1   TITLE: **Sex-linked inheritance of diapause in the Green-veined White, Pieris napi**

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3   RUNNING TITLE: Sex-linked inheritance of diapause

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5   Peter Pruisscher[1]; Helena Larsdotter-Mellström[1,2]; Sören Nylin[1]; Christopher W. Wheat[1]; and

6   Karl Gotthard[1]

7

8   1: Department of Zoology, Stockholm University, Svante Arrhenius väg 18b, S-106 91

9   Stockholm, Sweden

10   2: Centre for Evolutionary Biology, The University of Western Australia (M092), 35 Stirling

11   Highway, Crawley WA 6009, Australia

12

13   peter.pruisscher@zoologi.su.se

14   helena.mellstrom@uwa.edu.au

15   soren.nylin@zoologi.su.se

16   chris.wheat@zoologi.su.se

17   karl.gotthard@zoologi.su.se

18

19   Corresponding author: Peter Pruisscher, Department of Zoology, Stockholm University, Svante

20   Arrhenius väg 18b, S-106 91 Stockholm, Sweden, peter.pruisscher@zoologi.su.se,

21   orcid.org/0000-0002-6987-5839

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ABSTRACT (max 250 words)

Many temperate insects survive harsh environmental conditions, such as winter, by entering a state of developmental arrest. This diapause state is predominantly induced by photoperiod. The photoperiod varies with latitude and has led to local adaptation in the photoperiodic induction of diapause in many insects. In order to understand the rapid evolution of the photoperiodic threshold it is important to investigate and understand the underlying genetic mechanisms. In this paper the genetic basis of photoperiodic diapause induction is investigated in the Green-veined White butterfly *Pieris napi*, by assaying diapause induction in a range of conditions for a Swedish and Spanish population. Furthermore, the inheritance of diapause induction was assessed in reciprocal F1 and F2 hybrid crosses between the two populations. The southern population showed a highly dynamic response to photoperiod, while the northern populations showed a high incidence of diapause, regardless of photoperiod. The hybrid crosses revealed a very strongly sex-linked decision of diapause, and a diapause incidence that was highly dependent on photoperiod, revealing the importance of assaying a range of conditions in diapause inheritance studies. The results indicate a strongly heritable diapause induction with a major component on the Z-chromosome, as well as a minor effect of the autosomal background.

Keywords (max 6): photoperiodic response, diapause induction, inheritance, Lepidoptera, sex-linked

INTRODUCTION (Full paper max 7500 words)

Life cycle regulation is vital for organisms that occur in seasonal habitats. In temperate areas, organisms that do not migrate must possess adaptations that allow them to survive long periods of harsh conditions such as winter. Most temperate insects persist under adverse conditions by entering a state of developmental arrest called diapause, which is associated with physiological changes related to accumulation of energy and increased cold tolerance (Tauber & Tauber 1976; Tauber *et al.* 1986; Pullin *et al.* 1991; Koštál 2006; Hahn & Denlinger 2010). In many species diapause is induced at a given life stage regardless of environmental input (Tauber *et al.* 1986) and this obligate diapause typically results in a life cycle with one generation per year. However, it is also very common that temperate insects have a facultative diapause that allows them to produce several directly developing generation per year with only the last generation entering diapause before the arrival of harsh conditions (Lees 1955). In these insects, diapause is an alternative developmental pathway that is initiated in response to seasonal cues in advance of the onset of adverse conditions, the most common of which are temperature and photoperiod (Hahn & Denlinger 2010). This plastic induction of alternative developmental pathways represents an important life history adaptation in temperate insects.

59

60 Photoperiod, defined as the hours of light in a day, is a reliable environmental cue that is

61 consistent between seasons and years, and consequently is the predominant cue for initiating

62 diapause in temperate environments (Tauber *et al.* 1986). The photoperiod varies with latitude

63 and this has led to local adaptation in the photoperiodic induction of diapause (Bradshaw

64 1976). Moreover, as the length of the favourable season typically is decreasing from south to

65 north many species show latitudinal variation in the number of generation produced per year,

66 expressed as variation in voltinism (Levy *et al.* 2015). These two factors lead to variation in the

67 photoperiodic threshold along latitudes, most notably for species in which diapause is

68 facultative. Common garden laboratory studies have demonstrated that these population

69 differences in voltinism typically are due to heritable variation in the photoperiodic threshold

70 for diapause induction (Bradshaw 1976; Kimura 1988; Hahn & Denlinger 2010; Paolucci *et al.*

71 2013; Levy *et al.* 2015; Aalberg Haugen & Gotthard 2015).

