

# Latitudinal variation in diapause duration and post-winter development in two pierid butterflies in relation to phenological specialization

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**Abstract** Diapause plays a central role in insect life cycles by allowing survival during adverse seasonal conditions as well as synchronizing life cycles with the period of mate and food availability. Seasonal timing is expected to be particularly important for species that are dependent on resources available during a short time window—so-called phenological specialists—and latitudinal clines in seasonality are expected to favor local adaptation in phenological timing. However, to what degree latitudinal variation in diapause dynamics and post-winter development due to such local adaptation is influenced by the degree of phenological specialization is not well known. We experimentally studied two pierid butterfly species and found that the phenological specialist *Anthocharis cardamines* had shorter diapause duration than the phenological generalist *Pieris napi* along a latitudinal gradient in Sweden. Moreover, diapause duration increased with latitude in *P. napi* but not in *A. cardamines*. Sensitivity of the two species to winter thermal conditions also differed; additional cold temperature during the winter period shortened diapause duration for *P. napi* pupae but not for *A. cardamines* pupae. In both species, post-winter pupal development was faster after longer periods of cold conditions, and more southern populations developed faster than northern populations. Post-winter development was also invariably faster at higher

temperatures in both species. We argue that the observed differences in diapause dynamics between the two species might be explained by the difference in phenological specialization that influences the costs of breaking diapause too early in the season.

**Keywords** *Anthocharis cardamines* · *Pieris napi* · Local adaptation · Voltinism · Development

## Introduction

Temperate insects are under strong selection to time their life cycles to the seasonal changes in abiotic and biotic conditions. This selection is likely to vary spatially, for instance along latitudinal gradients, leading to local adaptation in insect life-cycle regulation (Bradshaw and Holzapfel 2008). Such local adaptations allow insects to grow and reproduce when food and mates are available at a given location, as well as staying in diapause when conditions are unfavorable (Nylin and Gotthard 1998). For any temperate insect species, the induction and termination of winter diapause are crucial components of the life cycle. These traits have a strong impact on spring-time phenologies and will have a large direct influence on how climate change might affect life-cycle timing and species interactions at temperate latitudes.

Diapause is a hormonally controlled state of metabolic suppression and cessation of development, allowing insects to survive periods of adverse conditions. Diapause induction, intensity and termination are regulated by a variety of factors like photoperiod, temperature and humidity (e.g., Tanaka et al. 1987; Hodek and Hodková 1988; Nunes and Saunders 1999). However, the conditions during diapause influence not only its progression but also affect

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post-diapause survivorship, development, morphogenesis and fecundity (e.g., Denlinger 1981; Wiklund and Solbreck 1982; Ishihara and Shimada 1995; Ellers and van Alphen 2002; Matsuo 2006). Depending on microclimate (Tauber et al. 1986), food quality (Hunter and McNeil 1997) and the number of generations produced per year (voltinism) (Ishihara 2000), the timing of diapause induction may show substantial variation among individuals within a population, ranging from a few weeks and up to several months within the same growth season. Therefore, another important aspect of diapause is its ability to synchronize life cycles of individuals so they can fully exploit favorable periods of physical conditions and food availability as well as increase their chances of successfully competing for mates (Chippendale 1982; Wiklund and Solbreck 1982; Forsberg and Wiklund 1988).

This synchronizing role of diapause is particularly important for insects depending on food that is available only within a narrow time period of the season. For instance, some herbivores are specialized to feed on a particular phenological stage of the host plants such as buds and young leaves (Feeny 1968; Blüthgen and Metzner 2007), flowers (Childers and Achor 1991; Emery et al. 2009), fruits or seeds (e.g., White 1975; Ostergard and Ehrén 2005; Teixeira and Polavarapu 2005). Variation in the degree of food specialization can be an important driver of natural selection on diapause characteristics. In species dependent on a food source that is available only during a short time period each year, selection for life cycle timing to this period will have a great influence on diapause dynamics. As a result, such species are likely to evolve a univoltine life cycle with an obligatory diapause throughout the whole unfavorable period (Masaki 1961). In contrast, if a species feeds on a food source that is available throughout the growing season, other factors, such as season length and temperature, become more important and will have a stronger influence on the timing of the active and the dormant periods (Masaki 1961). One example of such differences in selection on timing are the geometrid moths *Megabiston plumosaria* and *Jankowskia athlete* that both feed on tea bushes. While *M. plumosaria* is a phenological specialist on newly emerged leaves, *J. athlete* also consumes old leaves. The specialist's life cycle is adjusted to match the short period when fresh leaves are available, and it has only one generation per year, spending the rest of the time in diapause (Minamikawa 1950). For the generalist, the food source is present for most of the year and cool winter conditions are the main limiting factor, allowing the moth to have three generations per year in Japan (Minamikawa 1951). Differences in selection on diapause dynamics between phenological specialists and generalists should also affect the pattern of local adaptation across climatic gradients. Populations of specialists should be obligatory

