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Abstract

Background and aims – The annual hemiparasitic plant genus *Rhinanthus* displays large variation in the date of onset of flowering, and ecotypes have been described from populations with different flowering times. Much less is known, however, about the variation in flowering time within populations of an ecotype, which is important for the adaptive capacity of a population. The number of nodes produced before the first flower is an important trait linked to flowering time differences among populations, and this trait and its relation with flowering date were investigated. Methods – Seeds from a natural, early-flowering population of *Rhinanthus angustifolius*, mown in early July, were used to establish a new field population in 2003, mown after summer, and to cultivate plants in the greenhouse in 2001 and 2004. The onset of flowering, node number and plant size were recorded in the field population in 2005 and in 2008. In the greenhouse, germination date, node number and flowering da...

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Within-population variation in the relation between node number and flowering time in *Rhinanthus angustifolius* (Orobanchaceae)

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Background and aims – The annual hemiparasitic plant genus *Rhinanthus* displays large variation in the date of onset of flowering, and ecotypes have been described from populations with different flowering times. Much less is known, however, about the variation in flowering time within populations of an ecotype, which is important for the adaptive capacity of a population. The number of nodes produced before the first flower is an important trait linked to flowering time differences among populations, and this trait and its relation with flowering date were investigated.

Methods – Seeds from a natural, early-flowering population of *Rhinanthus angustifolius*, mown in early July, were used to establish a new field population in 2003, mown after summer, and to cultivate plants in the greenhouse in 2001 and 2004. The onset of flowering, node number and plant size were recorded in the field population in 2005 and in 2008. In the greenhouse, germination date, node number and flowering date were recorded.

Key results – Flowering time was strongly correlated with node number in all years: the production of more nodes before the first flower was associated with a delay in flowering. There was always considerable variation around the median flowering date among plants with the same number of nodes, even in the greenhouse. Removing variation in the timing of germination in the greenhouse experiment did not reduce variation in flowering date. Part of the remaining variation was due to variation in plant size: larger plants flowered earlier. After five years, the relaxed selection on flowering time by mowing later had increased node number and delayed flowering in the new field population.

Conclusions – Both genetically determined (node number) and phenotypically plastic (plant size) traits contribute to variation in flowering time within populations, and even under strong selection against late flowering, wild populations may harbour enough variation to react to a decrease in this selection pressure by later mowing.

Key words – Adaptation, annual, ecotype, flowering time, node number, Orobanchaceae, phenology, plant size, *Rhinanthus*, selection.

INTRODUCTION

The transition to the reproductive stage is one of the key life-history events, and variation in age and size at maturity usually has large effects on fitness (Stearns 1992). One important element of timing is phenology, the timing of life-history events within a season (Rathcke & Lacey 1985, Forrest & Miller-Rushing 2010). Flowering should take place when abiotic environmental conditions permit the development of flowers, when there is enough time left for fruit and seed formation, and when other plants of the same species are flowering as well, to achieve sufficient synchrony (Ollerton & Lack 1992, Fenner 1998). Biotic factors in the environment, such as pollinators and herbivores, may also put constraints on

flowering time and act as selective agents (Pilson 2000, Elzinga et al. 2007, Ehrlén 2015). Environmental factors such as temperature, photoperiod and humidity are used as cues to initiate the onset of flowering (Rathcke & Lacey 1985), but the extensive molecular-genetic research on flowering time regulation in *Arabidopsis thaliana* has shown that a number of genes are involved whose expression is controlled not by environmental, but by endogenous factors. These are considered to be part of the autonomous pathway (Mouradov et al. 2002). Variation in flowering time is often brought about by the plant itself: bigger plants develop faster and flower earlier than smaller plants, especially in perennials (Ollerton & Lack 1998), and the timing of vegetative development can

put constraints on the timing of subsequent flowering events (Sola & Ehrlén 2007).

Differences in flowering phenology can be achieved in three ways: by starting development earlier or later, by developing quicker or slower, or by changing the stage in which flowering takes place (Diggle 1999). The latter seems to be the case in the genus *Rhinanthus* (Orobanchaceae), hemiparasitic annuals of hay meadows and extensive pastures. Seeds are dormant and require cold stratification during winter in order to germinate, so seedlings will only emerge in early spring (ter Borg 2005), but development time after germination can vary widely. Within each species, ecotypes are found with hugely different flowering times, ranging from vernal ecotypes that flower in May up to autumnal ecotypes that flower only in August–September (ter Borg 1972, von Soó & Webb 1972, Zopfi 1993a, 1995). These are thought to have evolved in response to different mowing/grazing regimes, with vernal ecotypes occurring in meadows mown in July and autumnals growing in sites where mowing is much later or absent (ter Borg 1972). The ecotypes have a strikingly different morphology, which in former days has led to the description of separate species for each ecotype and staggering numbers of species within the genus. Nowadays, these ecotypes, which also occur in other genera within the Orobanchaceae, such as *Euphrasia* (Zopfi 1998a, 1998b) and *Melampyrum* (Štech 2000, Štech & Drábková 2005, Dalrymple 2007), and in genera such as *Gentianella* (von Wettstein 1901) are recognised as subspecies, varieties or forms (von Soó & Webb 1972). *Rhinanthus* is a well-studied genus in this respect, and several studies have been published on the morphological differentiation between the ecotypes in several species (Campion-Bourget 1970, 1982, ter Borg 1972, Zopfi 1993a, 1993b, 1995). Using genetic markers, it has been shown that the subspecies status of the different ecotypes is often not justified: populations of the same morphological ecotype are not genetically related in *Rhinanthus minor* L. in the United Kingdom (Houston & Wolff 2012) and *R. alectorolophus* (Scop.) Pollich in southwestern Germany (Pleines et al. 2013).

