



Sexual reproduction traits of *Holcus lanatus* L. and *Parnassia palustris* L. in response to absolute and relative supply of nitrogen and phosphorus

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ABSTRACT

Plant investment in sexual reproduction is affected by absolute and relative nitrogen (N) and phosphorus (P) supply. Reduced investment in reproductive traits has been suggested as an important adaptation to strong P-limitation. We experimentally tested how absolute and relative nutrient supply affected sexual reproduction traits of two grassland species.

Seedlings of a common grass (*Holcus lanatus*) and an endangered forb (*Parnassia palustris*) were grown from seed from a dune area with low relative P-availability. Plants were grown in a full factorial experiment with three N:P supply ratios (5, 15, 45) and two absolute supply levels of N and P. After one year, a range of traits was measured as a proxy for investment in sexual reproduction. We found that N:P supply ratio did not affect sexual reproduction at low nutrient supply; at high nutrient supply, investment in sexual reproduction was significantly less at higher N:P supply ratios. For *Holcus lanatus*, N:P supply ratio 45 restricted the increase in sexual reproduction upon increasing nutrient supply. *Parnassia palustris* survival and flowering were low, especially at N:P supply ratio 15 (no results were available for *P. palustris* at N:P supply ratio 45 due to the high mortality).

Our results highlight that at low nutrient supply N:P ratio rarely affected investment in sexual reproduction traits but at high supply low relative P-supply restricted plants' sexual reproduction investment.

1. Introduction

Plant species diversity in natural ecosystems is influenced by nutrient supply as well as by nutrient stoichiometry, i.e., the ratio of available nutrients in relation to their consumers' requirements (Tilman, 1980; Cardinale et al., 2009), with nitrogen (N) and phosphorus (P) as the two main nutrients that frequently limit vegetation productivity (Elser et al., 2007; Fort et al., 2015). In general, fast-growing plants dominate in nutrient-rich environments, whereas slow-growing species may persist in nutrient-poor conditions, where fast-growing species cannot dominant (Grime and Hunt, 1975; Lambers and Poorter, 1992) leading to differences in species composition between N-limited and P-limited sites (Verhoeven and Schmitz, 1991; Tomassen et al., 2003; Wassen et al., 2005; Roeling et al., 2018). N-limited grassland ecosystems frequently have higher productivity and harbor species such as *Phragmites australis* and *Holcus lanatus* (Wassen et al., 2005; Fujita et al., 2014; Roeling et al., 2018). In contrast, P-limited herbaceous ecosystems are mostly characterized by low productivity and slow-growing species such as *Succisa pratensis* and *Parnassia palustris* (Roeling et al., 2018). Many of these species are endangered (Wassen et al., 2005) or have narrow geographical ranges (Brown,

1984; Lahti et al., 1991).

P-limited systems are characterized by species that have evolved functional traits to acquire the scarce P resources, e.g., cluster roots, symbiosis with arbuscular mycorrhizae, and high phosphatase activity, which enables them to assimilate enough P for growth in a low P environment (Güsewell, 2005a, b; Lambers et al., 2008; Ceulemans et al., 2011). Another adaptation of these species to a low P environment is that their nutrient use efficiency is high (Aerts, 1999), enabling them to survive in extremely infertile habitats. Since the highest percentage of all acquired P is required for sexual reproduction (van Andel and Vera, 1977v; Fenner, 1986), lower investment in sexual reproduction appears to be an effective way of saving on P. Recently it has been shown that species adapted to P-limited environments, especially if endangered, have a shorter flowering period, smaller seed mass and lower seed number than other species (Fujita et al., 2014), indicating that these species do indeed invest less in sexual reproduction. These findings are in line with previous findings that endangered species have shorter flowering periods (Lahti et al., 1991), smaller seed mass (Murray et al., 2002), and poorer dispersal ability (Farnsworth and Ogurcak, 2008). Other studies have shown that deficiency of P leads to a strong reduction in the number of flowers produced (Brouwer et al., 2001).