univoltine everywhere across latitudes, while populations of generalists should differ in voltinism depending on the duration of the warm season, resulting in a decrease in the number of generations as the season shortens at higher latitudes (e.g., Corbet et al. 2006). Such differences in voltinism are typically manifested as differences in the mechanisms of diapause induction between obligate and facultative diapausing species (Saulich and Musolin 1996). However, to what degree differences among phenological specialists and generalists in diapause strategy also pertain to the mechanisms for its length and termination is largely unknown.

The mechanisms of winter diapause termination in temperate insects are not well understood, although it appears typically to happen during the cold winter months when low temperatures impede resumption of development (Hodek 1996, 2002). Diapausing individuals then enter a state of quiescence (exogenous inhibition of development; Košťál 2006) that allows them to resume development as soon as environmental conditions, in particular temperature, become favorable (Košťál 2006). As for the inherent duration of diapause (endogenous developmental arrest; Košťál 2006), an intuitive prediction is that it should be longer in areas where winters are longer, therefore increasing with latitude. However, Masaki (1961) addressed this idea by also including variation in the number of generations in local populations and identified two patterns of geographic variation. When populations express the same number of generations, the length of diapause should decrease with increasing latitude (or from warmer to cooler climates); because of the need to fit the same number of generation into a shorter season, diapause is terminated earlier at higher latitudes to allow fast development as soon as the conditions become favorable. However, if there is a change in voltinism (e.g., from two to one generations per year) along the latitudinal gradient, the diapause duration is expected to increase at higher latitudes, since there is plenty of time to produce one generation and selection due to time stress is relaxed. Importantly, this pattern is expected only for phenological generalists that benefit from terminating diapause as soon as thermal conditions become favorable and maximize the number of the subsequent broods. For phenological specialists, however, this is not necessarily the case, since they are adapted to match a suitable stage of their host plants. For instance, diapause duration in the cherry fruit fly *Rhagoletis cerasi*, the apple maggot *R. pomonella* and the blueberry maggot *R. mendax* were locally adapted in relation to the flowering time of their host species and even to late or early flowering cultivars in different areas (Teixeira and Polavarapu 2002, 2005; Moraiti et al. 2014). Because of the stronger influence of season length on the life cycle of phenological generalists, it can be expected that latitudinal variation in diapause

duration among populations should be more pronounced in phenological generalists than in specialists.

In this study, we experimentally explored local adaptation in life cycle timing to the onset of spring along a latitudinal gradient by investigating diapause duration and post-winter development in two butterfly species. The green-veined white, *Pieris napi*, is a phenological generalist, which consumes green leaves of various Brassicaceae species throughout the warm season, while the orange tip butterfly, *Anthocharis cardamines*, is a phenological specialist that feeds preferably on flowers and growing siliquae of Brassicaceae species, which are available for a period of approximately 1 month in May–June in Sweden (Wiklund and Friberg 2009). *P. napi* is known to be typically bivoltine throughout the sampled range (Eliasson et al. 2005), while *A. cardamines* is obligatory univoltine throughout Europe (Eliasson et al. 2005). Both species overwinter as diapausing pupae and eclose as adults in spring. According to the hypothesis of Masaki (1961), we expect that the duration of winter diapause to decrease with latitude in *P. napi* as they will have to fit the same two generations into a shorter season length in the north. An alternative hypothesis is that the duration of winter diapause is longer at higher latitudes simply because the winters are longer. Based only on the constant number of generations, the predictions for *A. cardamines* should be similar to that of *P. napi*. However, since *A. cardamines* must track the timing of host flowering, diapause duration should be selected indirectly through the host plants' phenological timing. This implies that typical expectation of latitudinal variation in diapause duration may not be relevant for the phenological specialist *A. cardamines*. In general we expected the phenological generalist, *P. napi*, to show a more pronounced latitudinal pattern in these timing adaptations as its life cycle is directly related to season length. Since the timing of the butterflies' spring emergence is determined both by diapause duration and post-diapause development, we also investigated their subsequent post-winter pupal development. In previous studies, both species showed cogradient latitudinal patterns in post-diapause development throughout Sweden (Posledovich et al. 2014; Stålhandske et al. 2014). However, the relationship between winter duration and the subsequent post-winter pupal development has not been studied. Due to selection for optimal spring emergence, we hypothesize that post-winter pupal development is slower if pupae experience a relatively short winter. This would keep them from emerging too early in spring and potentially protect them from late spring frosts.