One of the most persistent differences between the ecotypes is the number of nodes produced on the main stem before the first flower appears, which is thought to be directly linked to the change in the developmental stage in which flowering occurs (Diggle 1999): when flowering is postponed, a plant will produce more sterile nodes before the first node with flowers. Early-flowering ecotypes produce few sterile nodes (5–10), with long internodes, while late-flowering ecotypes produce many sterile nodes (up to 25) with very short internodes. Common garden experiments (ter Borg 1972, Champion-Bourget 1982, Zopfi 1993b) have demonstrated that differences among populations in flowering time and node number persist under identical environmental conditions.

Much less is known, however, about the relationship between node number and flowering among individuals within a population. Zopfi (1993b, 1995) showed that the relationship also holds among plants within populations of *Rhinanthus alectorolophus* and *R. glacialis* Personnat, but he used means for plants that flowered in the same week and not individual data points. If little variation in flowering time is

found within node numbers, and flowering time is largely genetically determined by node number, this would mean that pollination would mainly happen between plants with roughly the same number of nodes, especially when plants produce few flowers and have a short flowering duration (Devaux & Lande 2008). This would increase the degree of assortative mating and induce a segregation between early and late-flowering plants in the population (Fox 2003, Weis & Kossler 2004, Devaux & Lande 2008). Selection on flowering time will have a much faster response when variation in flowering time is largely genetic (i.e. the number of nodes under the first flower). But if other factors cause a large variation in flowering time even among plants with the same node number, then mating will be more random and the response to selection slower.

The aim of this paper is to determine the relationship between node number and flowering time within a population of *Rhinanthus angustifolius*, both under natural, outdoor conditions and in the greenhouse, and to quantify variation in flowering time among plants with identical node numbers. In our greenhouse experiments, seeds are germinated in a cold room before being planted, so we know the date at which the seedlings emerge. This stands in contrast to our field studies, where it is not possible to accurately record the date of germination or even emergence, since seedlings are often hidden in the vegetation at first. If differences in germination date are responsible for additional variation in flowering time (within node number classes), we should find less variation in flowering date in the greenhouse than under field conditions.

MATERIALS AND METHODS

Study species

Rhinanthus angustifolius C.C.Gmel. is an annual hemiparasitic plant of hay meadows. It is widely distributed across Europe (von Soó & Webb 1972), but since it does not support intensive agricultural management with application of fertilisers and early mowing, it is mostly restricted to nature reserves in the west of Europe and non-intensively used meadows elsewhere, with late mowing and no fertiliser application, as other *Rhinanthus* species (Westbury 2004). It can parasitise a wide range of herbaceous host plants, with the highest flower and seed production on grasses and legumes (ter Borg 1972). It is pollinated by bumblebees, which visit the flowers frequently for their nectar (Kwak et al. 1985, Natalis & Wesselingh 2012). The ecotypes occurring in the Netherlands have been studied in detail by ter Borg (1972). Seeds germinate in early spring (February–March) after stratification by winter cold (ter Borg 2005), and the cotyledons emerge after development of the radicle and start forming the aboveground stem. Attachment to host roots takes place at this early stage. Each node carries a pair of buds, one on each of two opposite sides of the square stem. The nodes alternate in the use of the four stem sides, so that two opposite sides of the stem only have buds at even nodes, and the other two sides at uneven nodes. The first node is the one that carries the cotyledons, and subsequent nodes first produce only vegetative buds, which may or may not develop into branches (fig. 1). After a certain number of vegetative nodes, the transition to flowering is made and no

more vegetative buds are formed hereafter on the main stem. The first reproductive node can either have one or two fully developed flower buds, or two early-aborted flower buds, in which case this node is sterile and called an intercalary node (Campion-Bourget 1970). Flowering commences with the first fully formed flowers of the main inflorescence and proceeds upwards, each pair of flowers opening about 1–3 days after the previous pair, depending on the ambient temperature. The inflorescence is an indeterminate spike, and the number of nodes with flowers varies with the nutrient status of the plant. If the plant develops branches, these will start producing flower buds after a few vegetative nodes, and these flowers usually open after the main inflorescence has finished flowering, although there can be some overlap. In large plants, the vegetative nodes on first-order branches can

develop second-order flowering branches, and higher-order branches can be found as well.

Observations in a semi-natural meadow population

A population of *Rhinanthus angustifolius* has been present since 2004 in a meadow next to the university greenhouses in Louvain-la-Neuve. This meadow is mown once a year, at the end of August at the earliest, and contains a diverse vegetation of grasses and forbs that is allowed to develop naturally. In December 2003, several thousands of *R. angustifolius* seeds, harvested earlier that year in a natural population of the aestival ecotype in nature reserve Doode Bemde (Heverlee, Belgium) had been sown on the site, which previously did not have any *Rhinanthus*. The size of the population, estimated as the number of flowering plants at peak flowering, was estimated in 2006 and 2007 as 2,600 and 3,500 flowering plants, respectively.