72

73 Diapause induction exhibits strong patterns of local adaptation and appears to evolve rapidly in

74 response to climate change (Bradshaw & Holzapfel 2001), and in invasive species that

75 experience novel conditions (Gomi 2007; Urbanski *et al.* 2012). This rapid response is a

76 consequence of strong selection on life cycle regulation, but may also be influenced by the

77 explicit genetic architecture of the trait. Evolution of a trait can be governed by an oligogenic

78 architecture where selection acts on few genes with large effect sizes (Roff 1996), or

79 alternatively a polygenic architecture with an infinitesimal number of genes of small effect sizes.

80 As the photoperiodic threshold in diapause induction in general seem to follow a continuous

81 gradient over latitude, it is possible that the latter architecture is most likely for diapause

82 induction. Furthermore, the location of the loci on the genome has an effect; selection is able to

83 act more strongly on beneficial mutations occurring on sex-chromosomes, as recessive

84 mutations are exposed to selection in the heterogametic sex in comparison to recessive loci

85 residing on autosomes, where they remain hidden from selection unless two parents carrying a

86 recessive locus pass it on to their offspring (Charlesworth *et al.* 1987). Thus, selection can act

87 upon sex-linked variation faster than the autosome, and this faster X-effect of sex-linked

88 mutations allows for them to spread through a population faster than mutations present on

89 autosomes, causing potentially rapid evolution of sex-linked traits and population divergence

90 (Meisel & Connallon 2013). As many traits are disproportionally sex-linked in Lepidoptera

91 (Sperling 1994), this can be a potentially powerful force in shaping the genetic architecture of

92 diapause induction. But, in order to understand the rapid evolution of the photoperiodic

93 threshold it is important to investigate and understand the underlying genetic mechanisms.

94

95 The inheritance of diapause induction has been studied in many species through reciprocal
96 crossing of divergent populations and species in an attempt to understand its underlying
97 genetic architecture. Evidence from crosses suggests different genetic architectures responsible
98 for adaptive clines in photoperiodic diapause induction, expressed as single locus Mendelian
99 inheritance (Doležel *et al.* 2005; Suwa & Gotoh 2006; Han & Denlinger 2009; Kawakami *et al.*
100 2010), polygenic or oligogenic inheritance with incomplete dominance (McCoy *et al.* 1968;
101 Kurahashi & Ohtaki 1977; Sims 1983; Söderlind & Nylin 2011; Raina *et al.* 2011; Xia *et al.* 2012;
102 Chen *et al.* 2012; Lehmann *et al.* 2016), and in several species there is evidence of oligogenic
103 inheritance with a clear sex-linked component (Lumme & Keränen 1978; Kimura & Yoshida
104 1995; Ikten *et al.* 2011; Chen *et al.* 2014). The inheritance of diapause induction can also be
105 modified by other components, such as maternal effects (McWatters & Saunders 1997). Recent
106 papers have also revealed strong gene by environment interaction effects, where inheritance
107 patterns and dominance are dependent on which photoperiod insects are reared in (Fu *et al.*
108 2015; Xiao *et al.* 2015). Fu *et al.* (2015) argue this would ensure that the right developmental
109 pathway is chosen even if there would be gene flow between populations. The different modes
110 of inheritance of diapause induction show the complexity and the need for further study to
111 understand this phenotype.
112
113 In this study we investigate the inheritance of diapause induction in the Green-veined White
114 butterfly *Pieris napi* (Lepidoptera, Pieridae), which enters diapause in the pupal stage and uses
115 photoperiod as the predominant cue for regulating diapause induction (Lees & Archer 1980;
116 Hahn & Denlinger 2010; Kivelä *et al.* 2015). Using two distinct populations, one that
117 facultatively induces diapause to produce between two and three generations per year, and one
118 northern population that is univoltine and shows no direct development in the field, we aimed
119 to answer two questions. First, what is the photoperiodic response of these natural
120 populations? To answer this, we characterized the diapause incidence of these two populations
121 under different photoperiods in order to document the natural variation that is present in this
122 trait, and to investigate whether the univoltine population has retained the capacity to develop
123 directly. Second, what is the mode of inheritance of diapause induction? To address this, we
124 generated reciprocal F1 hybrid crosses and assayed them for their diapause incidence under
125 different environmental conditions to study the mode of inheritance. In addition, we generated
126 four F2 hybrid backcrosses where F1 hybrids were crossed back to the northern population,
127 and assayed at a single condition to further investigate the inheritance of diapause induction.
128
129 **METHODS**
130

