

# Evidence of genetic change in the flowering phenology of sea beets along a latitudinal cline within two decades

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vernalization requirement.

## Abstract

Sea beets grown from seeds collected in 1989 and 2009 along the coasts of France and adjacent regions were compared for flowering date under controlled conditions. Seeds from both collection years were sown simultaneously and cultivated under the same glasshouse conditions. Date of flowering onset and year of first flowering were recorded. There was an overall northward shift in flowering time of about 0.35° latitude (i.e. 39 km) over the 20-year period. The southern portion of the latitudinal gradient – that is, from 44.7°N to 47.28°N – flowered significantly later by a mean of 1.78 days, equivalent to a 43.2-km northward shift of phenotypes. In the northern latitudes between 48.6°N and 52°N, flowering date was significantly earlier by a mean of 4.04 days, corresponding to a mean northward shift of 104.9 km, and this shift was apparently due to a diminished requirement of exposure to cold temperatures (i.e. vernalization), for which we found direct and indirect evidence. As all plants were grown from seed under identical conditions, we conclude that genetic changes occurred in the sensitivity to environmental cues that mediate the onset of flowering in both the northern and the southern latitudes of the gradient. Microevolution and gene flow may have contributed to this change. There was no significant change in the frequency of plants that flowered without vernalization. The lack of vernalization requirement may be associated with environmental instability rather than with climate conditions.

## Introduction

The rising temperatures of the last decades have had diverse significant effects on species. In many species of the Northern Hemisphere, there has been a northward range shift or extension (Warren *et al.*, 2001; Parmesan & Yohe, 2003) or a shift in altitude (Chen *et al.*, 2011). Phenology is directly affected by climate change, as shown by northward shifts of 'southern' phenotypes (Cleland *et al.*, 2007; Forrest & Miller-Rushing, 2010; Gordo & Sanz, 2010; Pau *et al.*, 2011; Franks *et al.*, 2014). In many plant species, earlier spring flowering is reported in temperate regions (Walther *et al.*, 2002; Parmesan & Yohe, 2003; Root *et al.*, 2003; Tooke & Battey,

2010). In addition to flowering time, other recurring seasonal biological events have been affected by climate change, for example leaf bud burst (Cleland *et al.*, 2007; Franks *et al.*, 2014). In contrast, although increasing temperatures may hinder winter chilling and thus delay flowering (Menzel *et al.*, 2006), latitude shifts in the vernalization requirement have not yet been reported to our knowledge. Vernalization is mostly known as a mechanism for avoiding flowering in the year of germination, but it can also play a quantitative role in flowering induction: in some plant species, incomplete vernalization delays flowering (Van Dijk, 2009b). Flowering time and the vernalization requirement are both traits controlled by temperature and therefore potentially affected by climate change.

The issues at hand are whether observed phenological change is the result of (i) genetic change (Gienapp *et al.*, 2008; Hoffmann & Sgrò, 2011; Heard *et al.*, 2012) or phenotypic plasticity (Miller-Rushing *et al.*, 2007);

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(ii) adaptive change and/or (iii) a response to climate change or to another causal agent (Merilä & Hendry, 2014). Although there is currently little knowledge about its relative importance, the genetic response of plant populations to climate change is an important topic (Jump & Peñuelas, 2005; Skelly *et al.*, 2007; Franks *et al.*, 2014). To test the genetic contribution to trait changes, common-garden experiments are useful for drawing robust inferences (Merilä & Hendry, 2014). This type of experiment often reveals genetic-based variation in flowering time according to latitude or altitude cline patterns (Kollmann & Bañuelos, 2004; Stinchcombe *et al.*, 2004; Hall *et al.*, 2007; Kenta *et al.*, 2011; Huang *et al.*, 2012; Franks *et al.*, 2014). This variation generally appears to be selected for by a combination of several environmental factors, such as temperature, day length, vernalization and light intensity (King & Heide, 2009). Synchronic studies, in which different populations are sampled at approximately the same time, can reveal spatial associations between environmental factors and phenotypes and are relatively easy to implement (Merilä & Hendry, 2014). Allochronic studies, in which populations are sampled and compared over time, are the most promising because, when technically possible (e.g. long-lived seeds are available), individuals produced in different years, with different genetic compositions, can be directly compared in the same common-garden trial, thus limiting the risk of environmental variation between trials (Merilä & Hendry, 2014).

