another five-minute  
172 period was devoted to search for eggs and larvae on the ventral side of leaves. The two sites were  
173 visited on the same days and observations were mainly done during days with no precipitation  
174 and minimal wind. The number of counted individuals in the different life-stages was plotted  
175 over time.

176

177 *Critical day-length response for diapause induction– genetic difference between populations or*  
178 *phenotypic plasticity to host-plant quality?*

179 The aim of this experiment was to: (1.) investigate if photoperiodic responses differ between *P.*  
180 *vulgatissima* beetles originating from plantations and natural willow habitats, and (2.) study if  
181 photoperiodic responses can be plastic in response to host-plant quality. From a previous study  
182 we know that *P. vulgatissima* respond to photoperiod for the induction of diapause. The critical  
183 day-length for the induction of diapause was estimated to be 18 hrs 10 min when the beetles  
184 were reared on greenhouse grown *S. viminalis* at 20°C in the laboratory (Dalin 2011).

185

186 Life history theory predicts that univoltine populations should have a longer CDL than bivoltine  
187 population at the same latitude and altitude (Roff 1980, Tauber et al. 1986). This is because  
188 univoltine populations need to enter diapause earlier in the season, at a time-point when day-  
189 lengths are longer, to avoid producing additional generations that may be unable to complete  
190 development to the diapausing stage before the onset of winter. Consequently, based on the  
191 observation that *P. vulgatissima* sometimes produce a second generation in plantations, we  
192 predicted that CDL should be longer in univoltine populations from natural habitats than in  
193 partially bivoltine populations from plantations.

194

195 An alternative hypothesis was that the induction of diapause can be influenced by host-plant  
196 quality. This phenotypic plasticity hypothesis predicts that CDL can be modified by host-plant  
197 quality. More specifically, we predicted that the propensity of diapause should increase when the  
198 insects were reared on a sub-optimal host-plant. To test this hypothesis, we reared the insects on  
199 two different willow species: *S. viminalis* which is frequently fed upon by *P. vulgatissima* in  
200 plantations, and *S. phylicifolia* which is a native willow growing along creeks and rivers in  
201 central Sweden but which is avoided by *P. vulgatissima* due to high concentrations of phenolic  
202 glycosides in the leaves (Kendall et al. 1996).

203

204 Stem cuttings were collected in January 2010 from *S. viminalis* (clone 78021, used in Dalin  
205 (2011)) growing in experimental plantations at the Ultuna campus, and from wild *S. phylicifolia*  
206 growing along the river Fyrisån near the campus. Stem cutting were planted in individual pots  
207 and placed in a greenhouse for shoot growth before the start of the experiment. When the plants  
208 had started to produce foliage (in February), we collected overwintering beetles from two

209 populations, one originating from a *S. viminalis* plantation (59°56'N, 17°28'E), and one from a  
210 natural *S. cinerea* stand located about 17 km east of the willow plantation. Beetles from the  
211 willow plantation originated from the same population that was used in Dalin (2011). The two *P.*  
212 *vulgatissima* populations were first reared for one generation under controlled conditions in a  
213 greenhouse (20:4 L.D; 15-20°C) to reduce potential influence of maternal effects on diapause  
214 incidence. The two populations were then reared for another generation in the experiment (from  
215 eggs to adults) on leaves of greenhouse grown *S. viminalis* and *S. phyllicifolia* under controlled  
216 conditions inside climate chambers (AB Ninolab, Upplands-Väsby, Sweden, Termaks Model  
217 KB8400L). We used a similar experimental procedure as in Dalin (2011), including four climate  
218 chambers with separate photoperiods (20:4, 19:5, 18:6 and 17:7 light:dark cycles) and constant  
219 20°C temperature. In the climate chambers, we reared the beetles in groups of 50-100 larvae  
220 inside transparent plastic containers (19x19x11cm). We used two replicate containers per  
221 photoperiod, host-plant and population treatments (16 containers in total). The containers were  
222 sealed with a mesh net over the open top to provide ventilation. The number of emerging adult  
223 beetles was counted every 2-3 days when fresh leaves were provided to ensure that larvae always  
224 had a surplus of food. Pieces of wet oasis were placed at the base of leaf petioles to provide  
225 moisture to the leaves. A layer of potting soil mixed with sand was added to the bottom of the  
226 containers to be used as pupation substrate by larvae.

227

228 Emerging adult beetles were removed and kept in separate containers provided with fresh leaves  
229 under the same experimental conditions as the beetles had been raised from eggs. The adults  
230 were allowed to feed and mate for approximately 14 days. Female beetles were then dissected  
231 under a microscope to confirm reproductive status (diapause or reproductively active).