Thus, we tested the hypotheses that (1) latitudinal variation in diapause duration—either increasing or, alternatively, decreasing with latitude—should differ between phenological generalist and specialist species. In addition, we explored (2) if winter length has a similar effect on

post-winter pupal development in the two species as well as (3) the capacity of winter severity to alter both diapause duration and post-winter development rate.

## Materials and methods

All experimental manipulations were performed in two series in 2012 and 2013. Two butterfly species—*A. cardamines* and *P. napi*—were collected as eggs or larvae in the field 1 year before they were used in the experiments (i.e. in 2011 and 2012). Three locations on the eastern coast of Sweden were chosen for sampling a southern population (Skåne province, 40 km area around Degeberga; 55°49'N, 14°05'E), a central population (Uppland province, Ljusterö island; 59°30'N, 18°35'E) and a northern population (Ångermanland province, 45 km area around Docksta; 63°03'N, 18°19'E) of the species, with approximately 900 km between the southern and the northern areas. The collected and newly hatched larvae were reared on their natural host plants in short day (12L:12D) conditions (for induction of pupal diapause) until pupation.

### Diapause duration

Diapause duration was measured as probability (i.e. proportion) of pupal eclosion after the diapausing pupae spent 3, 4 or 5 months in cold winter conditions. All diapausing pupae were kept at +1 °C for a period of 3, 4 or 5 months (in 2012) or at +2 °C for 3 or 5 months (in 2013) (groups 3m, 4m and 5m). At the termination of the cold period, pupae were transferred to thermally controlled conditions with average temperatures of 13 (13.5 ± 1.6; mean ± SD), 15 (15.8 ± 1.8) and 17 (17.6 ± 2) degrees in 2012 and 17 °C (17.7 ± 0.7) in 2013; and incubated until their eclosion. Both years the photoperiod during the warm treatments was 12L:12D.

Individuals that did not eclose as adults and showed no signs of development, but were clearly not dead (determined by rate of pupal weight loss and color) by the day when the last pupa hatched, were considered to be still in diapause.

In 2012, the sample sizes of the southern and the northern populations of *A. cardamines* were insufficient to divide them between all the treatment combinations, so they were used only in the 5 months diapause treatment. The central population of *A. cardamines* was tested after 3 and 5 months of diapause only. In 2013, all three populations of *A. cardamines* were tested together with two of the *P. napi* populations (southern, from Skåne, and northern, from Ångermanland). The sample size of the survived pupae as well as all the different treatment combinations are shown in Table 1.

**Table 1** Number of pupae of the three populations of *Pieris napi* and *Anthocharis cardamines*, tested in different treatments after accounting for mortality in 2012 and 2013

	Southern population			Central population			Northern population		
	+/-	Total	Dead	+/-	Total	Dead	+/-	Total	Dead
2012									
<i>P. napi</i>									
3m									
13 °C	9/31	40	0	0/17	17	1	6/84	90	2
15 °C	18/22	40	0	0/17	17	1	9/80	89	4
17 °C	21/15	36	2	1/12	13	5	10/79	89	4
4m									
13 °C	38/1	39	1	4/14	18	0	6/33	39	1
15 °C	40/0	40	0	6/11	17	1	4/35	39	1
17 °C	36/2	39	1	10/7	17	1	8/32	40	
5m									
13 °C	35/1	36	1	35/1	36	3	25/14	39	1
15 °C	40/0	40	0	30/4	34	4	25/12	37	3
17 °C	39/0	39	1	34/1	35	5	24/11	35	5
<i>A. cardamines</i>									
3m									
13 °C				15/0	15	0			
15 °C				13/0	13	0			
17 °C				15/0	15	0			
5m									
13 °C	17/0	17	1	38/0	38	1	20/0	20	0
15 °C	19/0	19	0	38/0	38	2	20/0	20	1
17 °C	17/0	17	1	39/0	39	0	20/0	20	0
2013 (only 17 °C temperature treatment was used)									
<i>P. napi</i>									
3m	73/6	79	10				101/39	140	21
3m extra cold	38/2	40	9				53/11	64	19
5m	74/0	74	26				135/9	144	16
<i>A. cardamines</i>									
3m	21/2	23	0	25/0	25	0	14/0	14	0
3m extra cold	17/6	23	0	25/0	25	0	13/1	14	0
5m	44/0	44	0	68/0	68	0	24/0	24	0

+/- number of hatched/non-hatched pupae

### Post-winter pupal development

For those individuals that terminated diapause and eclosed as adults in the diapause duration experiments in 2013, we investigated the rate of post-winter pupal development in relation to duration of cold winter conditions (groups 3m, 4m and 5m) and population of origin. Post-winter pupal development was calculated as pupal developmental rate (DR), which is the inverse of developmental time (the number of days from when the pupae were placed into the warm treatments until adult eclosion). Temperature inside the climate cabinets were recorded with the help of temperature loggers every half-hour throughout the whole experimental period.