Sea beet *Beta vulgaris* ssp. *maritima* (L.) shows a genetically based latitudinal gradient for flowering time along Western European coasts, with significant heritability for flowering time, critical day length and the vernalization requirement (Van Dijk *et al.*, 1997; Van Dijk & Hautekèete, 2007; Van Dijk, 2009b). Sea beet exhibits a special relationship between latitude and flowering date under common seminatural glasshouse conditions (Van Dijk *et al.*, 1997). Compared with plants from western Brittany, plants from further south and from further north both flower later, resulting in a 'V'-shaped relationship between flowering date and latitude (Van Dijk *et al.*, 1997). Plants from southern France often do not have any vernalization requirement, and then photoperiod is the only determinant of flowering induction (Van Dijk & Hautekèete, 2007). However, incomplete vernalization can potentially explain the later flowering onset in the northern part of the gradient. The vernalization requirement, that is, the length of the cold period needed to flower, strongly increases with latitude in sea beet (Boudry *et al.*, 2002). The percentage of flowering plants from a given origin is lower in more southern experimental fields and after shorter cold periods. The relatively mild (i.e. short) winters in the glasshouse are therefore assumed to retard flowering due to incomplete vernalization, with flowering date increasing with increasing latitude of

origin (i.e. the further north the origin of the plant, the later the flowering date in the common-garden experiment). If the cold period is too short, flowering is completely inhibited. However, in an experimental population, delays in flowering onset due to slightly shortened cold periods can be compensated for by artificially increasing the photoperiod (Van Dijk, 2009b).

In this study, we present a common-garden experiment to test the hypotheses that genetic changes in flowering time and the vernalization requirement for flowering have occurred over the last two decades in the sea beet *Beta vulgaris* ssp. *maritima* (L.). We compared populations sampled at two different times in the past which is possible in this species with long-lived seeds (Desprez, 1980). In all, 73 populations from 43 to 52°N were collected both in 1989 and 2009 in exactly the same places by the same person and were sown simultaneously in 2009 in a glasshouse. This experimental design is novel and promising because it combines synchronic and allochronic approaches (*sensu* Merilä & Hendry, 2014). We first tested the hypothesis that a genetic change has occurred between 1989 and 2009 in sea beet in the direction of a northward shift of flowering time phenotypes (by analogy to species range shifts). We specifically examined whether plants grown from seeds collected in 2009 flowered in the common garden at the same time as plants grown from seeds collected in 1989 in lower latitudes. We also tested the hypothesis of genetic change in the quantitative vernalization requirement over time; more specifically, that the required length of exposure to a cold period has decreased in the northern part of the gradient. Lastly, we tested the hypothesis that there is a lower frequency of plants not requiring vernalization in 2009 compared with 1989.

## Materials and methods

### Study species and plant material

Sea beet, *Beta vulgaris* ssp. *maritima* (L.), is an iteroparous, self-incompatible, wind-pollinated species with a geographically variable lifespan (see Hautekèete *et al.*, 2002). Flowering requires long days and, especially in the northern part of the species distribution, it also requires winter chilling. Vernalization occurs optimally between 3 and 8 °C (Smit, 1983), whereas the required length of the cold period varies strongly within the species (Smit, 1983; Boudry *et al.*, 2002).

Sea beet is mostly found on Mediterranean and European Atlantic coasts. In August 2009, 73 of the 93 populations sampled in August 1989 (Van Dijk *et al.*, 1997) were sampled again by the same person (the first author) at identical locations (Fig. 1). Some of the populations, especially in the southern study area, no longer existed. On the Channel Islands, only two of the original nine sites were revisited. Seeds were sampled



**Fig. 1** Map of sampled populations. White filled circles, populations used for the 'absence of vernalization requirement' only ( $n = 13$ ); grey filled circles, populations used in the 'absence of vernalization requirement' and the 'first flowering date' experiment ( $n = 24$ ); black filled circles, used in the 'absence of vernalization requirement' experiment, the 'first flowering date' experiment and in the 'quantitative vernalization requirement' experiment ( $n = 36$ ).

separately on each individual, with six to eight plants sampled per population. Plants grown from seeds collected in 1989 and 2009 from the same population did not show any visible systematic differences in germination rate, growth rate or phenotype.

### Growth conditions

Seeds sampled in 1989 and 2009 were sown simultaneously in September 2009 on commercial soil (Neuhaeus Huminsubstrat N3: 90% peat, 10% clay; pH 6; NPK 14:16:18,  $1.3 \text{ kg m}^{-3}$ ; conductivity  $35 \text{ mS m}^{-1}$ ) in a glasshouse with a constant temperature of  $20 \text{ }^\circ\text{C}$  and a 16-h photoperiod using 400-W high-pressure sodium lamps, with photosynthetically active radiation (PAR) of  $200 \mu\text{mol m}^{-2} \text{ s}^{-1}$  in the spectral range of 400–700 nm (hereafter called 'standard growth conditions'). After germination, plants of all populations and both years, hereafter called '1989 plants' and '2009 plants', were grown under these standard conditions in 1.7-L pots to carry out the experiments described below. Every 3 weeks, 1 g of fertilizer (NPK 12:12:17) was added.