4

131   In short, wild-caught Abisko, Sweden (NxN) and Barcelona, Spain (SxS) populations were reared

132   under six different photoperiods to assay diapause incidences in 2013. An independent set of

133   Abisko individuals was reared into diapause and crossed with a newly caught population

134   sample of Barcelona in 2014 to generate F1 hybrids under two environmental conditions, as

135   well as produce another generation of within-population samples. In 2015, the F1 hybrids and

136   pure population samples were used to generate F2 backcrosses, as well as pure population

137   controls. Furthermore, an independent set of F1 hybrids was generated in two environmental

138   conditions from newly caught Barcelona and Abisko individuals to further characterize the

139   diapause response. The experimental setup is described in detail below.

140

141   Mated *P. napi* adult females were collected in summer 2013 from Spain (Barcelona, 41°N,

142   hereafter designated as southern), and northern Sweden (two adjacent localities Abisko and

143   Kiruna, 68°N, hereafter designated as northern or Abisko). Females were kept individually in

144   one-litre cups where they were allowed to lay eggs on *Alliaria petiolata*. The southern

145   population was caught earlier in the season and was reared into direct development for one

146   generation in order to synchronize with the northern population. Offspring of both populations

147   were fed a mixture of wild *A. petiolata* and *Armoracia rusticana* and randomly assigned to one of

148   six treatments of LD 8:16, LD 11:13, LD 14:10, LD 17:7, LD 20:4, or LD 23:1 at 20°C to estimate

149   the effect of photoperiod on diapause incidence. Total sample and family sizes are recorded in

150   Table 1.

151

152   An independent sample of northern females (Abisko, Sweden 68°N) was also collected in 2013,

153   and again from the southern population (Barcelona, Spain, 41°N) in summer 2014. Eggs and

154   offspring were generated as described above. Larvae of the 2013 northern population were

155   reared under continuous light (LD 24:0, 23°C) and individuals that entered diapause were

156   stored in cold conditions until the following year to synchronize with the 2014 southern

157   individuals. This design created one generation of selection for high diapause incidence in order

158   to reduce polymorphism in the diapause response of the northern population used in the

159   subsequent crosses, as the initial survey of the pure populations showed some incidence of

160   direct development under these extreme conditions in the northern population (~25%, data not

161   shown).

162

163   Offspring of each line were crossed to generate southern SxS (crosses are designated female

164   first, then male) and northern NxN offspring and reared under LD 8:16 at 20°C for use in

165   subsequent crosses. Adults were also used to generate two types of F1 hybrids: SxN and NxS.

166   Hybrid offspring were reared under a split brood design, where half of the individuals were

167 reared for assessing diapause induction under LD 23:1 and 20°C, and the other half were reared

168 under LD 8:16 and 17°C to ensure diapause development. Preliminary analysis showed a very

169 asymmetric response on these F1 hybrids and indicated the need for an extra experiment to

170 properly test any Z-linked influence on diapause induction. Adult females were therefore

171 collected again from the same populations in summer 2015, and their offspring were reared

172 into diapause under LD 8:16, 17°C. In the following spring SxN and NxS hybrids were generated

173 and reared under LD 18:6, 23°C, as well as LD 12:12, 17°C. These conditions were selected to

174 further explore in which conditions offspring from the crosses entered diapause.

175

176 Individuals from the initial F1 cross in LD 8:16 at 20°C were taken out of diapause conditions

177 following spring 2015. Eclosing adults were mated in order to generate four reciprocal F2

178 backcrosses to the northern population (NxNS, NSxN, NxSN and SNxN) while also generating

179 SxS and NxN crosses to control for rearing effects. Larvae were reared in groups of ± 100

180 individuals synchronized by two laying days in L40xW30xH50 cm Pyjama cages, while being fed

181 ad-lib *A. petiolata* and *A. rusticana.* Families within and between crosses were randomly

182 distributed in cages placed in a climate controlled room at LD 23:1 and 20°C. In Lepidoptera,

183 females are the heterogametic sex and females exhibit no recombination (Traut & Marec 1997;

184 Sahara *et al.* 2012). Therefore, it is only in crosses with male hybrids (i.e. NxNS and NxSN) that

185 there will be recombination between the northern and southern chromosomes and any

186 potential Z-linked effects will be most apparent, allowing an assessment of whether diapause

187 incidence is inherited in a sex-linked manner or governed by additive autosomal elements.