Field observations were performed in the meadow population in 2005 and 2008. In 2005, all plants that reached anthesis during the first week of flowering were marked (63 plants), and after that a random subsample of around thirty plants that started flowering in a given week, up to 10 June. The last fifteen plants were marked between 11 and 23 June. All plants were individually marked with a unique number on a piece of masking tape around the stem, and sketches were made of their relative positions to facilitate their localisation. After fruit set, all plants were harvested, and node numbers (number of vegetative nodes and number of intercalary nodes) were determined. The diameter of the square stem was measured in the two directions in the middle of the fifth internode to the nearest 0.01 mm with digital callipers, and the two measures were multiplied to obtain stem section area in mm². This measure is highly correlated with the number of flowers (2005: $n \text{ flowers} = 3.987 - 0.024 \text{ area} + 0.630 \text{ area}^2$, adjusted $R^2 = 0.8928$, $F_{2,168} = 709.1$, $P < 0.0001$; 2006: $n \text{ flowers} = 1.498 - 3.157 \text{ area} + 0.173 \text{ area}^2$, adjusted $R^2 = 0.9161$, $F_{2,104} = 567.6$, $P < 0.0001$) and with the number of seeds produced (2006: $n \text{ seeds} = -9.463 + 34.116 \text{ area} + 0.605 \text{ area}^2$, adjusted $R^2 = 0.8637$, $F_{2,99} = 313.7$, $P < 0.0001$), and is thus a good proxy for both biomass and fitness.

In November 2007, seeds were sown in a part of the meadow that did not contain *Rhinanthus* in 2007. Seeds from several populations were sown in each of ten 40 cm × 100 cm plots, and the data presented here are for two of the populations used in that experiment. One of these plots was sown with 200 seeds from the source population Doode Bemde, collected in July 2007, hereafter referred to as DB, and the second with 200 seeds collected from the meadow itself in summer 2007, referred to as LLN. All the plants that emerged in each plot were individually marked and their flowering date recorded. All marked plants were harvested after fruit set, after which node numbers and stem section area were determined.

Observations in the greenhouse

In November 2000, 200 seeds collected in June 2000 in the same natural population as used for sowing the semi-natural population mentioned above, the nature reserve Doode Be-

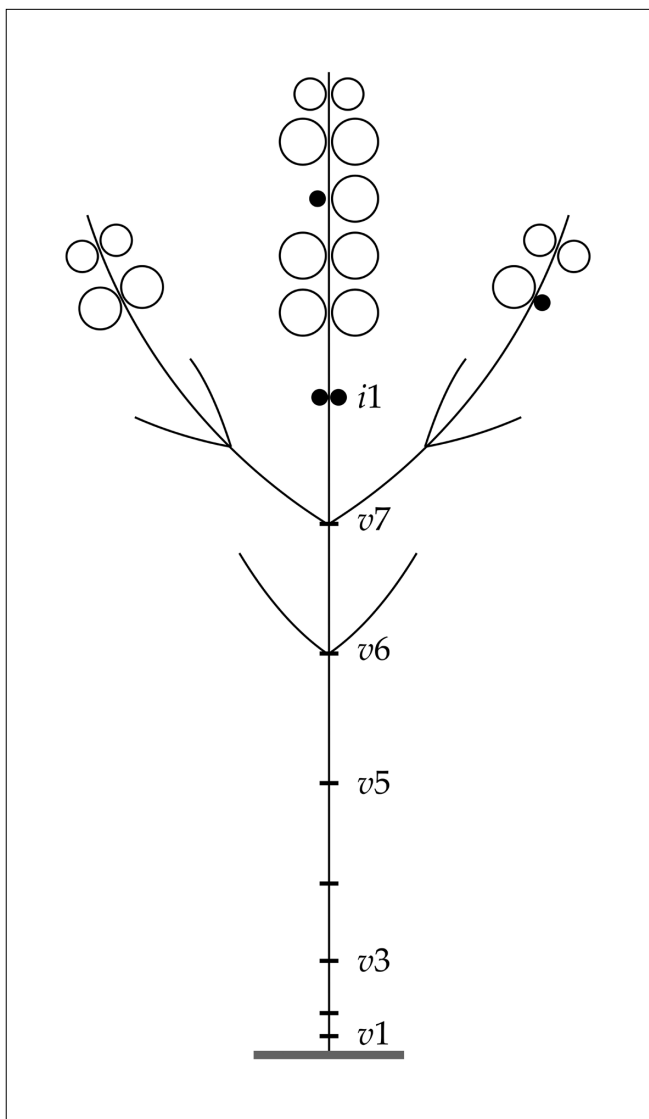


Figure 1 – Schematic representation of a *Rhinanthus* plant. Leaves and bracts are not shown. Open circles are fully developed flowers/fruits, small filled circles aborted flowers. Horizontal notches represent vegetative nodes, numbered $v1$ to $v7$, with the last two nodes bearing branches. Node $i1$ is an intercalary node, the total number of nodes under the first flower in this example is eight.

