RESEARCH PAPER

Atmospheric nitrogen deposition on petals enhances seed quality of the forest herb *Anemone nemorosa*

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ABSTRACT

- Elevated atmospheric input of nitrogen (N) is currently affecting plant biodiversity and ecosystem functioning. The growth and survival of numerous plant species is known to respond strongly to N fertilisation. Yet, few studies have assessed the effects of N deposition on seed quality and reproductive performance, which is an important life-history stage of plants.
- Here we address this knowledge gap by assessing the effects of atmospheric N deposition on seed quality of the ancient forest herb *Anemone nemorosa* using two complementary approaches.
- By taking advantage of the wide spatiotemporal variation in N deposition rates in pan-European temperate and boreal forests over 2 years, we detected positive effects of N deposition on the N concentration (percentage N per unit seed mass, increased from 2.8% to 4.1%) and N content (total N mass per seed more than doubled) of *A. nemorosa* seeds. In a complementary experiment, we applied ammonium nitrate to aboveground plant tissues and the soil surface to determine whether dissolved N sources in precipitation could be incorporated into seeds. Although the addition of N to leaves and the soil surface had no effect, a concentrated N solution applied to petals during anthesis resulted in increased seed mass, seed N concentration and N content.
- Our results demonstrate that N deposition on the petals enhances bioaccumulation of N in the seeds of *A. nemorosa*. Enhanced atmospheric inputs of N can thus not only affect growth and population dynamics *via* root or canopy uptake, but can also influence seed quality and reproduction *via* intake through the inflorescences.

INTRODUCTION

An ever-increasing number of anthropogenic stressors are acting on vegetation worldwide (Vellend *et al.* 2017). One of the key factors currently affecting plant biodiversity and ecosystem functioning is elevated atmospheric nitrogen (N) inputs (Bobbink *et al.* 2010; Simkin *et al.* 2016). Globally, atmospheric deposition of biologically reactive N more than tripled from 1860 to the early 1990s (Galloway *et al.* 2004), and remains high in many parts of Europe to date (Tørseth *et al.* 2012). The decades-long elevated N input is implicated with already

significant losses of terrestrial plant diversity in many ecosystems across the globe (De Schrijver *et al.* 2011; Dirnböck *et al.* 2014; Basto *et al.* 2015; Simkin *et al.* 2016). One of the main reasons for plant species losses after N enrichment is through differential and species-specific N uptake and growth and, as a result, changed competitive interactions (Hautier *et al.* 2009).

Atmospheric deposition of ammonia (NH_3), ammonium (NH_4^+), nitrogen oxides (NO_x) and nitrate (NO_3^-) can occur either as wet (with rainfall and snow) or dry (input of particulate and gaseous compounds) deposition. The most widely
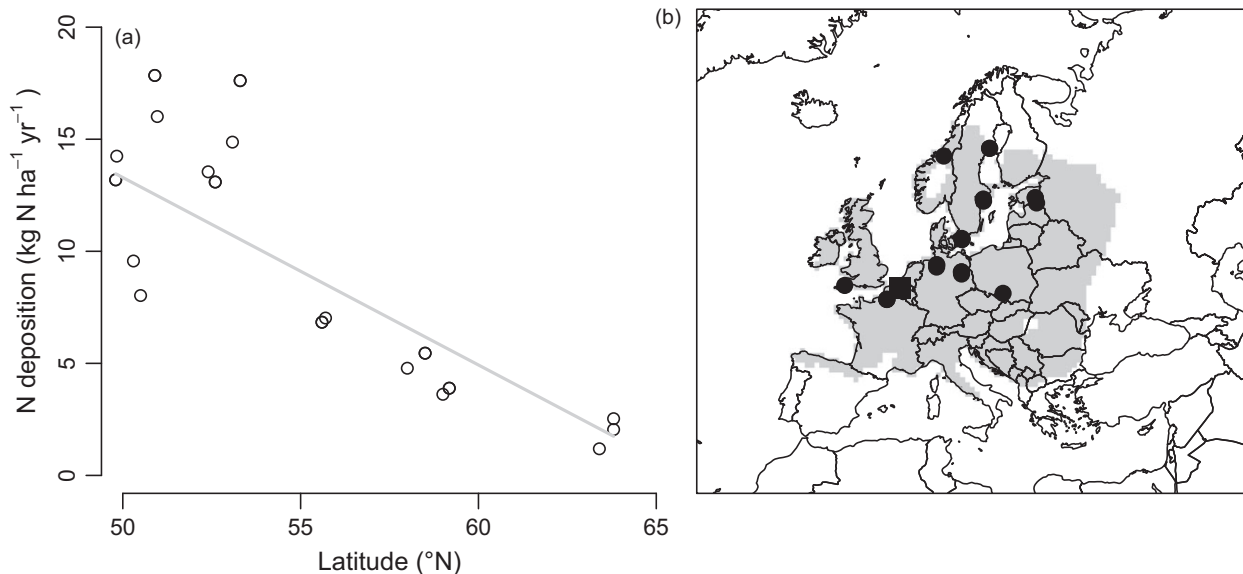
Fig. 1. Illustration of the N deposition gradient used in this study. (a) Relationship between latitude (°N) and N deposition rates (kg·N·ha^{-1}·year^{-1}) of the study sites. The fitted grey line denotes a mixed-effect model with random effect term 'region'. (b) Distribution range of *A. nemorosa* after Hultén & Fries (1986) (grey shaded area), sampling sites along the N deposition gradient (circles and square) and location of the experiment (square).