### First flowering date in the glasshouse

In December 2009, two 1989 plants and two 2009 plants (collected on different mother plants in both years) of 60 populations from the coasts of the Atlantic

Ocean, English Channel or around Dover Strait (Fig. 1) were placed in a seminatural glasshouse. This is a glasshouse without any additional [artificial] light and in general without heating although frost-free (hereafter called 'semi-natural glasshouse conditions'). Fertilizer was added every 3 weeks as described previously, except in January and February. For each plant, the date of opening of the first flower was recorded in spring and early summer of 2010.

### Vernalization requirement and flowering percentage

To investigate the possibility of a decreased vernalization requirement, four 1989 plants and four 2009 plants from 36 populations along the Atlantic coast (Fig. 1) were placed in December 2009 in a vernalization chamber at  $5 \text{ }^\circ\text{C}$  and natural day length, which varies between 9:00 and 10:40 h at this time of the year at the latitude of our experimental site ( $50.61^\circ\text{N}$ ). After 8 weeks, the plants were brought back to standard growth conditions in the glasshouse, which corresponds to a shortened cold exposure and thus insufficient vernalization for flowering of at least some of the plants (Boudry *et al.*, 2002). Flowering percentage was calculated from the number of plants flowering over the total number of plants.

### Absence of the vernalization requirement

From previous studies (e.g. Van Dijk & Desplanque, 1999), it is known that most sea beets from southern Europe do not need any vernalization to flower. Such plants initially form a rosette and bolt and flower after 1–3 months under standard conditions, whereas any plant with a vernalization requirement keeps its rosette form. To investigate the possibility of a decreased frequency in the requirement for vernalization, we recorded the number of plants in each population and each sampling year that flowered under standard growth conditions within 3 months. We expected an increase in the frequency of plants flowering without vernalization. Because of the fully standardized conditions, all previous data on plants grown from 1989 seeds could be included. Seed collection years 1989 and 2009 were compared by region, only using populations that were represented in both years and flowered without vernalization in at least one of the years.

### Data analysis

To track a potential northward shift of phenotypes, we computed latitude at the shortest flowering time in the seminatural glasshouse (the lowest point of the 'V') for 1989 and 2009 as the breakpoints of segmented regressions (package 'segmented') in R version 2.15.3 (R Core Team, 2013). Slopes of each part of the segmented regressions were compared among years by a test for

the equality of slopes following Paternoster *et al.* (1998). If 1989 and 2009 slopes were not significantly different, the shift in days could simply be converted into a shift in latitude and distance in km using the average values of the slopes over the two sampled years around the breakpoints. To make comparisons with data from the literature easier, we also converted shifts over the 20-year period into shifts per decade.

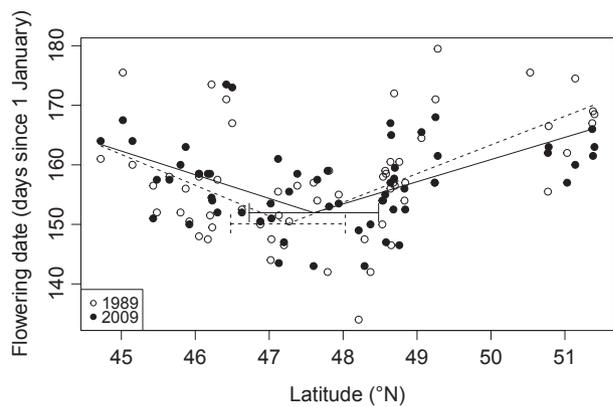
To test the hypothesis of earlier flowering in the southern latitudes of the gradient and later flowering in the northern latitudes of the gradient, we used a three-step analysis. First, we tested year and latitude effects using a generalized additive model (GAM) with flowering date as a dependent variable, year as a level factor and latitude as a smoother to determine the significance of both factors (package 'mgcv' in R, following Crawley, 2013). Second, we assessed the pairwise relationship between flowering date and year conditioned on latitude. We identified the parts of the gradient in which year had the strongest effect on flowering date using conditioning plots (package 'graphics'). Conditioning plots graphically show how the effect of a variable  $Y$  (i.e. year) on a dependent variable  $X$  (i.e. flowering date) is affected by another variable  $Z$  (i.e. latitude). Because latitude is a continuous variable, it was automatically split into overlapping intervals. Third, we tested for a year effect according to latitude. One-tailed Wilcoxon paired signed-rank tests were used to test for significant shifts in flowering time in the 2009 plants with respect to the 1989 plants in the areas of the gradient identified in the conditioning plots. Because we expected a shift towards higher latitudes, plants from the southern portion of the gradient were expected to display later flowering with time, and plants from the northern portion were expected to flower earlier.