### The effect of thermal conditions during diapause

In 2013, we also investigated diapause duration and post-diapause development in relation to the differences in overwintering temperature in two of the populations of *P. napi* (southern and northern) and all the three populations of *A. cardamines*. First, we exposed diapausing pupae to +2 °C for 1.5 months, then 1 month of -2 °C and then the last 0.5 month at +2 °C again. The timing of extra cold manipulation was chosen to simulate the period of the coldest winter temperatures in Sweden, which happens approximately at this stage of diapause. Altogether, this group of pupae experienced 3 months of cold winter conditions and after that they were incubated at 17 °C ( $17.7 \pm 0.7$ ) in climate

**Table 2** GLM (2012) and GLMM (2013) table for the proportion of *P. napi* pupae that developed into adults in relation to population of origin (southern/central/northern in 2012; southern/northern in 2013), length of cold winter period (3m/4m/5m in 2012; 3m/3m extra cold/5m in 2013) and warm temperature treatments (continuous variable of 13/15/17 °C treatments in 2012)

Parameter	Chi sq	df	P
2012			
Population (S/C/N)	270.48	2	$<2.2 \times 10^{-16}$
Cold period length (3/4/5m)	367.95	2	$<2.2 \times 10^{-16}$
Temperature	10.16	1	0.0014
Population $\times$ cold period length	47.83	4	$1.02 \times 10^{-9}$
2013			
Population (S/N)	17.47	1	$2.9 \times 10^{-5}$
Cold period length (3/3 extra cold/5m)	24.27	2	$5.4 \times 10^{-6}$

cabinets with photoperiod 12L:12D. Hence, the effect of the extra cold on diapause duration and post-winter development rate could be evaluated by comparison with pupae that spent 3 months at +2 °C.

### Statistical analyses

Statistical analyses were conducted with R 3.0.2 statistical software (R Development Core Team 2013). All the initial (full) models included all explanatory variables and their interactions. The best model structure was determined by step-wise removal of insignificant higher order interactions (and main effects) by using Akaike information criterion as a guideline. Goodness-of-fit of the final models was evaluated by visual investigation of the residual plots. When necessary, transformation of response variables was done based on Box–Cox transformation. Below, we describe the specific analyses carried out to test the respective hypotheses and questions.

#### Diapause duration

In 2012, the probability of pupal eclosion in *P. napi* was analyzed by fitting a generalized linear model with a binomial error distribution and a logistic link function to the data. The probability of pupal eclosion (developed or stayed in diapause) was set as a response variable and duration of the cold period (3m, 4m, 5m), population and average individual temperature (continuous variable) of the warm post-winter treatments as explanatory variables.

In 2013, since the warm temperature treatment of 17 °C was replicated in three climate cabinets, a generalized linear mixed-effect model (GLMM; function glmer; Bates et al. 2013) with a binomial error distribution, logistic link

function and cabinet identity as a random factor was fitted to the data on probability of pupal eclosion in *P. napi*. The proportion of pupae that developed was set as a response variable, while duration of the cold period as well as population and pupal weight were set as fixed effects. Since virtually all pupae of *A. cardamines* eclosed in 2013, we did not perform the same analysis for that species.

#### Post-winter pupal development

In 2013, pupal post-winter developmental rate (DR; square root-transformed) of both *P. napi* and *A. cardamines* was analyzed with linear mixed-effect models (LMM; function lme; Pinheiro et al. 2013), using cabinet identity as a random factor. The fixed effects included population, sex, initial pupal weight and duration of the cold period (3m and 5m).