188 Consequently, sample sizes were doubled in the NxNS and NxSN crosses compared with cross

189 with female hybrids, i.e. NSxN and SNxN. For this and all other experiments described here

190 developmental pathway (direct or diapause) was recorded for each individual as well as family

191 of origin. All cups and cages were checked daily, with newly pupated individuals collected and

192 placed in individual cups with a unique identifier. The directly developing individuals eclosed

193 within 2-3 weeks in their individual cups, while individuals that did not show any signs of

194 development after three weeks were deemed to be in diapause. Total sample and family sizes

195 for the F1 and F2 crosses are recorded in Table 2.

196

197 All statistical tests were performed using JMP v12.01 (SAS). To compare proportions of

198 diapausing versus direct developing individuals in the pure populations, a generalized linear

199 mixed model was used, assuming a binomial distribution (diapause or not), and a logit link

200 function. Photoperiod, population and their interaction were used as explanatory variables.

201 Family was added as random factor.

202

For the F1 hybrid crosses diapause incidence at each photoperiod was compared to expected diapause incidences under a Z-linked as well as autosomal additive genetic architecture of diapause induction. No dominance was assumed. Estimates were derived from the pure populations and compared by Fisher's exact test. If the diapause decision is completely sex-linked, the hemizygous females in the F1 hybrids should show a developmental choice in concordance with the paternal population of origin, while the males heterozygous for the Z-chromosome should show an intermediate response. In the case of additive autosomal inheritance, all hybrid individuals are expected to behave similarly and intermediate to the pure populations. The F1 hybrids reared under LD 18:6 did not have a corresponding sampling point in the pure populations and instead was compared to the average between the LD 17:7 and 20:4 points.

To test whether diapause incidences between the F2 crosses were different from each other, a generalized linear mixed model was used, assuming a binomial distribution (diapause or not), and a logit link function. Sex, Cross, and their interaction were used as explanatory variables, while Family and Cage number were added as random factors. To further test the mode of inheritance of diapause induction in the F2 crosses, Fisher's Exact tests were used to compare the observed diapause incidences to expected diapause incidences derived from the simultaneously reared pure populations. Again, we tested any deviations from expectation of a completely Z-linked, as well as a completely additive autosomal inheritance pattern. The expected incidence was derived from the observed diapause incidence in the pure populations that were reared at the same time. For the females, who only have one copy of the Z-chromosome, an individual with a Southern Z chromosome was expected to show 100% direct development, while a female with a Northern Z chromosome was expected to yield a 90% probability to go into diapause. The males have two copies of the Z chromosome, and if individuals had two southern copies of the Z-chromosome the expectation was 100% direct development. When males had two copies of the northern Z-chromosome, they were expected to have an 87% probability of entering diapause. Males that were heterozygous for the Z chromosome were expected to show a diapause development probability of $(87+0)/2=43.5\%$, when the effect is additive, and no dominance is assumed. Numbers were rounded up to whole integers for the chi-square test. The autosomal background in the crosses is expected on average to be 75% northern, and 25% southern. Under the assumption that the southern genotype leads to direct development, and the northern to 87%-90% diapause incidence, the expected diapause incidence is 67.5% in the females, and 65% diapause for the males, and no difference in proportion between the crosses. The different reciprocal F2 backcrosses will give further insight into sex-linkage, as the average autosomal backgrounds of the F2 backcrosses