studied pathway of how the elevated input of atmospheric N is acquired by plants is either through the root system or through aboveground foliar surfaces (canopy exchange; Adriaenssens *et al.* 2012). However, much less is known of how other plant organs contribute to the uptake of atmospherically available N (Carlo & Norris 2012). Recently, it was found that N ion uptake through permeable petal surfaces can be significant (up to 44% mass recovery rates), and N is subsequently allocated to ovaries and developing seeds (Carlo & Norris 2012). This newly found pathway of petal N absorption and incorporation into seeds can be especially important for plants with a high petal surface area to (total) seed mass ratio. Seed N concentration (percentage N per unit seed mass) and content (total N mass per seed) are key proxies for seed quality: they are positively associated with seedling fitness, establishment and survival, defence against herbivores (*e.g. via* synthesis of N-containing secondary compounds such as alkaloids) and reproductive success (Parrish & Bazzaz 1985; Hanley *et al.* 2004; Naegle *et al.* 2005; De Frenne *et al.* 2011c). This pathway is thus of potentially high ecological importance although it has never been tested under realistic conditions of N deposition in the field.

Here we focus on the herbaceous forest understorey plant *Anemone nemorosa*, an important representative of the group of 'ancient woodland indicator species' in temperate Europe. The species is renowned for its typical slow colonisation rates and associated failure to colonise recently established post-agricultural forests from, even nearby and adjacent, ancient forest patches (Brunet & von Oheimb 1998; Verheyen *et al.* 2003; Baeten *et al.* 2010; De Frenne *et al.* 2011a; Brunet *et al.* 2012). *Anemone nemorosa* is also an excellent study species to examine the effects of N deposition on its regeneration from seed because: (i) it has relatively large petals (tepals) compared to its plant size and a relatively large flower surface area to capture both wet and dry deposition; (ii) it flowers during the spring (April–May), which is an active period for farmers spreading fertilisers on grasslands and croplands, with these activities

typically resulting in high atmospheric concentrations of NH$_3$ (Cools *et al.* 2016); (iii) being a vernal geophyte, it flowers before canopy tree leaves flush and is thus more exposed to wet N deposition due to the lower precipitation interception by the tree canopy at that time (cf. reduced 'shelter effect').

We here report the results of two complementary studies: first, we used a macroecological approach and sampled *Anemone* seeds over 2 years (2009 and 2013) across its European distribution range, thereby taking advantage of the spatiotemporal continental-scale gradients in N deposition rates (typically decreasing from south to north; Fig. 1a). Second, we performed a controlled N addition experiment in which a solution of ammonium nitrate was applied to the petals and leaves of *A. nemorosa* and to the soil. We specifically addressed the hypotheses that (i) enhanced N deposition results in increased seed quality as quantified by means of the seed mass, N concentration and N content in the seeds; and (ii) only N addition specifically to the petals, rather than leaves or soil, increases seed quality.

MATERIAL AND METHODS

Study species

Anemone nemorosa L. (wood anemone; Ranunculaceae) is a perennial vernal forest rhizomatous geophyte common in deciduous Eurasian woodlands. Within Europe, the species has a latitudinal range from northern Spain to northern Sweden, and longitudinally from Ireland to the Ural (Hultén & Fries 1986; Fig. 1b). Most closely associated with ancient woodlands (Verheyen *et al.* 2003; De Frenne *et al.* 2011a), *A. nemorosa* grows on not too nutrient-poor and acidic forest soils, from relatively dry to wet. Shoots emerge between March and May and flowering starts a few weeks later. Flowers (one per ramet) have typically six white petals (tepals), are hermaphrodite and mostly self-incompatible and insect-pollinated, although

selfing does occur (Müller *et al.* 2000). The achenes (mean 10–30 per flower) contain a single seed and are gravity-, slug- and ant-dispersed between May and June (Delatte & Chabrerie 2008; Türke *et al.* 2010). The indistinct elaiosome (appendix of the fruit, an adaptation to myrmecochory) of *A. nemorosa* is, in comparison to other myrmecochorous species, relatively rich in the fatty acids oleic acid and palmitic acid (Pfeifer *et al.* 2010), neither of which contain N. Achenes (henceforth referred to as 'seeds') do not form a persistent seed bank (Eriksson 1995) or only a small short-term seed bank (Plue *et al.* 2012). Vegetative spread through rhizomes is common (Philipp & Petersen 2007), but sexual reproduction is considered to be more important for population spread (Brunet & von Oheimb 1998; Müller *et al.* 2000). *Anemone nemorosa* typically has very slow colonisation rates (Brunet & von Oheimb 1998; Verheyen *et al.* 2003; De Frenne *et al.* 2011a; Brunet *et al.* 2012).