Comparisons in flowering percentage between the 2 years for the quantitative and absolute vernalization requirement experiments were carried out using one-tailed exact binomial tests with the expectation of a decreased vernalization requirement (alternative hypothesis: true probability of success, that is, the number of plants flowering without vernalization or with incomplete vernalization, is higher in 2009 than in 1989, respectively).

## Results

### First flowering date under common glasshouse conditions

To track a potential northward shift of phenotypes, we performed segmented regressions on 1989 and 2009 plants (Fig. 2; effect of latitude in 1989: left regression,  $P_{\text{left}} = 0.0056$ , right regression,  $P_{\text{right}} = 0.0001$ ; in 2009:  $P_{\text{left}} = 0.1782$ ,  $P_{\text{right}} = 0.0002$ ). Breakpoints of these segmented regressions occurred at latitude  $47.25^{\circ}\text{N} \pm$



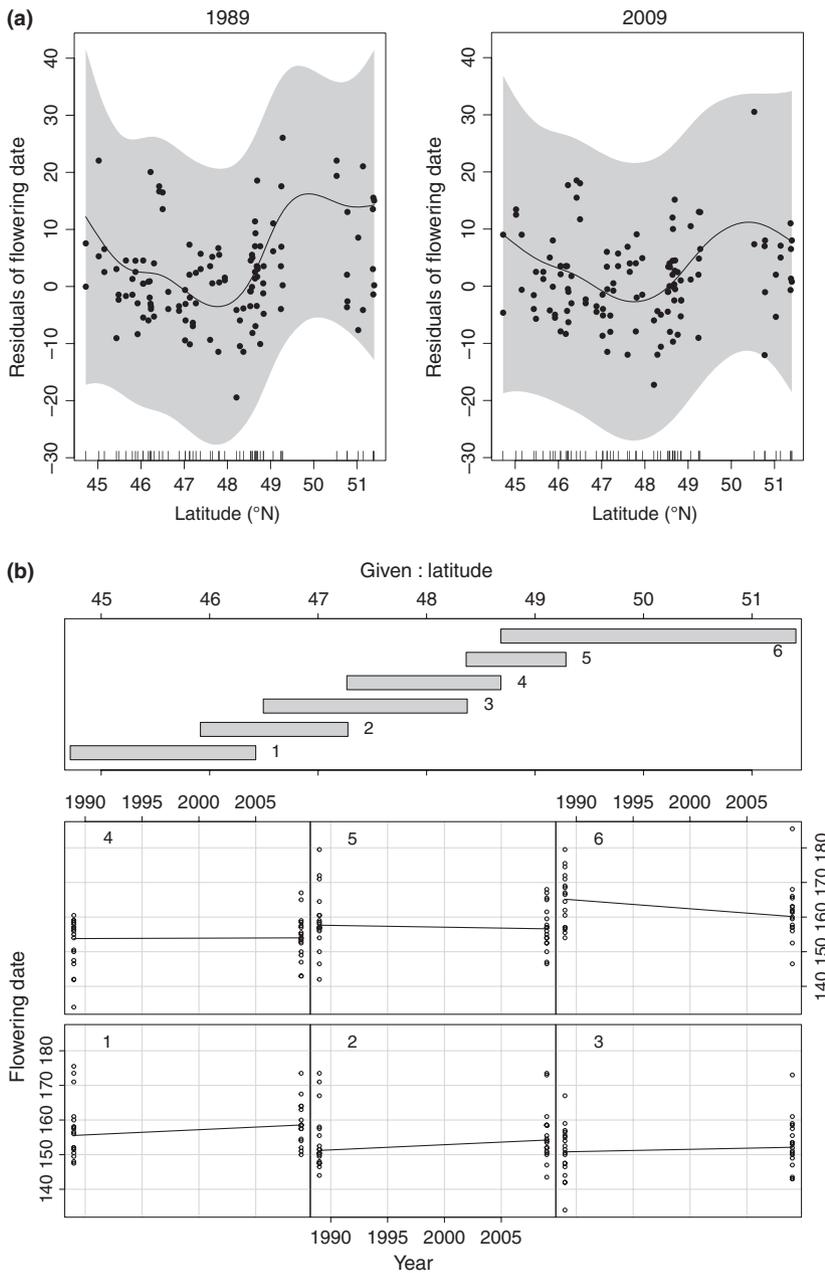
**Fig. 2** Segmented regressions of flowering date (number of days since 1 January) on latitude for the populations sampled in 1989 (white circles and dashed regression lines) and 2009 (black circles and solid regression lines). The confidence intervals of the breakpoints are shown.