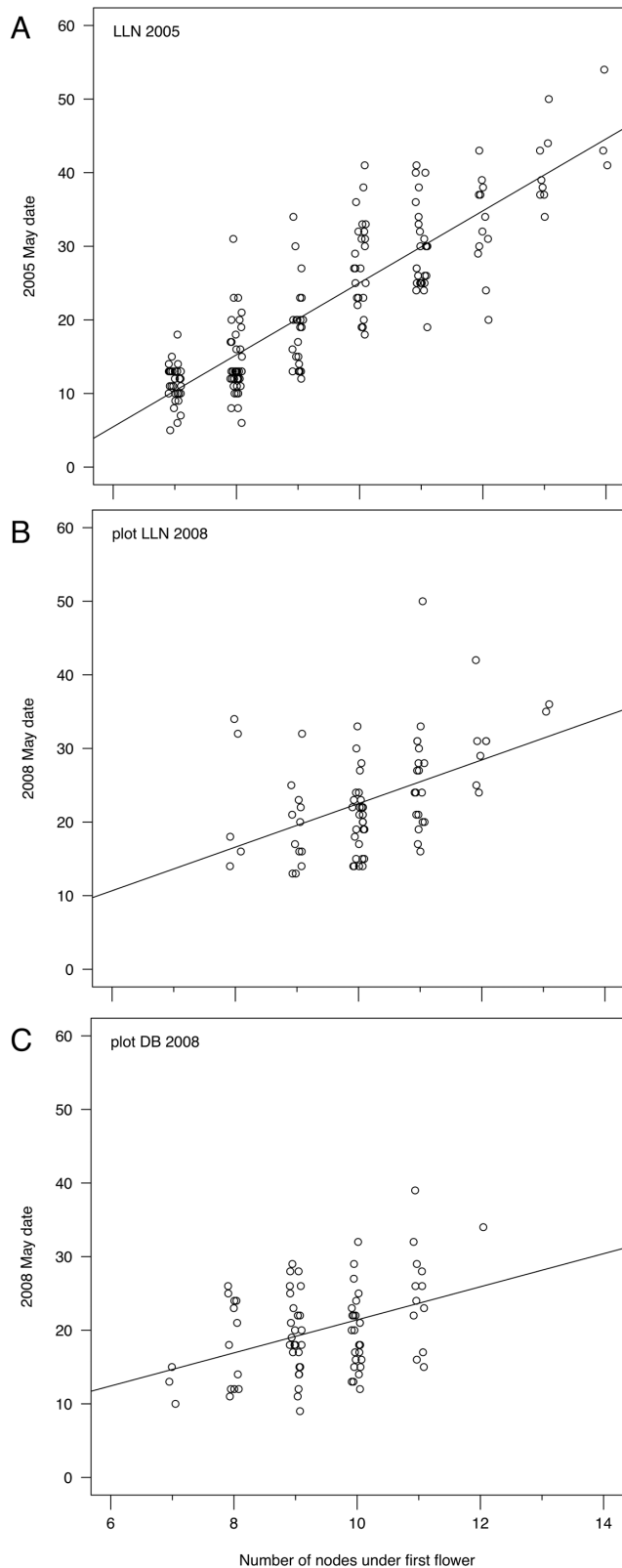


Figure 2 – The relationship between the total number of nodes under the first flower and the date of flowering (1 = 1 May) in: A, the field population in 2005, and the two plots in 2008 (B, Louvain-la-Neuve; C, Doode Bemde). The symbols are randomly scattered horizontally around their node number value to improve visibility of the individual data points.

mde (Heverlee, Belgium), were put in four Petri dishes, 50 seeds per dish, on moist filter paper and put in a refrigerator at $5 \pm 2^\circ\text{C}$ for stratification. Germination, which was considered to have taken place when the tip of the radicle protruded from the seed, started after 53 d, on 8 January 2001. Seedlings were kept in the fridge until both cotyledons had emerged from the seed coat, and then transplanted into pots in a heated greenhouse, at ages ranging from 24 to 49 days. Each pot contained one host plant (*Trifolium pratense* L.) and a maximum of two *Rhinanthus* plants. A total of 47 plants survived until flowering, which started on 12 April 2001. For each plant, the date of germination, the date of transplantation, the date of opening of the first flower, the number of flowers, the number of vegetative nodes and the number of intercalary nodes were recorded.

Seeds were collected in June 2002 in the same source population Doode Bemde and kept dry in closed containers in a refrigerator to preserve viability. On 9 January 2004, 100 seeds were put in two Petri dishes on moist filter paper in a refrigerator for stratification, and this was repeated on 16 January 2004 with two more Petri dishes with 50 seeds each. Germination started on 9 February 2004. For each seed, the date of germination was noted and seedlings were transplanted about 4 weeks (26–35 d) after germination into square pots (10 cm \times 10 cm \times 10 cm) containing 0.75 L of a 1:1 mixture of sand and potting soil placed in a heated greenhouse. Each pot contained one established host plant (*Trifolium repens* L.), which had been grown from seed sown on 9 January 2004. A maximum of two seedlings were planted in the same pot, in opposite corners. A total of 103 plants survived until flowering, which started on 29 April 2004. For each plant, the date of germination, the date of transplanting, the date of opening of the first flower, the number of flowers, the number of vegetative nodes and the number of intercalary nodes were recorded.

Data analysis

For the field data, flowering date was transformed to May date, the number of days after 30 April of that year. For the plants in the greenhouse, we used age at flowering, calculated as number of days after germination or the number of days after transplantation to the greenhouse. Linear regressions between total node number and May date (field data) or age at flowering (greenhouse data) were calculated for each year and plot separately. Differences between the two plots (field) and years (greenhouse separately) were tested with an initial linear model with node number as the covariate, year as a fixed factor, including the interaction between year and node number to test for heterogeneity of slopes. The model was then simplified if possible by removing the non-significant interaction, assuming homogeneity of slopes between years. The effect of biomass, estimated as stem section area at the 5th internode, on flowering time was investigated by calculating linear regressions between flowering date and stem section area within each node class in the 2005 field data set, and by including stem section area as a second factor in the linear model for flowering date. The deviation around the median date for each node number class in both field and greenhouse data was calculated using the median

absolute deviation (mad), since the flowering dates were not normally distributed within node numbers. Vegetative node number distributions in the field were compared between years and populations of origin with pairwise Kolmogorov-Smirnov tests. All statistical analyses were performed in R version 3.1.3 (R Core Team 2015).