239   are identical, but interact with different proportions of northern and southern sex-
240   chromosomes.
241
242   **RESULTS**
243   In order to understand the inheritance of diapause, we started by quantifying the natural
244   variation of photoperiodic induction in two natural populations of *P. napi*. Diapause incidence
245   was characterized for six different photoperiods at 20°C, in which the northern population
246   (Abisko, Sweden) showed a >90% incidence of diapause in all of the photoperiods examined
247   (Figure 1A). Diapause incidences for the southern population (Barcelona, Spain) differed
248   depending on photoperiod, showing an 82% diapause incidence at LD 8:16, with a steep
249   decrease until LD 14:10 where there was >85% direct development (Figure 1A). This was
250   corroborated by a GLMM, showing a significant effect of Population, Photoperiod, and their
251   interaction. (Full Model: χ^2=328.34, DF=3, p>0.001; Population: χ^2=36.83, DF=1, p<0.001;
252   Photoperiod: χ^2=242.51, DF=1, p<0.001; Population*Photoperiod: χ^2=10.51, DF=1, p=0.001).
253
254   *Inheritance of diapause induction in F1 hybrid crosses*
255   We examined the inheritance of diapause induction of F1 hybrids in four different conditions
256   (Figure 1B). F1 crosses reared in LD 8:16, 17°C showed high diapause incidences (NxS 100%,
257   SxN >98%), as well as in LD 12:12, 17°C (NxS >99%, SxN 100%). At LD 18:6, 23°C the NxS and
258   SxN males exhibited an intermediate diapause induction at 40-60%. Females of the NxS cross
259   showed 8.3% diapause and females of the SxN cross showed 100% diapause. At LD 23:1, 20°C
260   both the NxS and SxN crosses exhibited 0% diapause incidence.
261
262   We further examined the mode of inheritance of diapause induction in the F1 crosses reared at
263   LD 18:6 using Fisher's Exact Tests. Expectations of Z-linked diapause frequencies and expected
264   diapause incidences under autosomal control were calculated based on the combined diapause
265   incidences of the pure populations reared at LD 17:7, and LD 20:4. Diapause incidences for the
266   F1 females could not be distinguished from an expected Z-linked diapause incidence (NxS
267   p=0.7101, SxN p=0.239), and were significantly different from an additive autosomal
268   expectation (NxS p<0.001, SxN p<0.001). Observed male diapause incidences could neither be
269   distinguished from Z-linked nor autosomally controlled induction of diapause (NxS p=0.37, SxN
270   p=0.41), as the expected diapause incidences were identical between the two modes of
271   inheritance.
272
273   *Inheritance of diapause induction in the F2 hybrid crosses*

274 We also examined the mode of inheritance using F2 backcrosses, by crossing the reciprocal F1

275 hybrids back to the northern population. There was a large difference between the four

276 reciprocal F2 backcrosses, both within and between the sexes (Table 2). In the GLMM on the

277 backcrosses there was a highly significant effect of Cross and the interaction between Cross and

278 Sex (Full Model: χ^2=135.31, DF=7, p>0.001; Cross: χ^2=76.93, DF=3, p<0.001; Sex: χ^2=1.67, DF=1,

279 p=0.196; Cross*Sex: χ^2=40.53, DF=3, p<0.001).

280

281 Expectations of Z-linked, as well as expected diapause frequencies under additive autosomal

282 control were calculated using the diapause incidences of the pure controls reared in the same

283 conditions. Diapause incidence was 0% in the pure southern cross, and 90% for females and

284 87% for males in the northern cross (Table 2). For the backcrosses, in females the diapause

285 incidences adhered to the expected Z-linked distribution in three crosses (Table 3, Figure 2A).

286 However, in the NxNS cross the proportion of diapausing animals was significantly different

287 from a completely Z-linked inheritance and instead exhibited a higher diapause incidence close

288 to the autosomal expectation (Table 3). Diapause incidences in males followed the expectation

289 of a Z-linked inheritance for the SNxN cross (Table 3, Figure 2B). The NxSN cross adhered to the

290 expected autosomal and Z-linked distributions, which were identical and could not be

291 disentangled. The NxNS cross exhibited neither an autosomal nor a Z-linked expectation, and

292 the NSxN cross adhered to the autosomal expectation. Crosses not showing Z-linked

293 inheritances in general exhibited an excess of diapausing individuals.

294

295 **DISCUSSION**

296 Here the photoperiodic induction of diapause in two natural populations was investigated,

297 along with their F1 and F2 hybrid backcrosses. We found a difference in the incidence of

298 diapause of the natural populations that is dependent on the photoperiod experienced by the

299 insects. The southern population (Barcelona, Spain) showed a highly dynamic response to

300 photoperiod, while the northern population (Abisko, Sweden) showed a strong propensity to

301 enter diapause that was independent of the photoperiod in which the population was reared. F1

302 hybrids showed a strong response to photoperiod and indicated a highly sex-linked inheritance

303 of diapause induction. F2 backcrosses confirmed the pattern of sex-linked inheritance, and

304 revealed that a single locus, or several tightly linked loci, on the Z-chromosome could explain

305 the inheritance pattern of most of the crosses. We discuss the general findings in more detail

306 below.