$0.39$  (mean  $\pm$  SE) for 1989 and  $47.60^{\circ}\text{N} \pm 0.43$  for 2009 (Fig. 2), suggesting a northward latitudinal shift in flowering date of  $0.35^{\circ}$ . Slopes on the left side of the breakpoints were  $-5.185 \pm 2.377$  and  $-3.970 \pm 1.757$  in 1989 and 2009, respectively, and on the right side of the breakpoints  $4.821 \pm 1.120$  and  $3.729 \pm 1.065$  in 1989 and 2009, respectively. Neither the left nor right slopes differed significantly between years at  $\alpha = 0.05$  following Paternoster *et al.* (1998).

To test the hypothesis of later flowering in the southern latitudes of the gradient and earlier flowering in the northern latitudes of the gradient, we first tested year and latitude effects. The GAM (Fig. 3a) revealed a significant year effect ( $P < 2e^{-16}$ , e.g. steeper increase at around  $49^{\circ}\text{N}$  in 1989 than in 2009) and of the smoothing term latitude in both years (1989: estimated degree of freedom (e.d.f.) = 6.376,  $F = 5.212$  and  $P = 2.33e^{-05}$ ; 2009: e.d.f. = 5.010,  $F = 3.299$  and  $P = 0.0049$ ; 37.7% of the deviance explained, generalized cross-validation (GCV) score = 56.46,  $R^2$  (adj) = 0.311).

Second, we determined the areas of the gradient where year had the strongest effect on flowering date. Conditioning plots (Fig. 3b) showed positive effects of year on flowering date below latitude  $47.28^{\circ}\text{N}$  and a negative effect above latitude  $48.66^{\circ}\text{N}$ , as observed in the GAM (Fig. 3a).

Third, to assess the significance of these positive and negative effects, we carried out Wilcoxon paired signed-rank tests on flowering date on both ranges separately. Below latitude  $47.28^{\circ}\text{N}$ , a significantly later flowering date was found ( $n = 26$ ,  $z = -1.72$ ,  $P$  (one-tailed) = 0.04 and mean = +1.78 days). Above latitude  $48.66^{\circ}\text{N}$ , a significantly earlier flowering date was observed ( $n = 18$ ,  $z = 2.05$ ,  $P$  (one-tailed) = 0.02 and mean = -4.04 days). Therefore, 2009 plants from the southern latitudes of the gradient showed significantly



**Fig. 3** Effect of year on the relationship between flowering date and latitude. (a) Residuals of flowering date in a generalized additive model, with latitude as a smoothing term, by year (estimated degrees of freedom: 6.38 and 5.01, respectively). Shaded regions show the confidence bands. (b) Conditioning plots of flowering dates by year on six latitudinal ranges shown as overlapping grey bars. Panels read from bottom left to bottom right and then from top left to top right and correspond to latitudinal ranges shown by the overlapping grey bars above from bottom to top. Each panel shows the results for its correspondingly numbered latitudinal range (see grey bars).

later flowering onset in the glasshouse than 1989 plants, whereas those from the northern latitudes showed significantly earlier flowering onset.

Since 1989 and 2009 slopes were not significantly different, it was possible to convert the shift in days into a shift in latitude and distance in km using the average values of the slopes over the two sampled years on either side of the breakpoints (i.e. southern portion:  $-4.5775$  and northern portion:  $4.275$ ). Considering that, at this latitude,  $1^\circ$  equals 111 km, the shift in flowering date of  $+1.78$  days found in the southern portion of the gradient (below  $47.28^\circ\text{N}$ ) corresponds to

a northward shift of  $0.388^\circ$  or 43.2 km over the 20-year period, that is, the 2009 plants flowered in the glasshouse at the same moment as 1989 plants from about 43 km further south. The mean shift of  $-4.04$  days in the area north of  $48.65^\circ$  corresponds to a mean shift of  $0.945^\circ$  or 104.9 km further north.