232

233 The propensity of diapause in *P. vulgatissima* was analyzed using logistic regression (PROC  
234 GENMOD, binominal, logit; SAS Institute, 2008). Reproductive status (diapause or  
235 reproductively active) of individual female beetles was the dependent, binary response variable  
236 (1 for diapause, 0 for reproductively active). Thus, we pooled the results from the two replicate  
237 containers and treated each female as an individual observation in the analyses (Dalin, 2011).  
238 Photophase (hours of light), population origin and host-plant species were used as independent  
239 categorical variables. Logistic regressions with inverse predictions (PROC PROBIT  
240 INVERSECL, SAS Institute 2008) were used to calculate critical day-lengths ( $\pm 95\%$  confidence  
241 interval) (Dalin et al., 2010). Development time (i.e. the number of days it took for development  
242 from eggs to adult eclosion) was compared among treatments using two-way ANOVA and  
243 Tukey test for post-hoc treatment comparisons (PROC GLM, SAS Institute, 2008). The mean  
244 number of days to adult eclosion was calculated for each replicate container to be used as  
245 individual observations in the analysis.

246

#### 247 *Diapause incidence on coppiced versus mature willow plants*

248 A second laboratory experiment was conducted in 2011 to further investigate the effect of host-  
249 plant quality on diapause incidence in *P. vulgatissima*. The purpose of this experiment was to  
250 test whether diapause in adult females is reduced when the beetles are reared on leaves from  
251 previously coppiced and vigorously growing willow plants. For this experiment, we used a  
252 mixture of beetles collected from willow plantations and natural willow habitats. The beetles  
253 were exposed to three host-plant treatments: (1.) leaves from re-sprouting shoots of previously

254 coppiced *S. viminalis*, (2.) leaves from re-sprouting shoots of experimentally coppiced *S.*  
255 *cinerea*, and (3.) leaves from mature (un-coppiced) *S. cinerea* trees.

256

257 The beetles were collected in the field as eggs in May 2010. The proportion of eggs collected  
258 from plantations and natural habitats was approximately 50:50. Larvae were reared to adulthood  
259 under controlled conditions in a greenhouse to reduce maternal effects. Eggs from the second  
260 generation were then distributed between nine (3x3) rearing containers (see above) inside a  
261 climate chamber with constant 18.50 hours of light (photophase) and 20°C. One container per  
262 host-plant treatment was placed on three separate shelves (top, middle and bottom shelf) inside  
263 the chamber. The groups of containers located on different shelves were treated as blocks in the  
264 statistical analysis (described below). The specific photoperiod condition was chosen based on  
265 the previous experiment indicating that diapause incidence will vary among individuals when  
266 reared under this condition. Thus, we wanted to avoid all individuals becoming either  
267 reproductively active or in diapause.

268

269 The beetles were fed fresh leaves collected from plants in the field every 2-3 days. The coppiced  
270 plants used in the experiment had been coppiced (complete removal of shoots and branches) in  
271 the previous year. The coppiced *S. cinerea* plants were located less than five meters away from  
272 the mature *S. cinerea* to receive similar growth conditions of the two *S. cinerea* treatments. The  
273 *S. viminalis* plants were growing in experimental plantations near the Ultuna campus (see  
274 description of site above). Leaves collected in the field were immediately transported to the  
275 laboratory and fed to larvae. We used similar methods described above for the rearing and testing  
276 of reproductive status in female beetles.

277  
278 The propensity of diapause in relation to host-plant treatments was analyzed using logistic  
279 regression (PROC GENMOD, binominal, logit; SAS Institute 2008). Reproductive status of  
280 individual female beetles was again used as the dependent, binary response variable (1 for  
281 diapause, 0 for reproductively active) and host-plant treatment and block the independent  
282 categorical factors. We also scored the amount of fat-bodies in the abdomen of diapausing  
283 females as either small or large amounts. Chi-square tests were used to compare fat-bodies  
284 among host-plant treatments. In the analyses of fat-bodies, we pooled results from the three  
285 blocks. The total number of females included in the analysis of fat-bodies was 15 for *S.*  
286 *viminalis*, 50 for coppiced *S. cinerea* and 71 for mature *S. cinerea*. The size of adult females was  
287 also estimated by measuring the width of the thorax using a scale in a microscope (9x  
288 magnification lens). Data from 19-31 females per treatment were included in a one-way ANOVA  
289 (PROC GLM; SAS Institute 2008) with host-plant treatment the independent factor.

290

## 291 **Results**

### 292 *Life-cycle development and diapause induction of field populations*

293 In the field, we found that first-generation adults of *P. vulgatissima* became reproductively active  
294 if they enclosed from pupation before August in 2009 (before Julian date 205 in Fig. 1).

295 Although most beetles emerged later (around mid-August) and were in diapause, we observed  
296 mating by first-generation adults in July on recently coppiced plants of both *S. viminalis* and *S.*  
297 *cinerea* in the two habitats. However, the natural habitat mainly consisted of mature (un-  
298 coppiced) *S. cinerea* plants on which we did not observe any mating. Overall, diapause induction  
299 occurred earlier in the willow plantations with un-coppiced plants (five year-old shoots) and in

300 the natural habitat (late July), whereas first-generation adults remained reproductively active at  
301 least until early August in the recently coppiced *S. viminalis* plantation (Fig. 1).