307

308 The dynamic response to photoperiod of the southern population was consistent with the two

309 to three generations per year observed in the field, where there is strong selection pressure to

310 enter the correct developmental pathway over the course of the year as conditions change. The
311 northern population occurs above the Arctic Circle and is univoltine in the field, as the season
312 length generally does not permit a second generation. This lack of expression of the direct
313 developmental pathway creates the possibility for relaxed selection on the photoperiodic
314 threshold, and may eventually lead to the evolution of obligate diapause (Van Dyken & Wade
315 2010; Aalberg Haugen & Gotthard 2015). However, the fact that there is a subset of individuals
316 in the northern population developing directly does show that the capacity to do so is not lost in
317 this population, which may reflect the likely colonization history of this high latitude in the early
318 Holocene.
319
320 In the F1 hybrid crosses at short day lengths all individuals entered diapause, and under long
321 day lengths all F1 hybrids developed directly, regardless of the direction of the cross.
322 Interestingly, there is evidence of strong sex-linked inheritance of diapause under the more
323 intermediate photoperiod LD 18:6. The differences in the photoperiodic response of the F1
324 hybrids suggest a strong gene by environment interaction, where the diapause decision is
325 highly dependent on the photoperiod. At LD 18:6 more than 90% of females of the NxS cross,
326 which carry a southern Z chromosome, developed directly, and SxN females that instead carry a
327 northern Z chromosome entered the diapause pathway. Males, which are homogametic and
328 heterozygous for fixed differences between the populations at the Z chromosome, exhibited a
329 more intermediate diapause incidence with a tendency to follow the paternal phenotype. This
330 paternal effect has been observed in several other insect species, and it is as of yet unknown
331 what the reason is (Fu *et al.* 2015; Lehmann *et al.* 2016).
332
333 Diapause incidence in the F2 crosses was strongly affected by the direction of the cross. There is
334 a strong sex-linked component, as in a majority of the crosses diapause incidence could be
335 explained by a single Z-linked factor. This is in concordance with the patterns observed in the
336 F1 hybrids. As there is variation in diapause incidence between families and crosses that carry
337 the same overall proportion of northern and southern alleles in their autosomal and Z
338 chromosome background, it is likely that there are more genes involved, and there is genetic
339 variation in these genes. It appears that neither in the F1 nor in the F2 crosses there is evidence
340 of a major effect linked to the mitochondrial makeup of individuals, as then diapause would
341 have been expected to be inherited maternally, with no difference between sex in the offspring.
342
343 Studies that have investigated the inheritance of diapause have found vastly different patterns
344 of inheritance, and inferred characteristics such as dominance and preliminary expectations
345 about the architecture of the trait, although most often these studies only investigate one

particular photoperiod. It has become clear from recent studies (Fu *et al.* 2015; Xiao *et al.* 2015), and the results of the F1 hybrids presented here that the entire reaction norm should be taken into account. Considering only the initial results found in the long day photoperiods would have lead us to incorrectly conclude that direct development is dominant over diapause, and conversely that diapause is dominant over direct development had we only considered the short-day photoperiods. Rather, the inheritance of diapause as affected by hybridization appears to influence the position of the photoperiodic threshold. The combination of the northern and southern alleles in the F1 have moved the critical photoperiod, which in the southern population occurs around LD12:12, to an intermediate point at LD18:6, in between the reaction norms of the original populations. The autosomal background appears to be largely additive, as it represents an intermediate response, corroborated by the diapause incidence in the male hybrids. In the females, there is a very strong added effect of the Z-chromosome around the critical photoperiod. This indicates that the Z-chromosome contains a genetic factor with a major effect on diapause that, when homozygous, is dominant over the autosomal background. There appears to be a disproportionately large number of Z-linked linked traits in Lepidoptera, ranging from wing colouration to egg weight (Sperling 1994). Furthermore several other cases of Z-linked diapause induction have been discovered (Rockey *et al.* 1987; Hagen & Scriber 1989; Chen *et al.* 2014). Other diapause traits, such as termination and post-diapause development and post-diapause development have also been associated with the Z-chromosome (Levy *et al.* 2015). It remains unknown why there is such a strong tendency of these traits, which have a major impact on the life cycle, to be sex-linked. It has been speculated that this pattern might relate to sexual conflict over optimal life cycles {Nylin:1994ww, Soderlind:2011iq}, but it could instead, or also, be a result of the more rapid fixation of alleles on sex chromosomes when populations adapt to local conditions and diverge {Charlesworth:1987kq, Meisel:2013bk}.