RESULTS

Flowering time in the meadow population

In 2005, flowering started on 5 May, and complete data were obtained for 171 plants, the last one starting flowering 49 days later, on 23 June. The relationship between total node number and flowering date was highly significant (linear regression: $May\ date = 4.890\ nodes - 23.867$, adjusted $R^2 = 0.754$, $P < 0.0001$; fig. 2). The median absolute difference for each node number class was fairly low for the 7- and 8-node classes (1 and 2 days, respectively, despite large sample sizes; fig. 3) and reached its maximum, 4.5 days, in the 10-node class.

Within each node class, part of the variation in flowering date could be attributed to plant biomass, estimated by stem section area (fig. 4). Plant biomass generally decreased with flowering date, except in the two extreme node classes 7 and 14, and the relationship was significant in node classes 10 and 12 and near significance in node class 11. This indicates that larger plants flowered more rapidly, while small-

er plants took longer (linear regression: $May\ date = 4.921\ nodes - 0.911\ area - 22.033$, $n = 171$, adjusted $R^2 = 0.778$, $P < 0.0001$).

In 2008, flowering started on 9 May in the DB plot, in which a total of 80 plants were observed (last plant starting flowering on 8 June), and on 13 May in the LLN plot (last plant on 19 June), which yielded complete data for 75 plants. The relationship between node number and flowering date was again highly significant (fig. 4). The initial linear model included the interaction between node number and plot, which was not significant. The simpler model obtained by removing this interaction (linear regression: $May\ date = 2.607\ nodes + 0.971\ LLN - 4.4497$, $n = 155$, adjusted $R^2 = 0.2149$, $P < 0.0001$) had a parameter estimate of 0.971 for the LLN plot, indicating that plants in this plot, at equal node numbers, flowered around one day later in this plot than in the DB plot. The slope of the regression line of flowering date on node number was 2.607, so each extra node produced was associated with a further delay in flowering by around 2.6 days. Again, including stem section area increased the variance explained by the linear model (linear regression: $May\ date = 2.1566\ nodes + 2.288\ LLN - 2.6992\ area + 4.9996$, $n = 155$, adjusted $R^2 = 0.4665$, $P < 0.0001$), with an advance of 2.7 days of the flowering date per extra mm^2 stem section area. At equal node number and stem section area, the plants in the LLN plot flowered on average more than two days later than those in the DB plot.

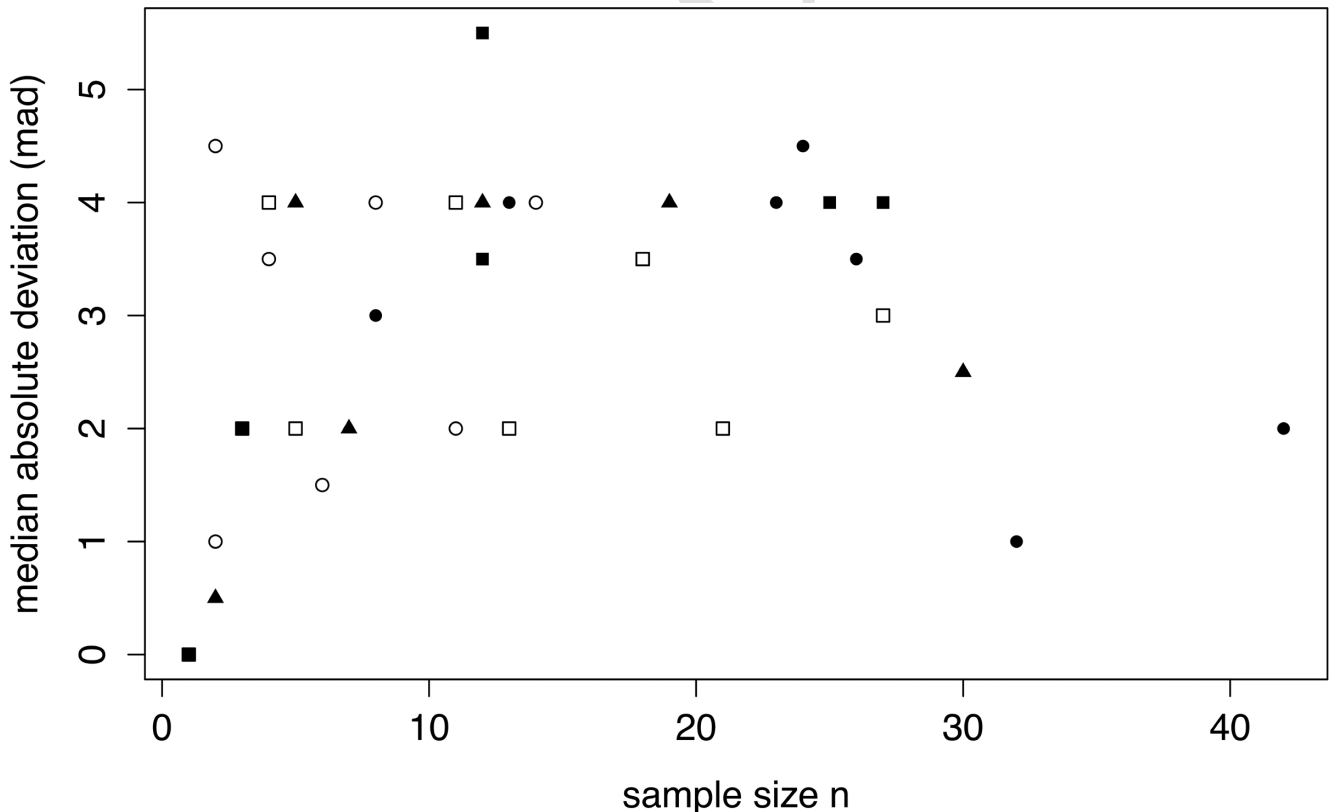


Figure 3 – The median absolute deviation as a function of sample size, given separately for each node number class in the field samples (black symbols) in 2005 (● black circle), plot LLN (▲ black triangle) and plot DB (■ black square), and in the greenhouse experiments (open symbols) in 2001 (○ open circle) and 2004 (□ open square).