302  
303 In the subsequent year (2010), we did not find any differences in the phenology of adult  
304 emergence from overwintering in the spring, or in the development of the first-generation broods  
305 during the summer, or in the phenology of when first-generation adults emerged from pupation,  
306 between the willow plantation and the natural habitat studied (Fig. 2). However, we found that  
307 first-generation adults produced a small partial second generation in the willow plantation by late  
308 July-August in 2010 (Julian date 225-240), which was not observed in the natural habitat (Fig.  
309 2).

310  
311 *Critical day-length responses for diapause induction— genetic difference between populations or*  
312 *phenotypic plasticity to host-plant quality?*

313 The laboratory experiment showed significant effects of photophase (hours of light) and host-  
314 plant species on diapause incidence in *P. vulgatissima* (Table 1). Fig. 3 shows that the proportion  
315 of females in diapause decreased with increasing day-length, and that a higher proportion of  
316 females entered diapause on *S. phyllicifolia*. Diapause incidence was also marginally affected by  
317 population origin ( $P = 0.049$ ), with beetles originating from the natural willow habitat having a  
318 higher diapause incidence than beetles from willow plantations. The non-significant Population x  
319 Photophase interaction suggests however that the two populations responded similarly to day-  
320 length for the induction of diapause (Table 1). We found a significant Host-plant species x  
321 Photophase interaction, indicating different day-length responses of *P. vulgatissima* on the two  
322 willow species (Table 1, Fig. 3). CDL was estimated to be 18.08 hours (95% confidence interval:

323 17.94-18.22 hrs) or 18.20 hrs (18.06-18.33) on *S. viminalis* (estimations for beetles originating  
324 from willow plantation and natural willow habitat, respectively); and 18.77 hrs (18.53-19.03) or  
325 19.03 hrs (18.66-19.53) on *S. phlyicifolia*.

326

327 Developmental time was significantly affected by day-length, but not by host-plant species or  
328 population origin (Table 2). Fig. 4 shows a significant reduction in developmental time for  
329 beetles reared under the shortest day-length treatment (17 hours of light).

330

### 331 *Diapause incidence on coppiced versus mature willow plants*

332 Diapause incidence in female *P. vulgatissima* was significantly affected by host-plant treatments  
333 ( $\chi^2 = 58.88$ , *d.f.* = 2, *P* < 0.001; Fig. 5). The proportion of females in diapause was  $95 \pm 5\%$  (*n* =  
334 75) on leaves from mature *S. cinerea* plants (means  $\pm$  standard errors calculated from three  
335 replicate rearing containers),  $60 \pm 11\%$  (*n* = 83) on coppiced *S. cinerea*, and  $39 \pm 9\%$  (*n* = 72) on  
336 coppiced *S. viminalis*. Diapause incidence was unaffected by the placement of containers within  
337 the climate chamber, as shown by the non-significant block effect ( $\chi^2 = 3.87$ , *d.f.* = 2, *P* = 0.15).

338

339 The amount of fat-bodies stored in the abdomen of diapausing females was higher in beetles  
340 reared on the two coppiced treatments: coppiced *S. viminalis* vs. mature *S. cinerea* ( $\chi^2 = 19.77$ ,  
341 *d.f.* = 1, *P* < 0.001), coppiced vs. mature *S. cinerea* ( $\chi^2 = 25.94$ , *d.f.* = 1, *P* < 0.001). No  
342 difference was found between the two coppiced treatments of *S. viminalis* and *S. cinerea* ( $\chi^2 =$   
343 1.60, *d.f.* = 1, *P* > 0.20). Host-plant treatments did not affect the size of adult females, as  
344 measured by the width of the thorax ( $F_{2, 69} = 0.69$ , *d.f.* = 2, *P* = 0.50).

345



346 **Discussion**

347 The leaf beetle *P. vulgatissima* sometimes initiate a second generation in short-rotation coppiced  
348 willow plantations in central Sweden. During 2010, we found that the beetles produced a partial  
349 second generation in a *S. viminalis* plantation but not in a nearby natural *S. cinerea* habitat. This  
350 second generation could not be explained by different phenology or development of first-  
351 generation broods between the two habitats. However, the seasonal timing of diapause was found  
352 to differ among leaf beetle populations in the field with diapause occurring 1-2 weeks later in  
353 coppiced willow plantation than in mature (un-coppiced) willow stands. A postponed (later)  
354 diapause induction could explain why the beetles sometimes initiate a second generation in  
355 plantations.

356

357 Using climate chamber experiments, we did not detect any difference in the critical day-length  
358 (CDL) response for diapause induction between beetles originating from plantations and natural  
359 habitats. However, the propensity to enter diapause was significantly affected by host-plant  
360 quality and was reduced when the beetles were reared on leaves from re-sprouting shoots of  
361 previously coppiced willow plants than when reared on leaves from mature plants. Moreover,  
362 diapause incidence was significantly reduced on the willow *S. phyllicifolia* compared with *S.*  
363 *viminalis*. The results suggest that host-plant quality influenced diapause induction in *P.*  
364 *vulgatissima*. Willow plantations are coppiced for woody biomass every 3-4 years which  
365 stimulates compensatory plant growth. The shoots of re-sprouting willows continue to elongate  
366 and produce new leaves over the course of the season whereas mature plants cease leaf  
367 production around mid-summer (Nakamura et al. 2005, P. Dalin pers. obs.). This implies that  
368 coppiced willow may provide new leaves during an extended period of time, which may support

369 the development of a second beetle generation. Although we lack information about the  
370 performance of second-generation larvae, the results suggest that the current harvesting regime,  
371 where willow plantations are coppiced every 3-4 years, can induce postponed diapause of *P.*  
372 *vulgatissima* resulting in a second generation.