Overall, our results demonstrate that there are one or several loci on the Z chromosome that influence the tendency to enter diapause in *P. napi.* These loci show a strong genotype by environment interaction in the F1 crosses and it seems likely that they influence the position of the entire photoperiodic threshold for diapause induction. In addition, the results suggest that there are also effects of loci on the autosomes as in several of the crosses we see significant deviations from a pure sex-linked inheritance pattern. Performing population crosses in *Pieris napi* is the first step in unravelling the mechanism underlying photoperiodic induction in this species, as well as improving the understanding of this phenotype as a whole. Future genomic analyses on these crosses will be able to link genetic variation in specific genes to the phenotype studied here.

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FIGURES AND TABLES
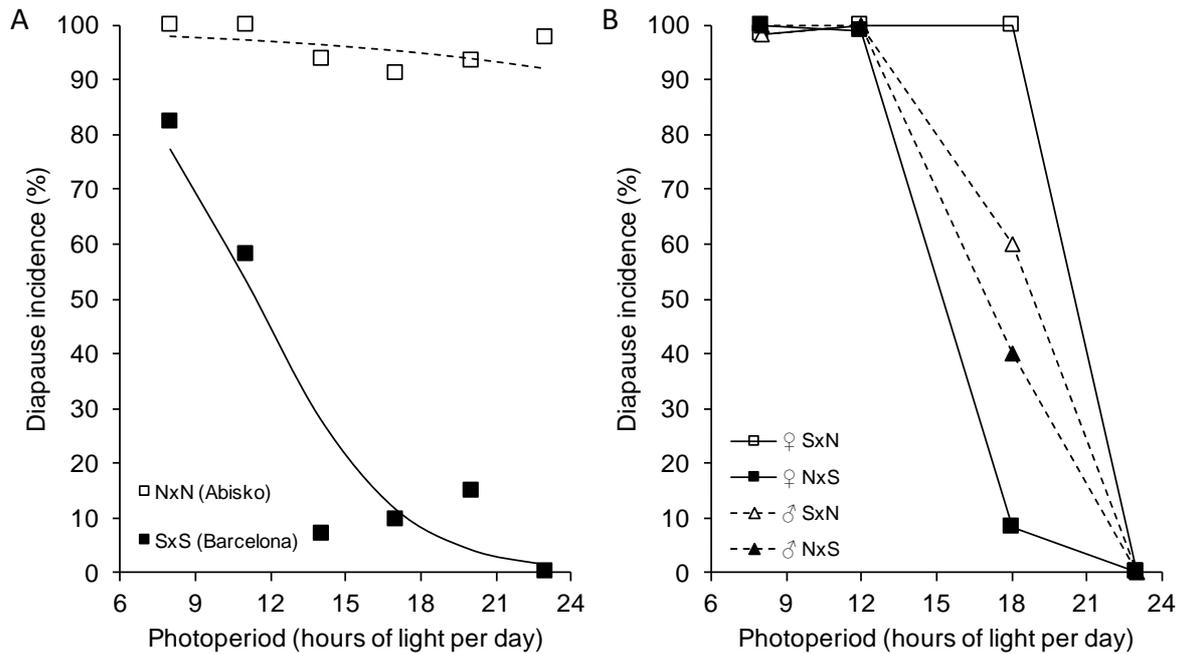
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494 Figure 1 – (A): Diapause incidence for the pure Spanish and Swedish populations measured in

495 six different photoperiods at 20°C. Solid line and squares represent the mean diapause

496 incidences and logistic fit for the SxS (Barcelona, Spain) population. Dotted line and open

497 squares represent the northern population (Abisko, Sweden). Lines represent the GLMM model

498 fit. (B): Diapause incidences for the F1 hybrid crosses. Open squares and solid line represent

499 SxN females, and open triangles and dotted line represent SxN males. Solid squares and solid

500 line represent NxS females, and filled triangles and dotted line represent NxS males. At the

501 lower photoperiods, all crosses entered diapause regardless of direction, and at the highest

502 photoperiod measured all crosses developed directly. However, at LD 18:6 strong sex-linked

503 induction was exhibited in the females, while the males showed an intermediate phenotype

504 with a slight skew depending on paternal phenotype. Note that for the F1 hybrids LD 8:16 and