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Together, these results indicate that reproduction traits and dispersal capacity are influenced not only by absolute supply levels but also by nutrient stoichiometry. The effect of nutrient stoichiometry on reproduction and dispersal has, however, received little attention to date.

Given that human activities have altered elemental global cycles considerably (Falkowski et al., 2008; Seitzinger et al., 2005; Rockström et al., 2009), the absolute availability of N and P, as well as their relative availability are changing in natural environments (Peñuelas et al., 2013; Bobbink and Willems, 1991; Hölzel and Otte, 2003). How these changes will affect plants' investment in sexual reproduction, and hence dispersal capacity, remains unknown. In one of the few studies that systematically analyzed the relation between reproduction traits and nutrient stoichiometry, Fujita et al. (2014) suggested that the low investment in sexual reproduction traits of endangered species adapted to P-limitation might have contributed to their current endangered status. Their analysis, however, was based on average trait values (Fujita et al., 2014), whereas trait plasticity is very common and has been identified as a key characteristic for adaptation to environmental change (Callaway et al., 2003; Berg and Ellers, 2010).

We therefore experimentally tested plant species' investment in sexual reproduction in a fertilization experiment in which we varied N and P supply levels and ratios for two species: one common (*Holcus lanatus*) and the other endangered (*Parnassia palustris*). We measured six sexual reproduction traits on plant individuals or populations. First, we included first flowering date (FFD) because it has been shown that nutrient fertilization in nutrient-poor conditions may lead to earlier flowering (Putterill et al., 2004); second, flowering period in individuals (FPI) and in the population (FPP) were included because it has been shown that length of flowering period was related to investment in sexual reproduction (Fujita et al., 2014); third, number of panicle or flower stalks per plant (NPS or NFS) was included because this type of trait was used as a good index of sexual reproduction in other studies (e.g. Burton, 1943); fourth, seed stalks and flower stalks: height (SSH or FSH) and length (SSL or FSL) were included because we assume that the greater the height at which the seeds or pollen are released, the greater the chance that the seeds or pollen will be dispersed over longer distances (Soons et al., 2010); fifth, seed panicle length and weight (SPL and SPW) were included because longer and heavier seed panicles may be indicative of more and heavier seeds; and lastly, total seed weight and number of seeds produced per pot (TSW and TNS). Our aim was to test the relative importance of N:P supply ratios compared to total nutrient supply levels for the investment of plants in sexual reproduction traits for these two species.

2. Materials and methods

2.1. Field area description

Seeds of *H. lanatus* and *P. palustris* were collected in October 2014 from the State Forestry Service Middenduyn nature reserve (52°24'N 4°35'E) in the west of the Netherlands. The area harbors herb-rich low-productive grassland, with occurrence of species such as *P. palustris* L., *Epipactis palustris* L., and *Rhinanthus minor* L. The climate is temperate, with annual temperatures between the lowest average of -2 °C and the highest average of 20 °C; mean annual precipitation is 939 mm/yr. Seeds of each species were collected from two sites. We measured the N:P ratio of the above-ground plant community in three samples at each of these sites, which were averaged. N:P ratios were 22.3 ± 2.1 and 40.5 ± 1.7 respectively. Total N concentration of plant material was measured with a Carlo Erba-NA 1500 NCS analyzer; total P concentration of plant material was measured with a Bruker S2 Picofox TXRF spectrometer. These values indicate P-limited conditions (cf Olde Venterink et al., 2003; Güsewell, 2005b; Wassen et al., 2005). The method of determining the type of nutrient limitation of the site by the N:P ratio in above-ground plant material has the advantage of measuring integratively over the seasons instead of only one snapshot over

time provided by water/soil N and P concentration (Fujita et al., 2014). However, the distinction between site MD 1 and site MD 2 was not made in the end of the experiment due to non-significant differences after statistical analysis. Hence, we feel safe to assume that the donor sites from which we obtained our seeds have a low relative P-availability (compared to N).