373

374 The fact that host-plant conditions can affect voltinism of herbivorous insects is not new and has  
375 been documented in a number of insect species (Tauber et al. 1986, Hunter and McNeil 1997,  
376 Wedell et al. 1997, Ishihara and Ohgushi 2006, Takagi and Miyashita 2008). However, host-  
377 plant quality may influence insect voltinism both directly and indirectly (Wedell et al. 1997),  
378 although few studies have been able to separate these effects experimentally. First, host-plant  
379 quality can have a “trivial” indirect effect on insect voltinism in the field by affecting the timing  
380 of when the insects reach the critical stage for diapause induction during development. In the  
381 current study, we did not detect any difference in the development or seasonal occurrence of  
382 naturally occurring leaf beetle populations between plantations and natural habitats, although the  
383 beetles produced a second generation in the plantation. We therefore believe that we can reject  
384 the “trivial-effect hypothesis” as an explanation to why the beetles sometimes produce a second  
385 generation in plantations.

386

387 Larval host-plants may also affect the propensity of insects to enter diapause. This may either  
388 occur as a direct response to cues from the host-plant or more indirectly via altered insect growth  
389 (Wedell et al. 1997). Such plasticity in diapause threshold could prevent insects from producing  
390 extra generations on a host-plant of poor or declining quality, a situation where their offspring  
391 may fail to complete the extra generation. To our knowledge, no study has been able to confirm

392 that insects respond directly to host-plant traits for the induction of diapause. This is because  
393 diapause propensity often co-varies with insect development, such as growth rates, which also  
394 may influence the choice of developmental pathway (Hunter and McNeil 1997, Wedell et al.  
395 1997). Several studies show that insects are more likely to exhibit direct development (e.g.  
396 produce another generation) when reared on host-plants that support rapid larval development  
397 (Hunter and McNeil 1997, Wedell et al. 1997, Ishihara and Ohgushi 2006). This “growth-rate  
398 hypothesis” predicts that insects can make use of their own growth rate as a cue to predict future  
399 conditions and for choosing developmental pathways (Wedell et al. 1997). Feeding on a high  
400 quality host-plant may, for example, indicate that the focal host-plant can support rapid  
401 development not only in the present, but also in the future, which then may allow another  
402 generation to develop within the same year.

403

404 We believe that our study reveals evidence that *P. vulgatissima* responded directly to cues  
405 signaling host-plant quality for the induction of diapause. This was because we did not detect any  
406 difference in developmental rate (time to adult eclosion) between beetles reared on *S. viminalis*  
407 and *S. phylicifolia* in the laboratory experiment, although the beetles were more likely to enter  
408 diapause on the latter plant species. The study is also one of the first to describe how the critical  
409 photoperiodic response changes in response to host-plant conditions. We found that CDL was  
410 prolonged by almost an hour when the beetles were reared on the willow *S. phylicifolia*. A  
411 population CDL that is 19 hours or longer will certainly decrease the likelihood for a second  
412 generation in central Sweden. Although these results in combination suggest that we can reject  
413 the “growth-rate hypothesis” as an explanation for longer CDL on *S. phylicifolia*, it cannot be  
414 ruled out that the insects may have responded to some other internal physiological process

415 (rather than an external cue from the plant) when “choosing” developmental pathway in the  
416 experiments. We found that adult beetles contained larger amounts of fat-bodies stored in the  
417 abdomen when they had been reared on new leaves from vigorously growing willow plants than  
418 when reared on old leaves from mature plants. This suggests that the beetles gained extra  
419 resources when developing on new leaves. This is speculative, but if the beetles are unable to  
420 gain enough resources during larval development, they may choose the developmental pathway  
421 leading to diapause. However, until this is investigated more rigorously, we will reject the  
422 original “growth-rate hypothesis” in its current form because the beetles would otherwise be  
423 expected to develop faster on *S. viminalis* than on *S. phylicifolia*.