Study 1: Seed sampling along an N deposition gradient – macroecological approach

Seed sampling

In 2009 and 2013, seeds were sampled in 11 regions across Europe thereby taking advantage of the spatiotemporal continental-scale variation in N deposition rates (Fig. 1a and b). All populations were large (>10,000 ramets) and situated in deciduous forest (mixed forests dominated by deciduous species in northern Sweden and Estonia) with 50–95% canopy cover unaffected by major recent disturbances (thinning, grazing, etc.). In 2009, two populations per region were sampled, each >1 km apart (eight regions, 15 populations in total; only one population was sampled in northern Sweden due to rarity in that region). In 2013, one population per region was sampled (11 populations from different regions). This resulted in a set of 26 populations × year combinations. At every collection site, we sampled and pooled the seeds of 30–50 randomly chosen ramets per population. Seed sampling was always performed at seed maturity, *i.e.* exactly at the moment when natural seed dispersal occurred (May–June).

Nitrogen deposition and climate data

Since we are not working in sites with high topographic heterogeneity and in close vicinity to important point emission sources, the best available standardised N deposition data at the continental scale were obtained from the European Monitoring and Evaluation Programme database (EMEP; http://www.emep.int). EMEP is the 'Co-operative Programme for Monitoring and Evaluation of the Long-range Transmission of Air Pollutants in Europe' and provides scientific information on the emission, transport and deposition of air pollutants in 50×50 km grid cells covering Europe (Tørseth *et al.* 2012). Atmospheric deposition rates were calculated by summing the modelled rates of wet and dry oxidised and reduced N and expressed as $kg \cdot ha^{-1} \cdot year^{-1}$. Data from 2009 and 2013 were used for the seed sampled in the respective years. For simplicity, we assumed a dry deposition factor equal to 1 (*i.e.* the factor by which dry deposition can be increased due to vegetation roughness; Erisman & Draaijers 2003). Data on N deposition ranged between $1.19 \, kg \cdot ha^{-1} \cdot year^{-1}$ (Trondheim, central Norway) and $17.85 \, kg \cdot ha^{-1} \cdot year^{-1}$ (Ghent, Belgium; Fig. 1a). Climate data were obtained from the CRU CL2.0 dataset using

Fetchclimate 2 (Microsoft Research Cambridge, http://fetchclimate2.cloudapp.net). We extracted the mean annual and spring (February–May) temperature and precipitation for each population for 2009 and 2013, depending on the year of seed sampling. Only the mean annual temperature and precipitation were used in the analyses because these were strongly correlated with the spring temperature and precipitation (Pearson $r = 0.951$, $P < 0.001$ and $r = 0.911$, $P < 0.001$, respectively). The effects of soil characteristics (*e.g.* pH, soil P, Ca, K, Mg, N, P) were not considered here since their effects on seed mass and N concentrations were shown to be negligible in an earlier study (De Frenne *et al.* 2011c).

Study 2: N addition experiment

Establishment of the experiment

The second study was a controlled addition experiment in which an N ion solution was added with a paintbrush to the petals and leaves of *A. nemorosa* and to the soil using a design adapted from Carlo & Norris (2012). Rhizomes with emerging ramets were collected in 2013 before flowering from an ancient deciduous forest dominated by *Fraxinus excelsior* and *Quercus robur* in central Belgium (square in Fig. 1b; 50.975° N, 3.804° E). The experiment was also performed at this location. Following collection, rhizomes were transplanted immediately into 1.5–l pots on 8 April 2013 using top 10 cm of soil from the collection site. Pots were installed in the forest but below transparent rain shelters (installed *ca.* 1 m above the plants) such that no natural precipitation could reach the pots, soil or plants. All pots were arranged randomly and watered throughout the experiment with distilled water as needed, carefully avoiding the plant tissues. We used five replicate pots for each of the nine treatment groups (control and a factorial combination of N application type and concentration, see next section). All individuals were flowering when monitored on 12 April 2013.

Treatments

Plants were treated with aqueous ammonium nitrate solutions using two complementary approaches. First, N was applied with a paintbrush (type Marabu Forte 6) by repeatedly inserting the paintbrush in the N solution and then using a constant number of paintbrush strokes to deposit the solution. Specifically, leaves were treated with five paintbrush strokes randomly spread across the three leaves surrounding each flower. Petals were also treated with five strokes randomly spread across the six petals of each flower (carefully avoiding the carpels and anthers). To also apply the N solution to the soil, a circular filter paper of *ca.* 20 cm² was installed on the soil surface immediately surrounding the plant stem and also treated with five paintbrush strokes (after which the filter paper was rinsed with distilled water such that the N was washed into the soil and became potentially available to the plant; Carlo & Norris 2012). Control plants were treated in exactly the same way, but using distilled water on the paintbrush. Second, the experiment included a treatment in which whole plants were sprayed with the same N solutions. This whole-plant approach mimicked a natural rainfall event by spraying all aboveground parts of the plants with a handheld sprinkler until water drops were visible on the entire plant. In all treatments, N solutions were applied over four occasions (on 15, 16, 23 and 26 April 2013).