#### The effect of thermal conditions during diapause

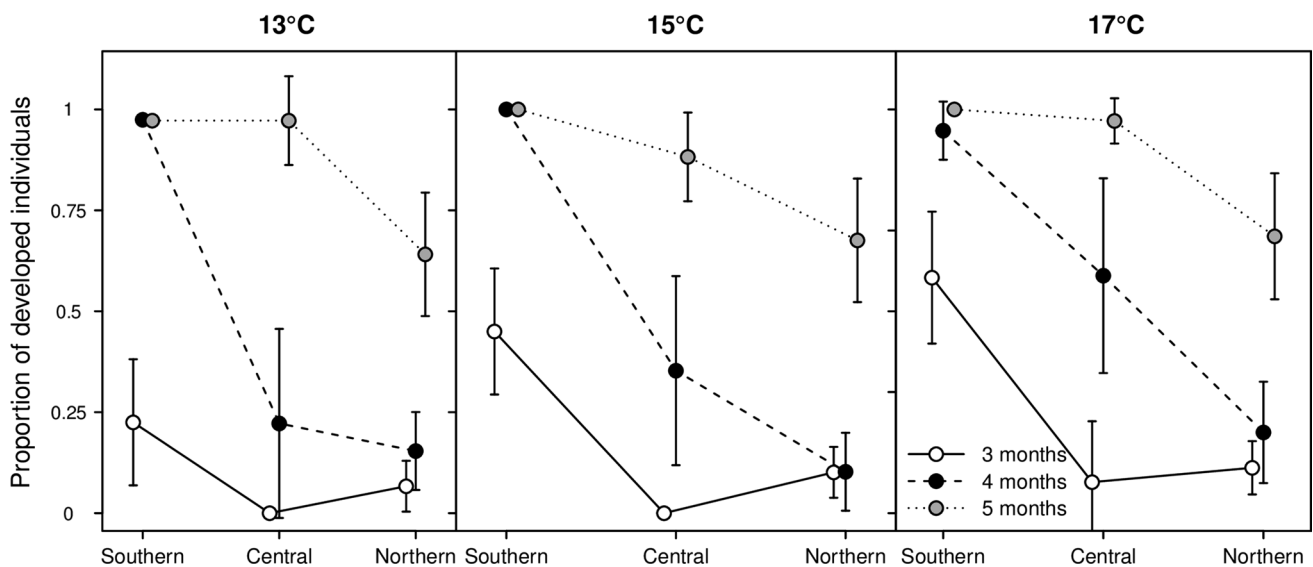
Probability of pupal eclosion in the extra cold treatment group of *P. napi* was compared with that of the control groups 3m and 5m using GLMM within the same model described in the diapause duration analysis above.

Post-winter DR in the extra cold treatment group of both butterfly species was tested against the control 3m group using LMM the same way as it was performed in pupal development (see above). In *P. napi*, analysis the variance function varIdent (Pinheiro et al. 2013) was used for modeling heteroscedasticity (different variances for each level of both the cold period groups and cabinet variable). Cubic root transformation was applied to DR.

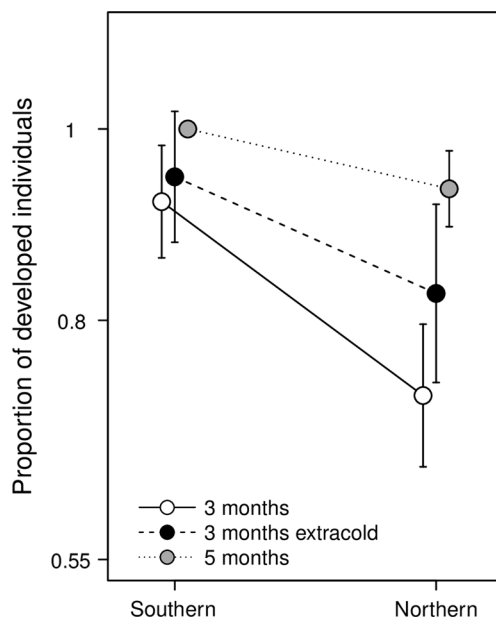
## Results

#### Diapause duration

The proportion of *P. napi* pupae that terminated diapause and developed into adults depended on the time spent in winter diapause (2012 and 2013), thermal conditions during the post-diapause pupal development (only tested in 2012) and population (2012 and 2013) (Table 2; Fig. 1). In all populations in the 2012 experiment, a longer cold period and a higher post-winter temperature increased the probability of breaking diapause and starting development. Moreover, the probability of breaking diapause decreased with latitude. While 4 months of cold was sufficient for allowing almost all individuals of the southern population to break diapause and start development, the central population needed 5 months of cold to achieve a similar developmental competence. In the northern population, a large proportion of pupae did not terminate diapause even after 5 months in



**Fig. 1** Proportions of developed specimens of *Pieris napi* after 3 (solid line), 4 (dashed line) and 5 (dotted line) months of cold period in the three different warm temperature treatments in 2012



**Fig. 2** Proportions of developed specimens of *P. napi* after 3 (solid line), 3 with extra cold (dashed line) and 5 (dotted line) months of cold period in the 17 °C incubation temperature in 2013

the cold. In *A. cardamines*, all the pupae from the central population developed into adults independently of the time spent in cold conditions (3m or 5m). For the southern and northern populations, we only had one treatment (5m), and in those cases all pupae also developed (Table 1).