424

425 For insects that develop on the leaves of woody plants, the quality of their food often decline  
426 over the course of the summer, which may reduce the growth and survival of individuals in  
427 subsequent generations (Ishihara and Ohgushi 2006, Nylin et al. 2009). The leaves often  
428 becomes tougher and accumulate higher concentrations of quantitative defense compounds after  
429 expansion (Feeny 1970, Strong et al. 1994). Many herbivorous insects have therefore  
430 synchronized egg hatch and the occurrence of young larval stages with the seasonal timing of  
431 bud break to be able to feed on the tender new leaves in the spring that also often are more  
432 nutritious than later in the season (Feeny 1970, van Asch and Visser 2007). In fact, many  
433 herbivorous insect species feeding on woody plants are always univoltine with an obligatory  
434 diapause that prevents them from producing additional generations (Tauber et al. 1986, Tammaru  
435 et al. 2001). Although the leaf beetle *P. vulgatissima* has a facultative diapause, the species is  
436 also normally univoltine in central Sweden (Dalin 2011). The first-generation completes  
437 development to adulthood before mid August when day-degree models predict that they should

438 be able to produce another generation in central Sweden (P. Dalin unpubl. data). Thus, it seems  
439 that the seasonal climate could allow two generations in Sweden. As far as we know, the species  
440 is univoltine at least down to central Europe where they may switch to a bivoltine life-cycle. One  
441 possible reason why bivoltinism is restricted to central and southern Europe could be because the  
442 quality of willow leaves declines over the course of the summer and, thus, can only support the  
443 development of one generation per year further north. One may therefore wonder why the  
444 species has a facultative diapause that can result in additional generations as far north as in  
445 Sweden. Willow plants may, however, sometimes provide high-quality food also later in the  
446 season that may allow a second generation. Willows are known to respond to disturbances, such  
447 as wind breaks and mammalian herbivory, by producing many lateral shoots that grow  
448 vigorously. These re-sprouting plants continue to produce new leaves throughout the summer  
449 that may be of high-quality for leaf beetles also when a potential second generation is  
450 developing.

451  
452 In summary, the results reveal that the leaf beetle *P. vulgatissima* has a facultative diapause that  
453 is influenced by both photoperiod and host-plant quality. We believe that this is the first study to  
454 confirm that herbivorous insects can respond directly to host-plant quality for the induction of  
455 diapause. This can allow the insects to produce extra insect generations under certain  
456 circumstances, such as in response to a sudden but unpredictable availability of high-quality food  
457 sources. It remains however to be investigated precisely what type of plant signal or cue the  
458 insects respond to for the induction of diapause.

459

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464 Eriksson for technical support and Xiao-Ping Wang for suggestions on experimental design.

465

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541

542

543 Table 1. Results of logistic regression (binominal, logit, type 3) investigating the effects of host-  
 544 plant species, population origin and hours of light (photophase) on diapause incidence in two  
 545 populations of *Phratora vulgatissima* originating from willow plantations and natural willow  
 546 habitats in central Sweden (59°56'N latitude).

Effect	$\chi^2$	<i>d.f.</i>	<i>P</i>
Host-plant species (Host)	35.1	1	< 0.001
Population origin (Pop)	3.9	1	0.049
Hours of light (Photophase)	425.2	3	< 0.001
Host x Photophase	31.2	3	< 0.001
Pop x Photophase	3.2	3	0.359
Host x Pop	0.4	1	0.511