Nitrogen solutions and concentrations

We used ammonium and nitrate as N forms because these are the most abundant N ions in precipitation: the concentration of ammonia and nitrate in bulk open-field precipitation in central Belgium amounts to 0.85 and 0.50 mg·N·l^{-1}, respectively (Verstraeten *et al.* 2012), adding up to 1.35 mg·N·l^{-1}. The long-term average precipitation during the flowering period of *A. nemorosa* (*ca.* 1 month) is 48 mm (1981–2010 April average for a weather station at 890 m from the experiment) resulting in deposition of 64.8 mg·N·m^{-2} (or 0.648 kg·N·ha^{-1}). This means that the N dose on the foliage of an average *A. nemorosa* ramet (27 cm^2 leaf area ramet^{-1} is the mean of this species across Europe; De Frenne *et al.* 2011b) amounts to 0.17 mg·N·ramet^{-1}. The N dose on the petals of an average *A. nemorosa* flower (9 cm^2 surface area flower^{-1} is the mean for this population) amounts to 0.06 mg·N·flower^{-1}. To determine the exact N dose, we then calculated the amount of water deposited on *A. nemorosa* petals and leaves by five paintbrush strokes. This amounted to 8.5 mg ± 1.0 (±SE; determined from eight replicate leaves) and 6.3 mg ± 1.0 (determined from eight replicate petals). To achieve a dose of 0.17 mg·N·plant^{-1}, spread over four applications each consisting of five paintbrush strokes, we thus needed to apply a solution with a concentration of 2.859 g·NH$_4$NO$_3$l^{-1} (1.0 g·N·l^{-1}): 8.5 mg·H$_2$O × 4 applications × 5 strokes × 1.10^{-6} g·N·mg^{-1}·H$_2$O. Our experiment also included a second scenario with a three-fold concentration of 8.576 g·NH$_4$NO$_3$l^{-1} (3.0 g·N·l^{-1}) that resulted in a dose of 0.51 mg·N·plant^{-1} (0.38 mg·N on petals). In sum, the average N doses during the entire experiment using the low concentration applied to the leaves and petals were 0.17 and 0.12 mg·N, respectively, and 0.51 and 0.38 mg·N using the highly concentrated solution. This experimental design resulted in a total of 45 plants: five control plants (using the paintbrush with distilled water), 30 plants in which either the flowers, leaves or soil were treated with the solution with the low or high N concentrations using the paintbrush, and finally ten plants that were entirely sprayed with the sprinkler with either the low or high N concentration. All seeds of each individual were sampled at seed maturity on 24 May 2013.

Seed traits

Seeds obtained from both experiments were air-dried to constant mass for *ca.* 1 week. We then determined mean seed mass by weighing all seeds (to the nearest 0.1 mg) per seed sample and dividing the obtained mass by the number of seeds. A subsample of seeds was then oven-dried to constant mass (50 °C for 72 h) and analysed for C and N using a CNS element analyser (expressed in %) and C:N ratios calculated from these results. The seed N content per seed (mg·N·seed^{-1}) was calculated by multiplying the N concentration (%) with the seed mass (mg). The response variables for both experiments were the seed mass, seed C and N concentrations and seed C:N ratio and N content since these are key variables reflecting seed quality and provisioning, and potential seedling survival and reproductive success (Parrish & Bazzaz 1985; De Frenne *et al.* 2011c). Seed mass is strongly correlated with germination percentages in this species (De Frenne *et al.* 2011c: $r = 0.656$, $P < 0.05$). We did not consider other seed nutrients such as K, Ca or P for this study because these are unlikely to strongly respond to petal N addition and did not show major variation

in this species with latitude (De Frenne *et al.* 2011c). Furthermore, no alkaloids were detected in *Anemone* seeds (De Frenne *et al.* 2011c). This is noteworthy since plants can invest a substantial part of their N in secondary compounds, especially in the Ranunculaceae. Thus, most probably all N in the seeds is present as free amino acids and/or proteins.

Data analyses

To explore whether and to what extent the seed traits varied in response to variation in N deposition rates and climate over time and space in Study 1, we applied linear mixed-effect models using the *lmer* function from the *lme4* package in R (R Core Team 2017). Random effect terms 'region' and 'population' nested within 'region' were added to the multilevel models to account for the hierarchical nature of the data and the spatiotemporal autocorrelation within populations and regions. We also added a non-nested random effect 'year' to account for the repeated sampling in 2009 and 2013 in the same populations (Zuur *et al.* 2009). To avoid multicollinearity issues of the explanatory variables temperature and N deposition within the same model, the effects of the predictors were always tested using separate models (one-by-one). Correlations among predictors were as follows: temperature *versus* N deposition: $r = 0.870$, $P < 0.001$; temperature *versus* precipitation: $r = 0.202$, $P = 0.284$; N deposition *versus* precipitation: $r = 0.0674$, $P = 0.723$.