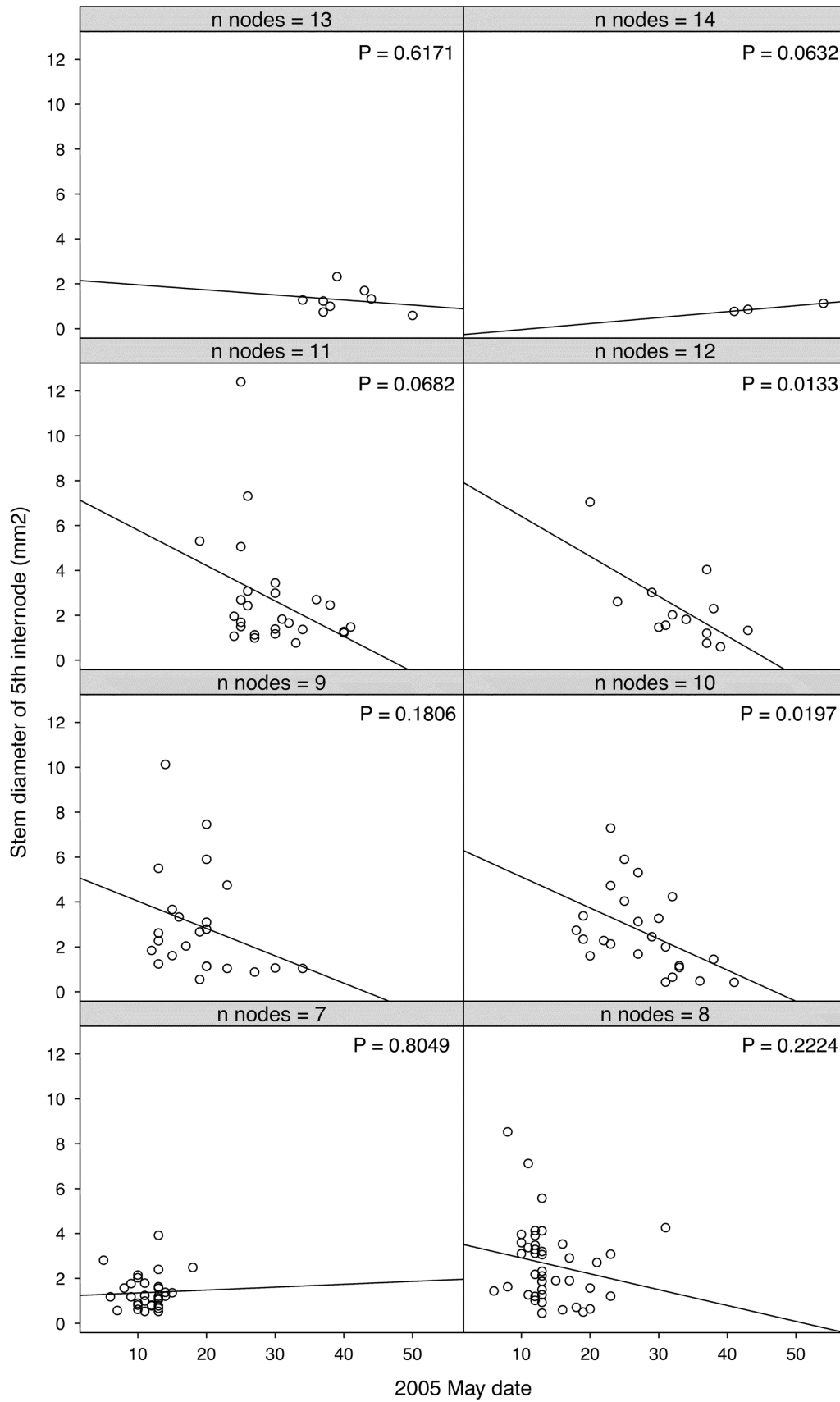


Figure 4 – The relationship between date of flowering and plant biomass, estimated by the stem section area at the 5th internode, per node class in the field population observed in 2005. P-values indicate the significance of the slope of the regression line.

Node number comparison among years

The frequency distributions of the number of vegetative nodes in the three field groups (fig. 5) all differed significantly from each other (LLN 2005 vs. plot LLN 2008: $D = 0.3895$, $P < 0.0001$; LLN 2005 vs. plot DB 2008: $D = 0.2311$, $P \approx 0.0059$; plot LLN 2008 vs plot DB 2008: $D = 0.3033$, $P \approx 0.0016$).

Flowering time in the greenhouse

In both greenhouse experiments, the age at flowering, measured as the number of days since transplantation, was strongly correlated with the number of nodes under the first flower (2001: $n = 47$, $age = 26.032 + 2.800 \text{ nodes}$, $R^2 = 0.325$; 2004: $n = 103$, $age = 24.560 + 2.289 \text{ nodes}$, $R^2 = 0.511$; fig. 6). The initial linear model showed that the slope was not significantly different between the two years (interaction year \times node number not significant), but there was a significant difference between the years. The model without the interac-

tion (and thus with an identical slope of 2.431 for both years) showed that the difference in intercept was 7.249 days: plants in 2001 were on average a week older at flowering than the plants in 2004. In both years, the relationship was stronger for the number of days since transplantation than for the number of days since germination (days since germination: adjusted $R^2 = 0.164$ in 2001; adjusted $R^2 = 0.432$ in 2004). The deviation around the median in each node class, as measured by the median absolute value, was comparable to the results from the field observations (fig. 3).