#### Vernalization requirement and flowering percentage

As expected, a considerable number of plants south of  $47.28^\circ\text{N}$  flowered after 8 weeks in the vernalization chamber, whereas almost none of the plants north of

48.66°N did so. There were thus three latitudinal ranges: < 47.28°N, 47.28°N – 48.66°N and > 48.66°N, and we tested for differences between the two sampling years (Fig. 4). There was a significantly higher proportion of plants flowering in 2009 compared with 1989 in the transition zone 47.28°N – 48.66°N of the 'V' using a one-tailed exact binomial test ( $P = 0.03623$ ). The two other ranges showed nonsignificant differences (<47.28°,  $P = 0.7954$  and > 48.66°,  $P = 1$ ).

### Absence of the vernalization requirement

All Mediterranean and inland populations, and 19 of the 66 Atlantic coastal populations (all south of 47.8°N) showed in one or both years plants that flowered with no vernalization. None of these categories showed significantly higher flowering without exposure to a cold period in 2009 than in 1989 (Table 1).

### Discussion

This study provides one of the rare cases of evidence for genetic change in response to climate change (Merilä, 2012; Merilä & Hendry, 2014) for both flowering date under glasshouse conditions and one of its underlying triggers, the vernalization requirement. The extreme longevity of sea beet seeds in controlled storage conditions provided the opportunity to distinguish genetic change from phenotypic plasticity; most studies are only able to demonstrate the latter source of variability (Gienapp *et al.*, 2008). The interyear comparison of flowering in the glasshouse revealed that flowering is triggered by new values of environmental stimuli, with a northward shift of phenotypes. The flowering date in 2009 plants corresponded to that of 1989 plants growing further south. This result suggests that there has been genetic change – and not just genetic drift – with selection among local genotypes and possibly gene flow, driven by climate change. The northward shift in flowering date and the vernalization requirement can be interpreted as a response to a warmer climate during the growing season. Microevolution might have occurred

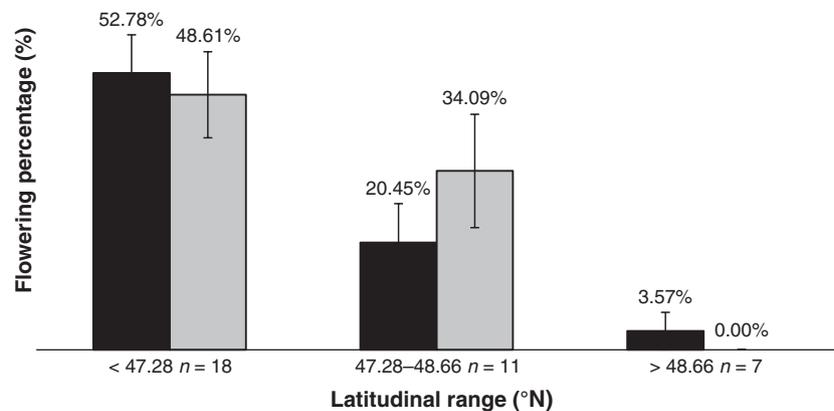
*in situ*, owing to the high heritability of the trait (0.33 within populations, Van Dijk *et al.*, 1997). However several mechanisms, other than microevolution, may be at work. Long-distance pollen flow is documented in the close relative *Beta vulgaris* ssp. *vulgaris* (Fénart *et al.*, 2007), but this species occurs inland, where winds can easily connect distant populations. Along the coast, dominant winds are rarely parallel to the shore; nonetheless, sea beet seeds are thought to be sea-transported (thalassochory). Long-distance gene flow is therefore probable in this species and may have participated in the observed genetic change. Maternal effects may also play a part, although the consequences of maternal effects on evolutionary potential are currently controversial (Ghalambor *et al.*, 2007; Danchin *et al.*, 2011).

Nevertheless, the changes observed in the glasshouse do not imply that plants have actually changed their flowering time *in situ*. Although common-garden experiments are useful tools for examining the genetic component of variation, the flowering times obtained in these experiments have no direct relationship with *in situ* flowering times (Kawecki & Ebert, 2004; Montague *et al.*, 2008; Novy *et al.*, 2013). It is possible that this genetic change only compensates for environmental change, resulting in maintaining flowering date *in situ* (which can be compared with the concept of counter-gradient genetic variation, *sensu* Conover & Schultz, 1995, but over time).

### The shift in flowering date under common glasshouse conditions

We found a northward latitudinal shift of flowering date phenotypes of 0.35° (around 38 km) in the glasshouse for 1989 vs. 2009 plants between latitudes 44.7 and 51.4°N. The 2009 plants from the southern latitudes showed significantly later flowering in the glasshouse compared with the 1989 plants, and significantly earlier flowering was observed in the most northern end of the latitude gradient.