The seed quality data from Study 2 were first expressed as response ratios with the natural logarithm of the ratio of the seed traits (x_i) divided by the mean seed traits of the control plants ($\bar{x}_{control}$) as $\log(x_i \sqrt{x_{control}})$. These response ratios were then analysed with two-way ANOVA with the treatment (soil, leaves, flowers or entire plant treated with N) and concentration (low *versus* high) as factors using the *aov* function in R (R Core Team 2017).

RESULTS

In Study 1, seed traits exhibited strong spatial variation matching closely the estimated N deposition rates across Europe (Fig. 2, Table 1). Seed N concentrations and N contents were strongly positively correlated with N deposition, while C:N ratios decreased with N deposition. For instance, N concentrations increased, on average, from *ca.* 2.8% seed N at N deposition levels of 1 kg·N·ha^{-1}·year^{-1} to *ca.* 4.1% at N deposition levels of 18 kg·N·ha^{-1}·year^{-1} (Fig. 2b, d, Table 1). In contrast, seed mass and seed C concentrations did not differ significantly in seeds sampled from regions with high *versus* low levels of N deposition. Second, temperature was also related to the seed traits along the latitudinal gradient, with significant positive effects on N concentrations and negative effects on seed C:N ratios. Yet, the AIC values of the temperature models were consistently higher for all seed traits than of the models with N deposition as predictor, which demonstrates the higher relative importance of N deposition for seed traits of *A. nemorosa* along the latitudinal transect (Table 1). Precipitation did not demonstrably influence any of the investigated seed traits. Nevertheless, to experimentally disentangle the effects of N deposition from potentially confounding effects of other environmental changes along the latitudinal gradient, we designed the experiment in Study 2.

Seed traits also responded significantly to the experimental N treatments in the controlled Study 2, but mostly to the
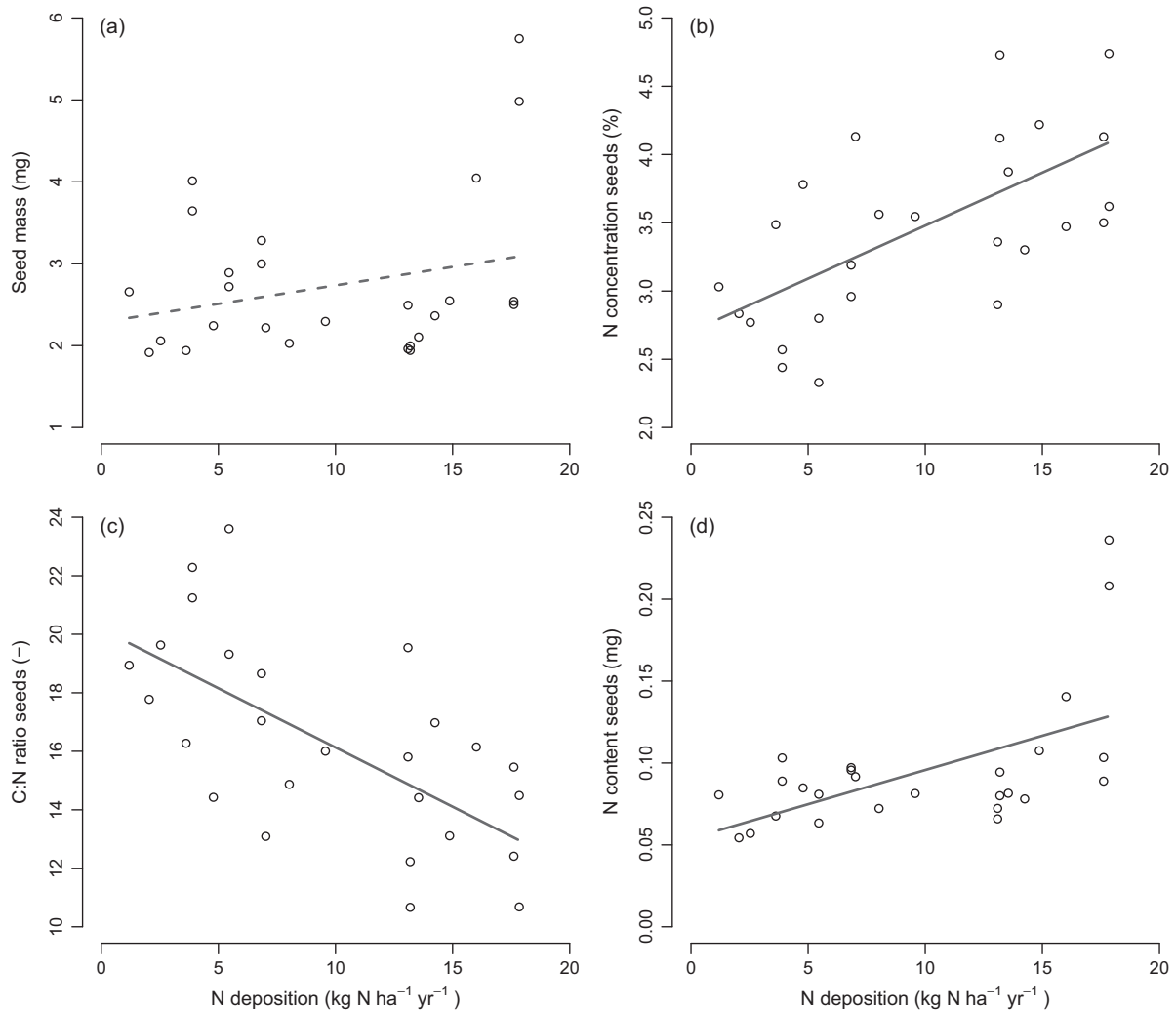
Fig. 2. Relationships between N deposition rates (kg·N·ha^{-1}·year^{-1}) and the measured seed traits of *A. nemorosa* seeds (a: seed mass; b: N concentration; c: C:N ratio; d: N content) sampled along an N deposition gradient in Europe (Study 1: macroecological approach). Fitted regression lines are the linear mixed-effect models shown in Table 1, dashed lines are not significant at $P > 0.1$, solid lines are significant at $P < 0.05$.