Also in 2013, the proportion of pupae that emerged after 3 months was significantly lower than after 5 months for both the southern and northern populations of *P. napi*

(Table 2; Fig. 2). As for *A. cardamines*, only two pupae (8 %) remained in diapause in the 3 months group in the southern population (Table 1). All other populations broke their diapause in both 3m and 5m groups.

#### Post-winter pupal development

Post-winter pupal developmental rate was higher in the 5 months treatment than in the 3 months treatment in all populations of both *P. napi* and *A. cardamines*. Pupae from more southern populations as well as males of both butterfly species developed at higher rates (Table 3; Fig. 3).

#### The effect of thermal conditions during diapause

The extra cold treatment increased the proportion of eclosed individuals in the two populations of *P. napi* compared to the 3m group that did not experience the extra cold month. The 3m extra cold treatment showed an intermediate response compared to the 3m and 5m treatments (Fig. 2). In *A. cardamines*, very few pupae also remained in diapause after 3 months of winter without the extra cold treatment and there was no effect of an extra cold treatment on development.

Pupae of *P. napi* from the 3m extra cold treatment developed significantly faster than pupae from 3m treatment, and had intermediate values compared to the 3m and 5m in both southern and northern populations (Table 4; Fig. 3). Males also developed faster than females in the 3m extra cold treatment. However, males from the southern population were more sensitive to the additional cold than females, while males and females from the northern population differed less (population  $\times$  sex  $\times$  cold period length



**Table 3** LMMs table for post-winter developmental rate of *P. napi* (southern/northern populations) and *A. cardamines* (southern/central/northern populations) in relation to length of cold winter period (3 m/5 m) and sex in 2013

Parameter	df	F	P
<i>P. napi</i>			
Intercept	1	106.09	<0.0001
Population (S/N)	1	23.28	<0.0001
Cold period length (3m/5m)	1	187.68	<0.0001
Sex	1	48.10	<0.0001
<i>A. cardamines</i>			
Intercept	1	502.29	<0.0001
Population (S/C/N)	2	28.94	<0.0001
Cold period length (3m/5m)	1	567.94	<0.0001
Sex	1	221.15	<0.0001

interaction in Table 4; Fig. 3). Developmental rate of *A. cardamines* populations was not affected by the additional cold treatment (Table 4; Fig. 3).

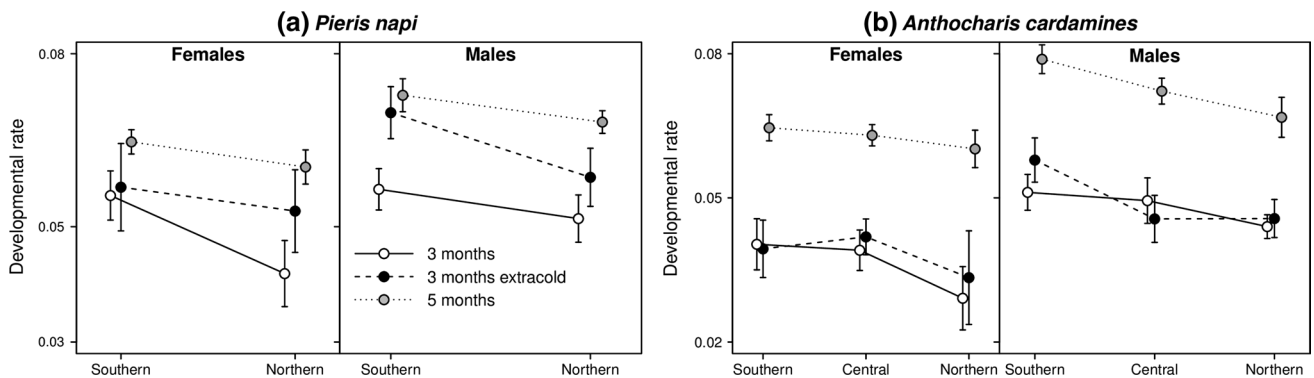
**Discussion**

The length of diapause and the mechanisms for termination differed between the two butterfly species. All three populations of *A. cardamines* needed a period of <3 months of cold temperatures to terminate diapause, and there were no differences among populations from different latitudes. In contrast, only a small proportion of *P. napi* pupae broke their diapause after 3 months, and the length of diapause increased with latitude.