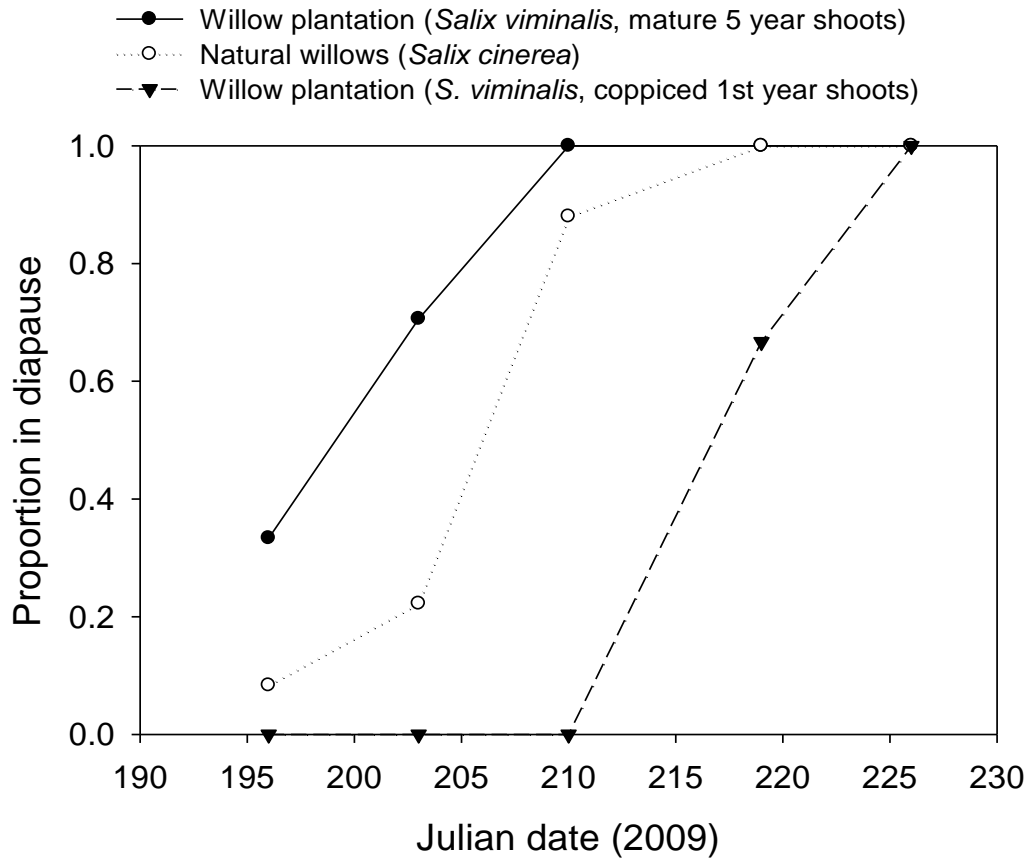
547  
 548

549 Table 2. Results of ANOVA investigating the effects of host-plant species, population origin and  
 550 hours of light (photophase) on development time (days) from eggs to adult in two populations of  
 551 *Phratora vulgatissima* origination from willow plantations and natural willow habitat in central  
 552 Sweden (59°56'N latitude).

Effect	<i>MS</i>	<i>d.f.</i>	<i>F</i>	<i>P</i>
Host-plant species (Host)	0.1	1	0.2	0.699
Population origin (Pop)	1.1	1	1.4	0.255
Hours of light (Photophase)	37.7	3	46.2	< 0.001
Host x Photophase.	0.2	3	0.2	0.926
Pop x Photophase	0.5	3	0.6	0.647
Host x Pop	0.5	1	0.6	0.443

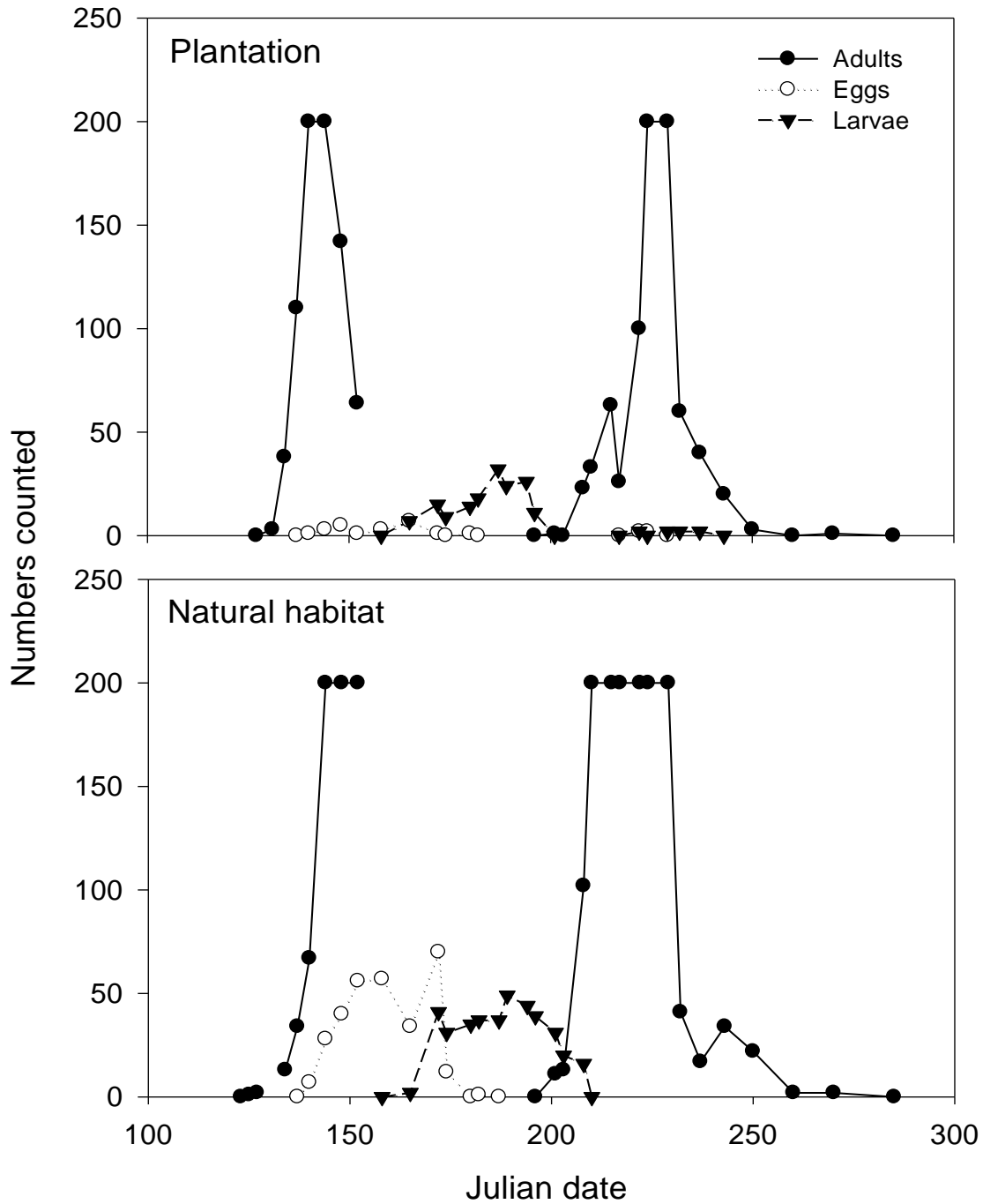
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554 Fig. 1. Field diapause induction of first-generation adult females of *Phratra vulgatissima* on *S.*  
555 *cinerea* in natural habitats and on *S. viminalis* in willow plantations in 2009.