highly concentrated solution (Fig. 3, Table 2). There were significant differences between the mass of seeds, seed N concentration, C:N ratios and N content in the contrasting treatments, with, for instance, seeds containing more N if petals received additional N (Fig. 3). Seed N concentrations varied significantly with treatments, increasing from *ca.* 2.8 to *ca.* 3.2% when petals were treated with N compared to the control treatment (Fig. 3b). Nonetheless, the concentration of the solution did not significantly affect this result. Finally, the seed N content responded both to the N addition treatment as well as the applied concentration (Fig. 3d). Seed C concentrations were not significantly affected (Table 2). Addition of N to the leaves and soil did not affect seed N concentrations, but spraying the entire plant resulted in similar responses of seed traits as addition of N to the petals alone (Fig. 3).

DISCUSSION

We detected consistent effects of N deposition on seed traits of *A. nemorosa*. Seed N concentrations and total seed N content

increased with large-scale N deposition patterns in the observational study across Europe (Study 1) and with experimental N addition (Study 2). We previously established a strong north–south gradient in seed N concentrations in this forest herb, but the causes behind this mechanism remained unknown (De Frenne *et al.* 2011c). With the observational and experimental results reported here, we identify the likely driver for observed latitudinal clines in seed N concentrations as we demonstrate that N deposition on petals during anthesis enhanced bioaccumulation of N in seeds. Deposition of N on the leaves and soil alone did not meaningfully change seed N concentrations, but when N was applied to the whole plant, patterns were similar to the petal treatments. Our results thus corroborate Carlo & Norris (2012) who showed that petals can act as permeable membranes through which N is readily incorporated into the reproductive structures, most likely *via* N transport to the ovaries and seeds *via* the phloem (Zhang *et al.* 2010). Additionally, water droplets might flow over the petal surface to reach the ovaries, or dry deposits may be wetted by rainfall and as such transported directly to the ovaries. The lack of significant

Table 1. Effects of N deposition rates, temperature and precipitation on seed traits of *A. nemorosa* across Europe. Results from Study 1 (macroecological approach) using linear mixed-effect models with N deposition rates and climatic variables as predictor variables (see Material and Methods).

Predictor	Response variable	Parameter estimate	Chi²-value	*P*-value[1]	AIC
N deposition	Seed mass	4.51×10^{-2}	1.10	0.294 (ns)	68.6
	Seed C concentration	-8.58×10^{-2}	1.66	0.198 (ns)	121.35
	Seed N concentration	7.76×10^{-2}	12.28	<0.001 ***	49.49
	Seed C:N ratio	-4.05×10^{-1}	12.19	<0.001 ***	134.92
	Seed N content	4.18×10^{-3}	5.52	0.019 *	−101.8
Temperature	Seed mass	7.19×10^{-2}	0.52	0.470 (ns)	69.21
	Seed C concentration	-1.10×10^{-1}	0.50	0.4796 (ns)	122.51
	Seed N concentration	1.72×10^{-1}	11.44	<0.001 ***	50.32
	Seed C:N ratio	-8.74×10^{-1}	10.45	0.0012 **	136.66
	Seed N content	6.84×10^{-3}	2.64	0.104 (ns)	−98.96
Precipitation	Seed mass	2.64×10^{-4}	0.05	0.817 (ns)	69.7
	Seed C concentration	-2.27×10^{-3}	0.85	0.356 (ns)	122.2
	Seed N concentration	7.60×10^{-4}	1.28	0.258 (ns)	60.5
	Seed C:N ratio	-4.39×10^{-3}	1.57	0.210 (ns)	145.5
	Seed N content	1.86×10^{-5}	0.15	0.696 (ns)	−96.5

[a]Significances are indicated: (ns): $P > 0.10$; (*): $P < 0.1$; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$.

effects of precipitation on seed traits might indicate non-negligible effects of dry deposition. This is confirmed by the N concentrations obtained in the experiment where we only manipulated wet deposition. N concentrations there reached *ca.* 3.2%, while maximum concentrations of more than 4.0% were obtained in the field in Study 1.