505 LD 12:12 crosses were reared at 17°C, LD 18:6 at 23°C and LD 23:1 at 20°C.

506
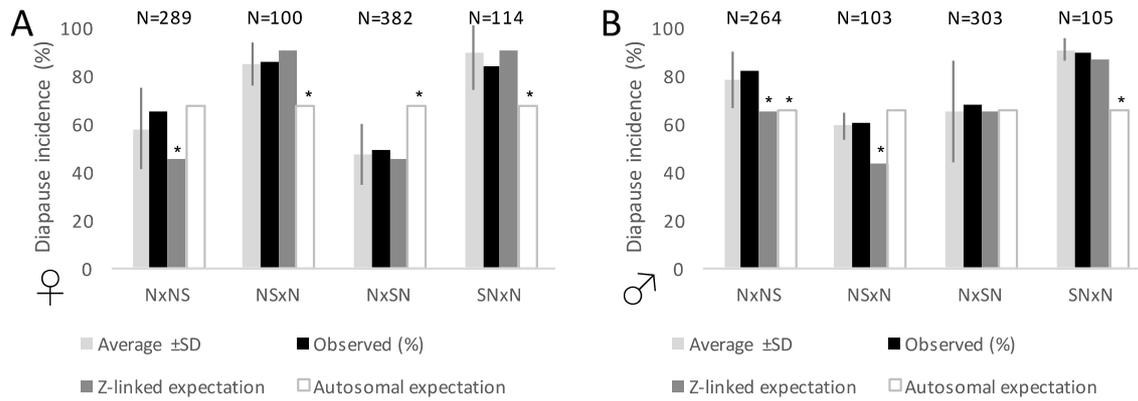
Figure 2 – Diapause incidence in the F2 backcrosses for females (A), and males (B). Light grey bars and lines represent family Means ±1 Standard Deviation for each cross. Black bars represent observed total incidences for each cross, dark grey bars represent the expected incidences for Z-linked diapause induction, and the hollow bars represent diapause incidence under expected autosomal control. The stars denote significance by Fisher's Exact Test, as reported in Table 2. Non-significant differences indicate the likely mode of inheritance, whereas significant differences indicate a non-likely mode of inheritance.

517   Table 1 – Diapause incidence of the pure populations for each photoperiod. Percentage of
518   diapausing individuals, the total number of individuals, as well as the number of families in each
519   cross are given.

	Cross ♀ x ♂	Diapause (%)	Total number	Number of families
P	S x S	82.1	39	8
LD 8:16	N x N	100	58	13
	S x S	58.1	31	8
LD 11:13	N x N	100	44	13
	S x S	7.1	28	7
LD 14:10	N x N	93.5	62	15
	S x S	9.5	21	6
LD 17:7	N x N	90.9	50	14
	S x S	14.7	34	8
LD 20:4	N x N	93.2	44	13
	S x S	0	32	8
LD 23:1	N x N	97.5	40	14

520

521

522   Table 2 – Diapause incidence for each hybrid cross. Percentage of diapausing individuals is split

523   by sex. The total number of individuals in each cross is given, as well as the number of families

524   in each cross.

	Cross ♀ x ♂	Diapause incidence (%)		Total number	Number of families
		♀	♂		
F1	N x S	100	100	93	1
LD 8:16, 17°C	S x N	98.3	98.3	226	3
F1	N x S	99.6	100	496	4
LD 12:12, 17°C	S x N	100	100	414	4
F1	N x S	8.3	40	76	1
LD 18:6, 23°C	S x N	100	60	84	1
F1	N x S	0	0	39	1
LD 23:1, 20°C	S x N	0	0	134	1
P	S x S	0	0	228	4
LD 23:1, 20°C	N x N	90.0	87.0	76	2
F2	N x NS	64.7	82.2	553	4
LD 23:1, 20°C	NS x N	86.0	60.2	203	3
	N x SN	48.6	67.4	586	7
	SN x N	84.2	89.5	219	2

525

526

527 Table 3 - Expected vs observed frequencies of diapause incidence for females under complete Z-
528 linked induction and no dominance. Based on the frequencies of the pure populations, expected
529 diapause incidences were calculated and compared to the observed values by Fisher's exact test.

F2 Cross	♀ Fisher's exact two-tailed P		♂ Fisher's exact two-tailed P	
	Z-linked	Autosomal	Z-linked	Autosomal
N x NS	0.0001	0.5386	0.0001	0.0001
NS x N	0.5146	0.0040	0.0254	0.5647
N x SN	0.4476	0.0001	0.6068	0.6068
SN x N	0.2326	0.0050	0.6707	0.0001

530