**Diapause duration**

For pupae to be able to respond to an increase in temperature, the diapause first needs to be broken. However,

terminating diapause too early is associated with certain costs. A longer duration of diapause can reduce pupal mortality due to late frosts in spring, as many insect species are known to be more cold tolerant in the state of diapause than in non-diapause (Hahn and Denlinger 2007). Moreover, terminating diapause and maintaining the state of quiescence may be costly, since insects face the risk of depleting their nutrient reserves as the metabolic rate increases in the post-diapause period (Feder et al. 1997; Hahn and Denlinger 2007; Ragland et al. 2009). Thus, breaking diapause early may be more costly for more northern populations of both species where the winter is more extended, compared to southern latitudes where spring comes earlier. However, our results suggest that the benefit of being developmentally competent early in the season, and thereby emerge when host plants flower, outweighs these costs in *A. cardamines*. The present results demonstrate that the initiation of pupal sensitivity to temperature typically occurs earlier for pupae of *A. cardamines* than for *P. napi*. Moreover, the pattern of latitudinal variation in diapause duration differed between the species, possibly because of the difference in phenological specialization. Climatic conditions in the locations of the tested populations (according to the data of the closest meteorological stations) differ, so that the first month when the average temperature is  $\leq 2$  °C is December in the southern location, mid-November in the central location, and the end of October in the northern location. This means that 3 months of winter diapause would be over in early March for the southern populations, the end of February for the central population, and in the end of January for the northern populations, well before the food becomes available. A study of *A. cardamines* in the United Kingdom showed that temperatures in the period from early March to early May were the best predictors of the average flight time of *A. cardamines* across the whole country (Phillimore et al. 2012). Most likely, the diapausing pupae had already broken their diapause by that time and responded to warm



**Fig. 3** Pupal developmental rate ( $\pm 95$  % CI) of **a** southern/northern populations of *P. napi* and **b** southern/central/northern populations of *A. cardamines* depending on the length of cold period and extra cold treatment in 2013

**Table 4** LMMs table for post-winter developmental rate of *P. napi* (southern/northern populations) and *A. cardamines* (southern/central/northern populations) in relation to length of cold winter period (3m/3m extra cold) and sex in 2013

Parameter	df	F	P
<i>P. napi</i>			
Intercept	1	27.50	<0.0001
Population (S/N)	1	19.07	<0.0001
Cold period length (3 m/3 m extra cold)	1	36.61	<0.0001
Sex	1	15.92	0.0001
Population × sex	1	0.01	0.9
Cold period length × population	1	1.62	0.2
Cold period length × sex	1	1.63	0.2
Population × cold period length × sex	1	3.55	0.06
<i>A. cardamines</i>			
Intercept	1	775.51	<0.0001
Population (S/C/N)	2	8.83	$3 \times 10^{-4}$
Sex	1	59.63	<0.0001

temperatures during that period by accelerating their post-diapause development. Since host plants of *A. cardamines* flower relatively early in spring, and the herbivore is able to respond to the timing of the flowering only indirectly, this butterfly species is likely to be under particularly strong selection to terminate diapause and become temperature sensitive early in the season in order to track the period of flowering. In contrast, the phenological generalist *P. napi* is not dependent on the early flowering of its hosts and is more affected by selection for emerging under optimal climatic conditions. This would result in a relatively longer period of temperature-insensitive diapause that is strongly dependent on geographical variation in the average start of spring along the species' range. Thus, the difference in diapause duration between the populations of *P. napi*, which is typically bivoltine along the examined latitudinal range, was the opposite of expectations from the theory of Masaki (1961), increasing from south to north. Instead, we found support for the idea that longer winters select for a longer duration of diapause, underscoring the evolutionary costs associated with early diapause termination.

#### The effect of thermal conditions during diapause

Winter thermal conditions had a significant effect on diapause duration for the two populations of *P. napi* examined in 2013, whereas there was no such clear effect in *A. cardamines*. A decrease in temperatures from  $+2^{\circ}$  to  $-2^{\circ}$  during one winter month increased the proportion of *P. napi* individuals that terminated their diapause after 3 months. This suggests that to some degree the duration of cold conditions can be substituted by lower temperatures in *P. napi* and that the butterfly's

post-diapause development may be expected to proceed more quickly in years with harsher winters. Lower winter temperatures were shown to be more effective in diapause termination in the cabbage root fly, but temperatures below zero did not add much to increase the proportion of developers (Collier and Finch 1983). Pupae of the cherry fruit fly *Rhagoletis cerasi*, a phenological specialist, were more likely to stay in diapause for one more year when winter temperatures were either higher or lower than optimal (Moraiti et al. 2014). Lowering the temperature to  $-5^{\circ}\text{C}$  also had a higher diapause terminating impact when applied in the middle of cold period (peaking between days 30 and 50 after diapause initiation) in the gypsy moth *Lymantria dispar* (Gray et al. 2001). In our study, the  $-2^{\circ}\text{C}$  treatment had a larger effect on diapause duration in the northern than in the southern population of *P. napi*, which may indicate its adaptive nature in the two populations to different winter conditions at their respective locations (average temperatures of the three winter months is about  $-0.1^{\circ}\text{C}$  in the south and  $-5.2^{\circ}\text{C}$  in the north). Unfortunately, the shortest winter period of 3 months was too long to determine the time when *A. cardamines* began to shift to post-diapause quiescence, and it is possible that the cold manipulation was applied when all the pupae had already done it. Still, our results suggest that the increase in winter temperatures, forecasted for the northern hemisphere under the present climate warming, may have different effects on these two butterfly species, as well as on the populations of *P. napi* originating from different latitudes.