Changes in seed size and quality as a result of enhanced atmospheric N deposition are likely to have important ecological implications. Enhanced N accumulation in seeds resulting from N deposition may increase resources available for embryo development, seedling nutrition and establishment. Most probably, this N is stored as free amino acids and proteins (no secondary N compounds such as alkaloids for defence against herbivores are present in *A. nemorosa* seeds, see Material and Methods). Bioassays on seeds of a wide range of species show that the usable internal N reserves were depleted fastest of all nutrients after germination (Fenner & Thompson 2005). This means that even small differences in seed N provisioning can improve seedling survival and fitness in adverse abiotic and biotic environments, such as shady or nutrient-poor habitats, during drought episodes and heatwaves, and under intense competition for resources (Parrish & Bazzaz 1985; Moles & Westoby 2004; Naegle *et al.* 2005; De Frenne *et al.* 2011c; but see Hanley *et al.* 2007). These processes can, in turn, influence plant population dynamics and community composition. Accordingly, community changes in response to fertilisation might not only be due to changes in above- and belowground competitive interactions for light or nutrients (Hautier *et al.* 2009), but may also result from altered seed provisioning and differential recruitment. N uptake through permeable surfaces of petals can also be advantageous to flowering plants in the absence of elevated atmospheric N deposition. For example, arthropod pollinators such as honeybees deposit significant amounts of ammonium and nitrate *via* their faeces (Mishra *et al.* 2013). Deposition of N *via* faeces on petals can thus efficiently be incorporated into the seeds. Finally, diaspores of *A. nemorosa* and numerous other forest herbs are ingested and/or dispersed by ants and slugs (Delatte & Chabrerie 2008; Türke *et al.* 2010) and changes in their chemical composition

may affect the probability of diaspore removal and ingestion (Reifenrath *et al.* 2012), and consequently, species colonisation and trophic interactions.

Scaling up to the population and species level, we expect a positive impact of elevated N deposition on *A. nemorosa* recruitment and population dynamics, especially in the longer term. Nevertheless, previous field studies found mixed results, which varied strongly according to the amount of annual N addition and the sampling period. For instance, in an experiment with addition of not less than 180 kg·N·ha^{-1}·year^{-1} in a forest in southern Sweden, Falkengren-Grerup (1993) detected a significantly reduced shoot length, flowering frequency, cover and biomass of *A. nemorosa* relative to the control after 6 years. Clearly, the applied N level was much higher than the levels of atmospheric N deposition in this study. In a field experiment in Belgium with application of 100 kg·N·ha^{-1}·year^{-1}, the percentage ground cover of *A. nemorosa* declined significantly by 32% over 6 years of N application (unpublished data, experiment described in De Frenne *et al.* 2015). In the last five decades, the frequency of occurrence of *A. nemorosa* in resurveyed semi-permanent vegetation plots within ancient woodlands across Europe also has not changed dramatically (on average this changed from 46.3% to 45.2%; De Frenne *et al.* 2013). Undoubtedly, other global environmental changes such as climate change, land-use change and management change interact with N deposition to affect population dynamics and the species' distribution, and thus play an important role in determining the ultimate response of populations at the continental scale (Baeten *et al.* 2010). The mixed results at the population level between previous N addition field experiments (Falkengren-Grerup 1993; De Frenne *et al.* 2015) and this study are likely due to N addition that was not added exclusively on *A. nemorosa* petals in the other experiments and thus such changes likely reflect negative effects of N addition on adult cover and biomass, and not on recruitment and reproductive success *via* petal uptake.