DISCUSSION

The data from the field show that variation in flowering date among plants can be explained by both node number and biomass, with an increase in node number adding to flowering time and an increase in biomass accelerating flowering. De-

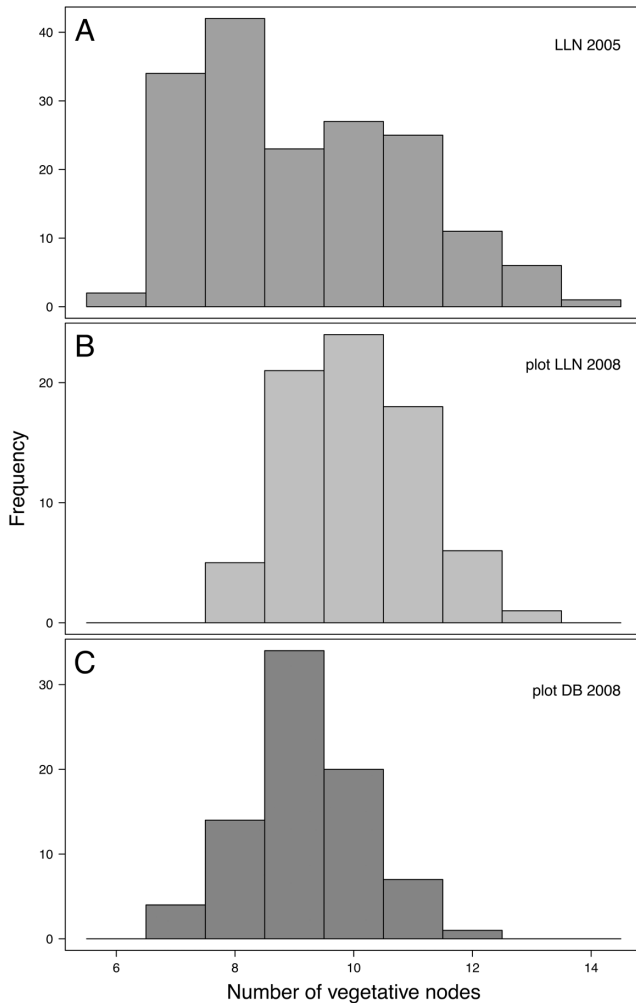


Figure 5 – Frequency distributions of the number of vegetative nodes in: A, the field population in 2005; B, the plot sown with seeds from Louvain-la-Neuve in 2008; C, the plot sown with seeds from the source population Doode Bemde in 2008.

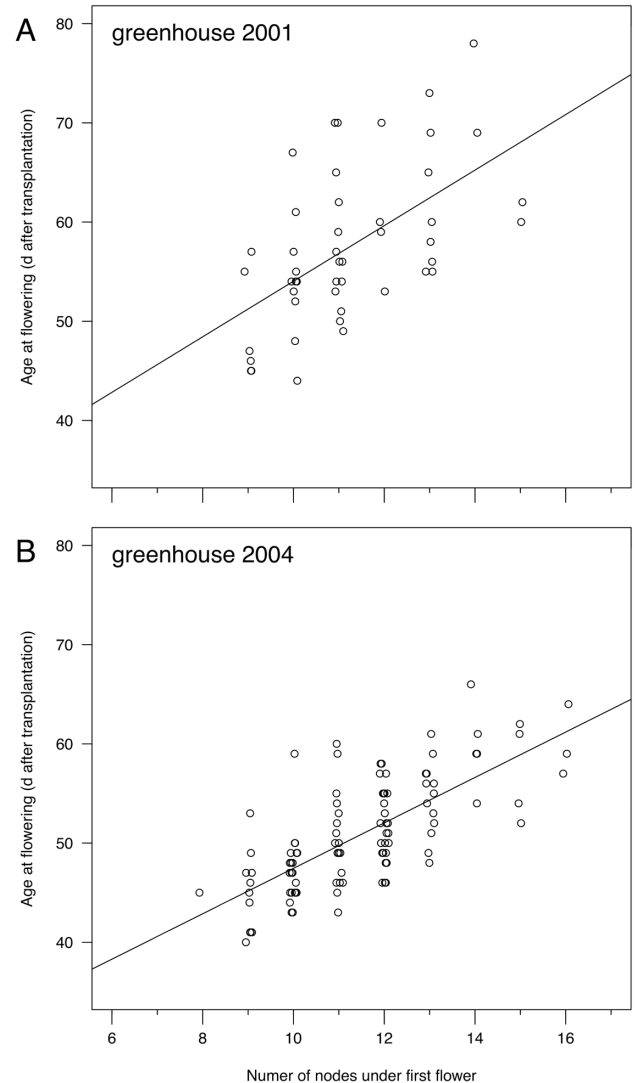


Figure 6 – The relationship between the number of nodes under the first flower and the age at flowering, counted as the number of days since transplantation of the seedling to the greenhouse, in the greenhouse experiments: A, 2001; B, 2004.

spite the fact that the emergence (transplantation) date was known in the greenhouse, the flowering dates in the greenhouse were not less variable than in the field, suggesting that emergence date plays a minor role in determining flowering time, either because emergence is highly synchronised in the field (which is not likely, judging from the spread in germination dates in our experiments), or because later emergence is at least partly compensated for by a more rapid development, potentially caused by higher temperatures later in spring. An alternative explanation may be that despite the standardized conditions in the greenhouse, differences in growing conditions (degree of attachment to the host) in the greenhouse may have caused variation in biomass and therefore in flowering time. Unfortunately, stem diameter was not measured in the greenhouse. A study in which emergence is recorded under outdoor conditions is clearly needed to elucidate the importance of seedling emergence time in determining flowering date under natural conditions.