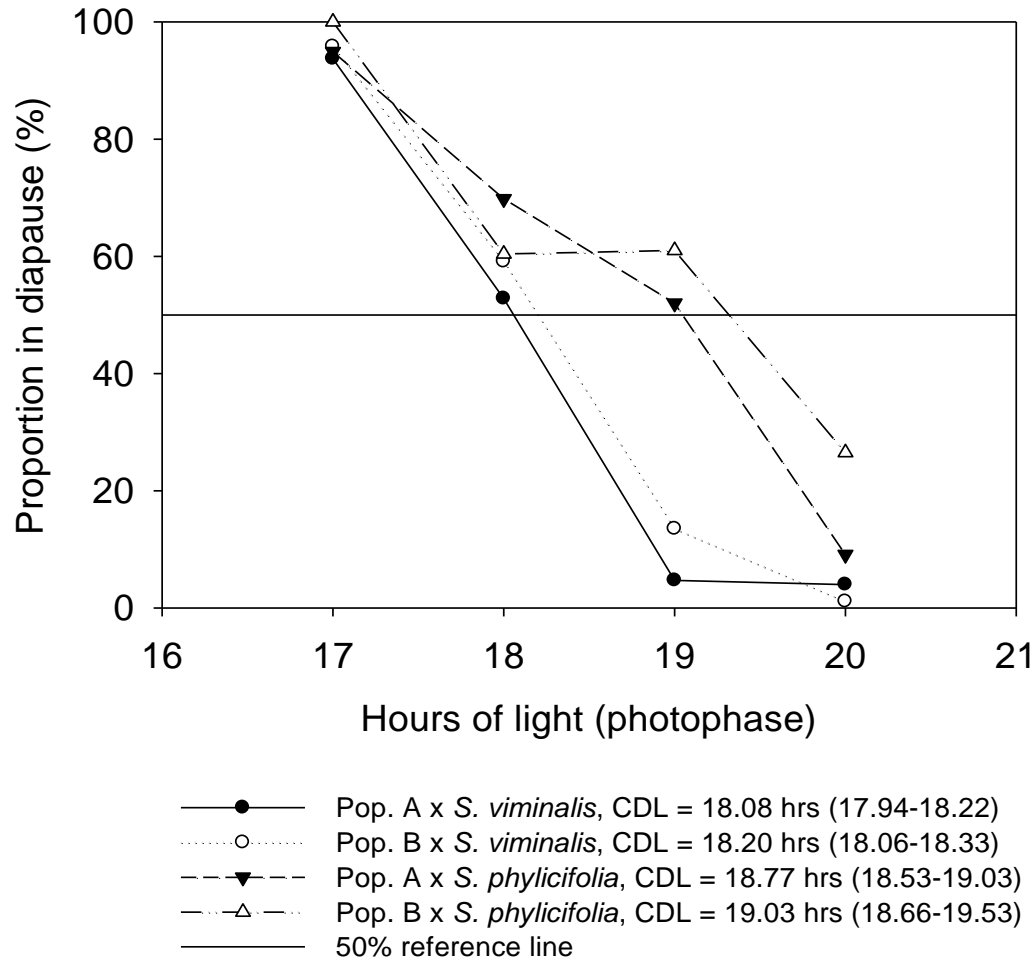
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558 Fig. 2. Phenology and life-cycle development of the leaf beetle *Phratora vulgatissima* in a  
559 willow plantation (*Salix viminalis*) and a natural willow habitat (*S. cinerea*) during 2010. The  
560 beetles produced a partial second generation in the plantation (Julian dates 225-240).