The observed shift in flowering date in the southern latitudes of the gradient was about 1.78 days later over



**Fig. 4** The percentage of flowering (and their standard errors) after cold exposure for 6 weeks in three latitudinal ranges < 47.28°N, 47.28°N to 48.66°N and > 48.66°N (see text). Black bars: 1989 plants; grey bars: 2009 plants. ( $n$ ) = number of populations per year (four tested plants per population per year).

**Table 1** Comparison of the percentage of plants flowering without any cold treatment in 1989 and 2009 by region (Mediterranean coasts, inland populations and Atlantic coasts), tested using a one-tailed exact binomial test (H0: 2009 shows the highest proportion of plants without any vernalization requirement).

Region	Number of populations	1989		2009		P
		No. of plants	No. of flowering plants (%)	No. of plants	No. of flowering plants (%)	
Mediterranean	4	46	35 (76)	30	26 (89)	0.1229
Inland	3	90	77 (86)	30	22 (73)	0.9778
Atlantic	19	646	54 (8)	191	15 (8)	0.6374

the 20-year period, corresponding to a northward shift of 43.2 km. In other words, the flowering date of 2009 plants was similar to that of 1989 plants located 43.2 km further south. The northern latitudes of the gradient, that is, north of 48.6°N, showed a significantly earlier flowering date of about 4 days over the 20-year period, corresponding to a mean northward shift of phenotypes of about 104.9 km. In the northern part of the gradient, the cold requirement plays a role that will be discussed below. The lifespan of sea beet under glasshouse conditions in this latitudinal range is estimated at 5–7 years (Hautekète *et al.*, 2002); however, the actual number of generations elapsed over the 20-year period is difficult to determine *in situ*. Therefore, we cannot calculate per-generation estimates.

The observed northward shift of about +0.194° or ca. 21.6 km per decade in the southern portion, and of +0.47° or 52 km per decade in the northern end of the gradient, is higher than the average observation of species distribution area shifts (ca. 6.1 km per decade; Parmesan & Yohe, 2003). This particularly high gradient shift rate may principally be due to the fact that genetic variation is locally high (Van Dijk *et al.*, 1997), allowing for short-term change. Moreover, gene flow is thought to be relatively high in sea beet, possibly bringing new genotypes from southern locations.

The observed northward shift offers new elements that have the potential to contribute to an ongoing debate on the relative roles of genetic change vs. phenotypic plasticity in phenotypic change due to climate change (Lavergne *et al.*, 2010). In particular, genetic change may mitigate, in some species and to some extent, the consequences of climate change. It is, however, not clear whether the rate of this genetic change is sufficient to mitigate completely the consequences of environmental change, especially in the near future. The observed change in the southern portion of the gradient was indeed about 1.78 days over a 20-year period, which is much lower than observations from phenological surveys in the field (around 2.5 days per decade; Tooke & Battey, 2010), which likely integrate both microevolution and phenotypic plasticity. However, in the northern end of the gradient, the change was about –2 days per decade, which is of similar magnitude to that reported in other studies.

### The role of the vernalization requirement

The flowering date experiment in the seminatural glasshouse provided indirect evidence of genetic change towards lower vernalization requirements in the northern latitudes of the study area. The 'V'-shaped relationship between flowering date and latitude in sea beet when grown under glasshouse conditions results from contrasting mechanisms. The negative relationship observed in the southern latitudes of the gradient is due to warmer conditions in the south, requiring higher critical temperature sum values to induce flowering at the right time of the year. The positive relationship between flowering date and latitude for the northern latitudes of the gradient appears to stem from a positive latitudinal gradient on the quantitative vernalization requirement (Boudry *et al.*, 2002). The effective duration of the cold period under glasshouse can thus limit flowering in plants from more northern latitudes as postulated in Van Dijk *et al.*, 1997; see Supplementary Material for a more formal demonstration). As suggested by Tooke & Battey (2010), climate change can have opposing effects: (i) in the southern latitudes of the gradient, flowering induction is mediated only by spring temperatures and (ii) in the northern latitudes of the gradient, winter chilling is a limiting factor. If the principal environmental factor controlling flowering onset in the southern latitudes of the gradient is the temperature sum, as it is reported for several species (Langlet, 1971; Solantie, 2004), there has been a systematic increase in this sum. If this mechanism is extrapolated to the northern latitudes, it can be assumed that the same pattern of a temperature sum requirement, decreasing with latitude but increasing over the course of the past few decades, also applies to the northern region, and we would expect later flowering in the north as well. The earlier flowering observed in the northern portion of the gradient is therefore probably due to a genetic change towards a decreasing vernalization requirement.