2.2. Species studied and seed collection

Holcus lanatus L. is a common velvety perennial grass species, often found in nutrient-rich environments throughout Europe. The hermaphroditic inflorescence flowers between May and September, with numerous seeds shedding from June to early autumn (Watt, 1978). Seeds shed easily, and are mainly dispersed by wind (Beddows, 1961a). Flowers of *H. lanatus* were reported to be cross pollinated by wind (Beddows, 1961a), and have been shown to be "highly self-sterile" (Beddows, 1961b).

Parnassia palustris L. is a perennial herb, with a basal cluster of leaves and several straight stems up to 30 cm high, each culminating in a hermaphroditic flower. Usually the stigma matures later than the stamen. The species flowers from June until October and seeds ripen between September and October. The tiny dust-like seeds are dispersed by water and wind. This species is insect- and cross-pollinated. Seed set is enhanced if pollinated by plants from different genotype fragments (Bossuyt, 2007). In the 20th century this species became less frequent, which has resulted in genetic isolation and an endangered status in many European countries (Bossuyt, 2007).

Seed collection was from June until September 2014. Seeds were stored at 4 °C for 10.7 weeks to break physiological dormancy (vernalization).

2.3. Nutrient treatments and sexual reproduction traits research

2.3.1. Seedling preparation

After germination in June 2015, seedlings were transplanted into separate pots per species (four per pot, 48 pots in total). Pots contained a mixture of quartz sand (Carlo Bernasconi, Zürich, CH, 0.1–0.7 mm) and dune sand at a ratio of 11:1. To create relatively natural conditions for plant growth, dune sand was collected from the Kennemerland National Park close to Middenduyn; it has low concentrations of N and P but contains a natural microbial community. Total N and P concentration in the water extraction of the pure and dune sand (which we treated as the plant-available N and P concentration (Kachi and Hirose, 1983)) were determined using HACH-LANGE colorimetry test cuvettes (LCK 138, LCK 349, Hach-Lange, Germany). Based on the measured N and P concentration in the sand mixture we could fine-tune our nutrient recipe, following Güsewell (2005a).

2.3.2. Plant cultivation

The nutrient treatments, which lasted from July 2015 until June 2016, were given in a greenhouse with 400 W/m² light from 9 a.m. to 4 p.m. From October 2015 until March 2016, temperature was manipulated to Dutch winter conditions (-1 °C to 14 °C), to stimulate vernalization (Wiebe et al., 1992); for the rest of the experimental period, temperature ranged between 14–30 °C. Each pot was placed on a tray, to prevent loss of water and nutrients. The pots were switched around regularly to randomize possible differences in light, temperature, and moisture conditions in the greenhouse.

A full factorial combination with six nutrient treatments was applied, with three supply ratios (N:P = 5 (Low relative N-supply), N:P = 15 (co-limitation), and N:P = 45 (Low relative P-supply)), and two absolute nutrient supply levels: low and high. The treatments have been defined by the total amounts of N and P applied per plant throughout the cultivation process (in mg), and were calculated as:

$$N = L \cdot \sqrt{N:P} \quad \text{and} \quad P = \frac{1}{\sqrt{N:P}}, \quad (\text{where } L = \sqrt{N \times P}) \text{ in which } L \text{ is}$$

the overall supply level (geometric mean of N and P supply). L was 13.4 mg for low supply level and 40.3 mg for high supply level, following the method developed by Güsewell (2005a) in the first year of her two years' fertilization experiment.

The experiment was carried out with seedlings grown in separate pots for each species; each pot contained four seedlings planted in its corners. The total number of pots was 2 species \times 3 nutrient supply ratios \times 2 nutrient supply levels \times 8 pot replicates = 96. Nutrient solutions were applied weekly. The nutrient supply was increased gradually during the growth process (Güsewell, 2005a): in weeks 4–6 the weekly supply of nutrients was double the dose in weeks 1–3; in weeks 7–9 it was 1.5 times the dose in weeks 4–6. After week 9 when growth stopped, nutrient doses were kept at those of weeks 4–6, to ensure that plants remained alive and healthy until the day when senescence started. Apart from receiving nutrient solutions, plants were watered frequently with demineralized water to prevent drying out; normally this was done once every 1–3 days, but in summer this was necessary daily.