#### Post-winter pupal development

Post-winter pupal developmental rates in *P. napi* were positively correlated with variation in diapause duration both between populations and winter duration treatments. In 2013, post-winter pupal development of both southern and northern populations proceeded faster after 5 months of cold period and much slower after 3 months. This pattern was a mere reflection of the proportions of the individuals that did break diapause (Figs. 2, 3a). This observation suggests that the co-gradient pattern of spring emergence in *P. napi*, reported in an earlier paper (Posledovich et al. 2014), could be due to a longer diapause duration in the northern populations as compared to populations further south. From a physiological point of view, such an influence of winter duration on the subsequent pupal developmental rate in the post-winter period suggests that the *P. napi* pupae that did develop after 3 months of winter were still in their final diapause termination stage, in which a gradual decrease in diapause intensity had already made the pupae sensitive to diapause terminating conditions (Košťál 2006). The earlier the pupae were in their diapause terminating phase, the longer it took them to develop into adults, which is why post-winter development time was the longest after



3 months of winter and the shortest after 5 months in *P. napi*. As for *A. cardamines*, although its diapause duration was shorter than 3 months and we could not detect any pattern between the populations, the difference in post-winter pupal development rate between the 3 and 5 months groups indirectly suggests that diapause duration in this species was approximately 3 months (Fig. 3b).

#### Concluding remarks

In conclusion, diapause duration and thermal requirements for diapause termination differed between the two butterfly species. This is most likely a consequence of differences in their life histories, where *A. cardamines* is obligatory univoltine and depend on being able to accurately track the period of host plant flowering at each of the geographic localities. In contrast, *P. napi* shows latitudinal variation in voltinism and is less dependent on the early emergence of hosts, which may decrease the benefits of early diapause termination. Instead, the latitudinal variation in diapause duration suggests that it is adapted to the typical length of the winter period. As a result of these differences, the balancing of the trade-off between avoiding late frosts as well as lowering energetic demands, and maximizing the availability of host plant resources differs between the two species. Thus, biotic conditions seem to play an important role in life-cycle regulation in *A. cardamines*, while abiotic factors are likely to be most important in *P. napi*.

Sensitivity of life-cycle timing in spring to different environmental cues may result in distinctly different expectations for different insect species under climate change. Temperature is not the only factor that affects diapause duration in insects. Photoperiod is another powerful modulator of diapause dynamics that can either have an effect on its own or be supplemented by thermal cues (Tauber and Tauber 1976; Danks 1987; Valtonen et al. 2011). For some species, there seems to be no specific stimulus that ends diapause (Tauber and Tauber 1976), but instead it must be due to some intrinsic timekeeping mechanism. As pointed out by Valtonen et al. (2011), the particular mechanism for diapause termination should determine a species' responsiveness to climate warming. Thermal control of diapause dynamics is expected to lead to a quick response in a species' spring phenology, while photoperiodic control would require time to evolve local adaptations (Valtonen et al. 2011). Neither *A. cardamines* nor *P. napi* are known to be sensitive to photoperiod at the pupal stage, and both species successfully terminate diapause when kept in constant darkness. Thus, taking into account their thermal nature of diapause control and the fact that they terminate diapause fairly long before the environmental temperatures become permissive for development (especially *A. cardamines* with only 3 months of diapause duration), both these species are

expected to advance their spring phenology under climate change because of thermal plasticity. Indeed, among Swedish butterflies, *A. cardamines* is one of the species that have shown the greatest advancement in spring flight during the last 20 years (Karlsson 2014). As plant phenology is typically influenced both by photoperiod and temperature, it seems unlikely that the host plant phenology will change in exactly the same way, and it is probable that a warming climate may influence the realized host use of this phenological specialist (Navarro-Cano et al. 2014, in press). The advancement of adult spring emergence in the generalist *P. napi* may, however, be advantageous within this northern part of its distribution, as it is likely to prolong the favorable season. In general, the effects of climate change on such species interactions are likely to be influenced by the exact characteristics of the organisms in question, and as these interactions make up an important part of biodiversity, it is of great interest to understand how they are likely to be affected by continued warming.

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