Winters are colder *in situ* than in the seminatural glasshouse, where conditions are relatively Mediterranean. This raises the question of whether vernalization intensity is really a limiting factor under natural conditions, although the gradient for quantitative vernalization

requirement suggests so and has also been observed in other species (Cook *et al.*, 2012). The principal role of vernalization is to prevent the plant from flowering too early. Spring temperatures, which are lower in the northern part of the study area than in the southern part, may induce young plants to flower in their first year (Van Dijk & Desplanque, 1999). The vernalization requirement coincides with a reduction in the duration of the growing season with increasing latitudes. Boudry *et al.* (2002) confirmed that the threshold value for the vernalization requirement increased with latitude. There is therefore strong selection pressure to decrease the vernalization requirement under a warming climate.

The quantitative vernalization requirement experiment led to a somewhat contrasting outcome (Fig. 4). Plants from latitudes above 48.6°N apparently did not reach the vernalization threshold within 6 weeks in the vernalization chamber, and they could not be used in our interyear comparison. On the other hand, an unexpected absence of change was found for the plants from latitudes south of 47.28°N. The vernalization requirement appears to stabilize, with about 50% of the plants flowering after 6 weeks of vernalization. The plants from the transition zone 47.28–48.66°N showed a significant shift from 20% in 1989 to 34% in 2009. In the transition zone, there apparently has been a genetic change towards a decreased requirement for exposure to cold temperatures, although it remains unclear why plants from the southern part of the study area did not show any genetic change. One reason could be that the selection pressure weakens above a certain temperature range.

### Absence of the vernalization requirement

The vernalization requirement is thought to control the timing of first reproduction, which should not happen too early in the life of an individual. Early reproduction could indeed have a negative effect on fitness, through either increased post-reproductive mortality or a detrimental effect on future reproduction. Environmental factors selecting for the vernalization requirement may be climate-related or not, but the fact that it is controlled by temperature and that the total absence of vernalization requirement is more frequent in the southern part of the study area (Van Dijk *et al.*, 1997; Boudry *et al.*, 2002) suggest that genetic change in this trait can occur with climate change.

The proportion of plants tested under standard conditions that did not show any cold requirement did not change significantly during the 20-year period in the study area. It is possible that the selection pressure favouring flowering in the first summer after germination derives from environmental instability rather than from effective season length (Hautekète *et al.*, 2009). Flowering without requirement for a cold period may

be important in human-disturbed situations such as arable fields and roadsides to promote spring germination even when no or little cold period is experienced. In southern regions, with early autumn or early spring germination (both are possible *in situ*; Wagmann *et al.*, 2010), immediate flowering in the first spring may be selectively favourable. A weak vernalization requirement is then sufficient, postponing flowering to the following year if germination occurs in late autumn. Under this hypothesis, in the southern area where the absolute vernalization requirement is apparently only marginally selected for, increasing temperature should not be a strong selective pressure. However, in intermediate or northern regions, maintaining the same life cycle may require a decreased vernalization requirement, and plants without any vernalization requirement may be more frequent. This was not supported by our results, suggesting that vernalization requirement is still selected for in the north, while fine-tuned adjustments are mediated by a change therein.

A remaining question is whether the disturbance level (e.g. due to human activities or climate, Hautekète *et al.*, 2002, 2009) has decreased during our study period, selecting for higher levels of the vernalization requirement, thus counter-balancing any climate change effect. Disturbance did not appear to have any effect according to our observations during sampling, although further investigations are required to settle this issue.

### Conclusion

Our general hypothesis of a shift in flowering induction towards northern latitudes was confirmed. Flowering occurred significantly later under standard glasshouse conditions in the southern latitudes of our study region, which may have been due to an increased temperature sum to induce flowering and maintain flowering date *in situ* in the same, most favourable part of the year. A more complicated picture was obtained for the northern latitudes due to the role of the vernalization requirement. Here, the change in flowering date in the glasshouse could well be due to a compensatory effect of an increasing temperature sum, as in the south, as well as a dominating decrease in the vernalization requirement. In the south, where the vernalization requirement was almost absent, a predominant role of disturbance may explain the absence of genetic change in this requirement.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Data S1** The effect of incomplete vernalization on flowering date. Each year from 1990 to 1999, seeds from all populations sampled in 1989 were sown in September under standard growth conditions and after 3 months transferred to the seminatural glasshouse where they were evaluated for flowering date for the rest of their lifetime as described in Van Dijk (2009a,b).

**Figure S1** The difference in flowering date between young and old plants to assess the effects of incomplete vernalization on flowering time independently of the effect of plant age on flowering time.

Data deposited at Dryad: doi:10.5061/dryad.1h0b7

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