The difference in slope between the two field studies (2.6 days per node in 2008 vs. 4.9 days per node in 2005) can be explained by a clear difference in growing conditions between the two years. The average temperature in May 2008 was 16.4°C, the warmest May month on record in Belgium (RMI 2015), while it was 13.4°C in May 2005 (only 12.9°C from 5 May onwards), which means that plants with higher node numbers took longer to reach anthesis after the first plants with lower node numbers had started flowering in 2005. In 2008, the number of days per node came close to what was measured in the greenhouse in both years (2.4 days per node), illustrating the strongly accelerating effect of higher ambient temperatures. The difference between the two greenhouse experiments (a delay of one week in 2001 compared to 2004) is also likely due to differences in temperature in the greenhouse, which was heated, but not cooled. Transplantation to the greenhouse started a month later in 2004 than in 2001, and the plants are thus likely to have experienced higher temperatures in 2004. These observations also provide information on the possible effect of anthropogenic climate change on flowering time in *Rhinanthus*. Where other species may lag behind because of minimum daylength requirements (Parmesan & Hanley 2015), *Rhinanthus* does not have such constraints: it can follow global warming with an accelerated vegetative development, leading to earlier flowering. There could however be a problem if winter temperatures are not sufficiently low for seed stratification, which takes at least eight weeks, or if the cold period is interrupted by sufficiently long warm periods to induce secondary dormancy (ter Borg 2005). In that case, germination would be delayed or even prevented completely. This could probably play a role at the southern limits of the geographical range of the species.

Similar results for the number of days per node were found across populations of different ecotypes of *Rhinanthus glacialis* (Zopfi 1995) and *R. alectorolophus* (Zopfi 1993b) grown in three common gardens in Switzerland: 4.30 and 4.83 days per node in Zürich (420 m a.s.l., earliest start of flowering 26 April and 5 May 1989, respectively), 3.92 and 3.58 days per node in Bilten (490 m a.s.l., 8 and 17 May 1989) and 4.32 and 3.79 days per node in Schwanden (700 m a.s.l., 10 and 21 May 1989). Since *Rhinanthus* plants usu-

ally open one pair of flowers (on one node) per day, and the number of nodes in the inflorescence can range up to 8 or 9, flowering times of plants with different node numbers are likely to overlap at some point, especially for larger plants that continue producing flowers on branches after the main inflorescence.

The total number of nodes under the first flower is a combination of the number of intercalary nodes and the number of vegetative nodes. The former is in part determined genetically (autumnal ecotypes have more intercalary nodes; Campion-Bourget 1970, Zopfi 1993b), but there is also phenotypic plasticity: plants that develop slowly and produce few flowers often have more intercalary nodes than their larger counterparts (unpubl. data from greenhouse experiments). The number of vegetative nodes is largely genetically determined (narrow-sense heritability estimate from parent-offspring regression $h^2 = 0.94$ for *R. angustifolius*, unpubl. data) and the main trait associated with ecotypic differentiation in flowering time among populations (Zopfi 1993b, 1995). There were clear differences in the frequency distributions of vegetative nodes between the population of origin (Doode Bemde) and the Louvain-la-Neuve population after two years (2005) and five years (2008). While the wider range of vegetative node numbers in 2005 compared to 2008 is probably due to the large difference in sample size between the two years ($n = 171$ vs. $n = 75-80$), the comparison between Doode Bemde and Louvain-la-Neuve in 2008 shows a clear shift towards higher node numbers (and concurrent later flowering), which is likely the result of a relaxation of the selection pressure imposed by mowing. The meadows in Doode Bemde are always mown in the first week of July, while in Louvain-la-Neuve, mowing does not take place before the end of August, and often later. Early mowing exerts a strong selection force against higher node numbers, since these plants flower too late to produce ripe seeds before they are mown. Later mowing will allow plants with more vegetative nodes, and hence also potentially more flowering branches, to develop fully and produce more seeds than the earlier genotypes, with often do not develop flowering branches. Later mowing will thus allow the persistence of more node number classes and genetic variation in flowering time within a population.

In conclusion, there is considerable variation in flowering time within the early flowering population, which can be partly explained by differences in node number and biomass. Where vegetative node number fixes the earliest possible flowering date relative to plants with other node numbers within the population, plant biomass modulates this date. Plants with low vegetative node numbers but low biomass flower later, also because they make more intercalary nodes, and they will be exchanging gametes with flowering plants with high node numbers and high biomass. This is likely to render strictly assortative mating for node number impossible. The difference in vegetative node number distribution with the source population after five years of relaxed selection shows that even strongly selected populations apparently still harbour sufficient genetic variation to allow an expansion towards higher node numbers. A selection experiment with differential mowing regimes applied on subpopulations founded from the same seed source is currently under way,

and will show how quickly the response is to increased selection for early flowering.

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The manager of the nature reserve Doode Bemde kindly gave permission to collect *Rhinanthus angustifolius* seed in the reserve. Véronique Ducarme, Jérôme Vrancken, Linda Dhondt and Hélène Ghyselincx helped with scoring the plants in the greenhouse, and Pauline Boslé counted nodes and measured stem diameters of the plants from the field experiment in 2008. This is publication number BRC356 of the Biodiversity Research Centre of the Université catholique de Louvain.

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