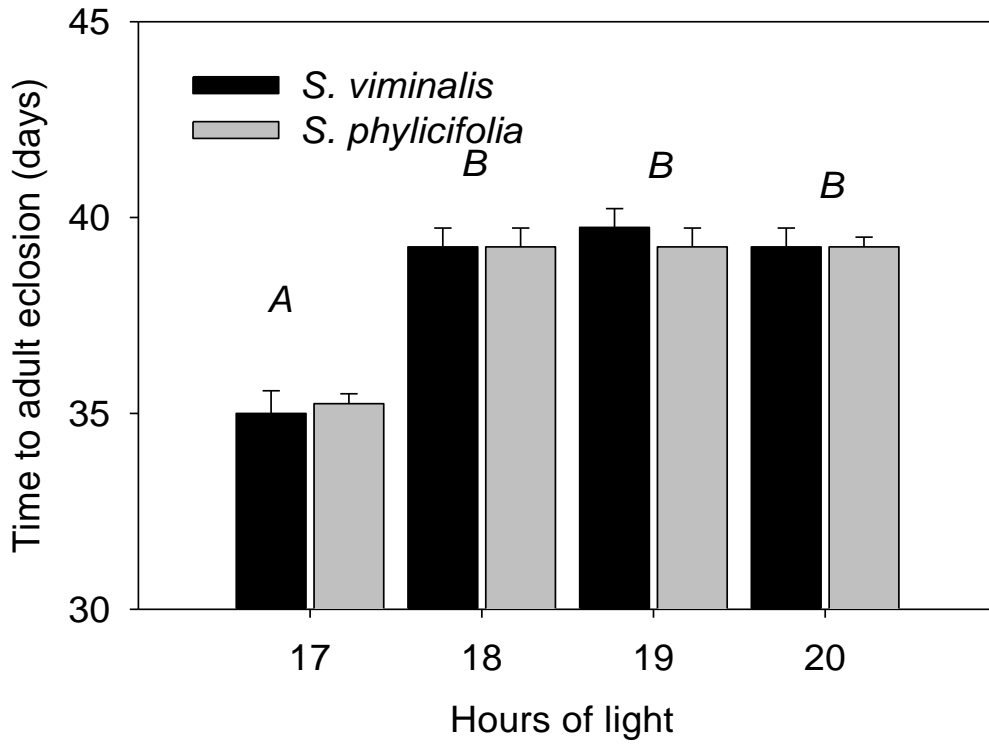
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563 Fig. 3. Diapause incidence of two populations of *Phratora vulgatissima* originating from the  
 564 same latitude (59°56'N), reared on greenhouse-grown plants of *Salix viminalis* (circles) and *S.*  
 565 *phylicifolia* (triangles), under four day-length (hours of light) treatments and constant 20°C. Pop.  
 566 A (filled symbols) represent beetles originating from a willow plantation (*S. viminalis*), whereas  
 567 Pop. B (open symbols) represents beetles from a natural willow habitat (*S. cinerea*).



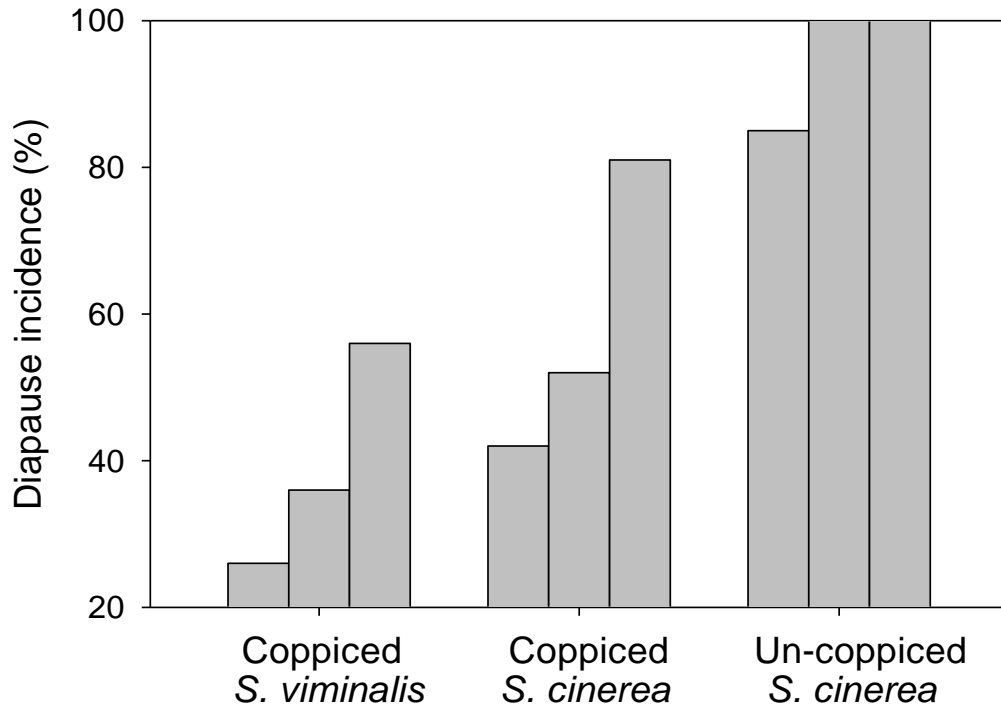
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570 Fig. 4. Developmental time from egg to adult of *Phratora vulgatissima* in relation to day-length  
571 (hours of light) when reared on *Salix viminalis* and *S. phylicifolia* at 20°C. Different superscripts  
572 represent significant different means among day-length treatments, as revealed by Tukey tests.



573

574 Fig. 5. Diapause incidence of *Phratora vulgatissima* when reared on leaves from three host-plant  
575 treatments: previously coppiced *Salix viminalis*, previously coppiced *S. cinerea*, and mature (un-  
576 coppiced) *S. cinerea* trees in the field. The figure presents the results from three replicate rearing  
577 containers per treatment with groups of larvae reared under controlled conditions (18.5 hours of  
578 light, 20°C) in a climate chamber.



579