Since N deposition rates across Europe correlate with other environmental changes from north to south (most notably, temperature), it is difficult to distinguish correlation from
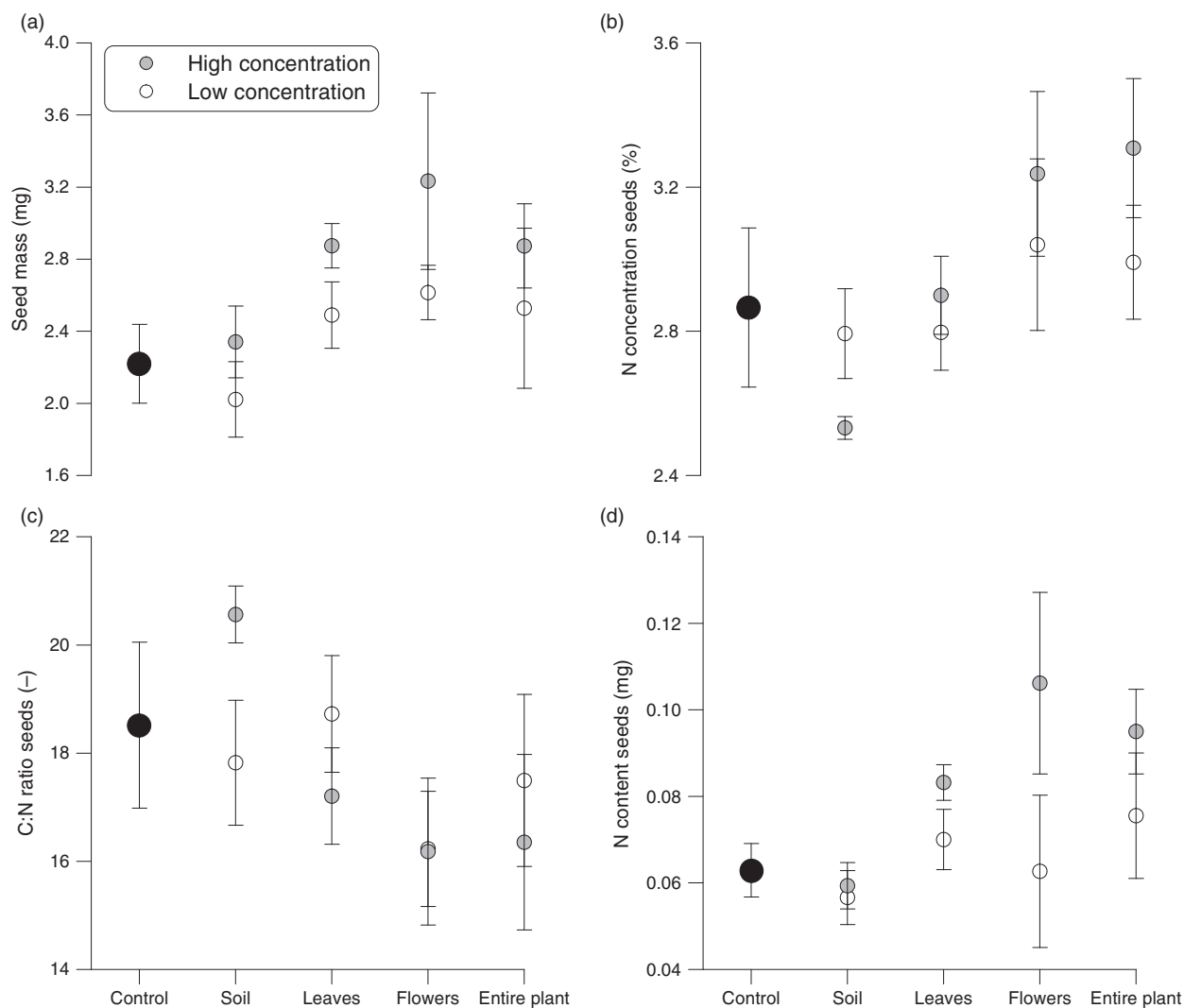
Fig. 3. Effects of N addition using a solution with low (open circles) and high (grey circles) N ion concentration to the soil, leaves or petals of flowers or the entire plant on seed traits of *A. nemorosa* (Study 2) (a: seed mass; b: N concentration; c: C:N ratio; d: N content). The control treatment is shown with a larger black circle. Error bars denote SE.

Table 2. Effects of the experimental N addition treatments (leaves, flowers, soil or entire plant treated with N as levels) and concentrations (low *versus* high as levels), and their interaction, on seed traits of *A. nemorosa*. Results from the experimental Study 2 using log-response ratios and two-way ANOVAS (see Material and Methods).

response variable	treatment	concentration	treatment: concentration
Seed mass	0.078 (*)	0.038*	0.998 (ns)
Seed C concentration	0.637 (ns)	0.339 (ns)	0.323 (ns)
Seed N concentration	0.011*	0.513 (ns)	0.301 (ns)
Seed C:N ratio	0.085 (*)	0.945 (ns)	0.314 (ns)
Seed N content	0.010*	0.039*	0.822 (ns)

Significances are indicated: (ns): $P > 0.10$; (*): $P < 0.10$; *: $P < 0.05$.

causation in Study 1. Nonetheless, in the controlled pot experiment of Study 2, only N addition was manipulated; all other biotic and abiotic environmental variables (temperature, soil moisture, soil nutrients, canopy cover, etc.) were kept as constant as possible. Taking our two sub-studies together, our results support a dominant role of N deposition on petals in explaining the observed patterns in seed traits of this species. More generally, we hypothesise that a wide array of plant species (specifically those with relatively large petals and small seeds) in regions with high levels of atmospheric N inputs currently display higher seed mass, N concentrations and content than before the historical onset of enhanced air pollution and N emissions. Whether enhanced atmospheric inputs of N *via* direct intake through petals can influence demography, and population and community dynamics, clearly warrants further research, for example, by subjecting seedlings resulting from seeds with contrasting N concentrations to stress and/or herbivory experiments and by performing seed dispersal and cafeteria experiments with seeds of contrasting N concentrations.

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