N was supplied as KNO_3 and $\text{Ca}(\text{NO}_3)_2$, P was provided as KH_2PO_4 . Both KNO_3 and KH_2PO_4 supplied part of the potassium (K), the rest of K was added by KCl (Güsewell, 2005a). The other essential macro-nutrients for example calcium (Ca) and micronutrients such as iron (Fe) and copper (Cu) were applied in non-limiting concentrations and were supplied in the same amounts to all treatments weekly. Details of the nutrient treatments are given in Appendix A. Cl was the only element that was not supplied in fixed amounts to all treatments. We have indicated the range of Cl addition to Appendix A.

To prevent nutrient toxicity (Fujita et al., 2010), each pot was leached every six weeks with demineralized water.

When pollination of *H. lanatus* started in May (13-5-2016), two fans were installed at the corners of the platform to create sufficient air movement for pollination. The position of the fans was changed regularly. *P. palustris* has bigger and fewer flowers, so pollination was done manually, using a small brush.

2.4. Data collection and analysis

2.4.1. First flowering date (FFD) and length of the flowering period (FPI or FPP)

First flowering date (FFD): The day on which the first flower appeared in a pot was taken to be the first flowering date for all four individuals within that pot. The FFD of the first pot was assigned the value 0.

Flowering period in individuals (FPI) and in the population (FPP): Since we did not find a clear definition of flowering period in the literature, we recorded the length of the flowering period at both individual level (FPI) and population level (FPP). The first approach (FPI) consisted of marking a random individual flower or panicle (how many depended on the total number of flowers or panicles in one pot and ranged between 1 and 8), and tracking the selected individuals to be able to define the start and end of the flowering period. The second method (FPP) consisted of calculating the difference between starting time as defined above (FFD) and the moment the last flower in that pot disappeared.

2.4.2. Number of panicle or flower stalks per plant (NPS or NFS)

Both *H. lanatus* and *P. palustris* carry one panicle or flower per stalk. Per pot we cumulatively counted the panicle stalks (number of panicle stalks: NPS) or flower stalks (number of flower stalks: NFS) as soon as they appeared. Later, the mean NPS or NFS per plant was calculated by dividing the number of panicle (or flower) stalks in one pot by the number of plants in that pot.

2.4.3. Seed stalks and flower stalks: height (SSH or FSH) and length (SSL or FSL)

The height of the stalks bearing seed and the flower stalks were

measured as a proxy for the dispersibility of seed of *H. lanatus* and of pollen of *P. palustris* (most plants of the latter did not set seeds in the experiment). We measured the stalk height (SSH or FSH) relative to the soil surface and also measured the stalk length (SSL or FSL) by straightening the stalks manually so they were vertical and then measuring the distance between the soil surface to the top of the seeds panicle or flower (Heady, 1957). The first method is indicative of seed or pollen release height, while the second one (which is a more standardized measurement) is indicative of a plants' investment in the structure of stalks.

2.4.4. Seed panicle length and weight (SPL and SPW)

SPL (the straight length of panicles) was measured from the bottom to the top of each seed panicle. Panicles were weighed with all the seeds on them (SPW).

2.4.5. Total seed weight and number of seeds produced per pot (TSW and TNS)

Collection of seeds of *H. lanatus* lasted from May to July 2016. All the seed panicles were collected individually immediately upon ripening. The collected seeds were dried in an oven at 30 °C for four hours and stored in a sealed box with silica. Average weight of an individual seed from each pot was determined by weighing 1000 random seeds in each pot and then dividing by 1000. To obtain total seed weight in each pot (TSW), all seeds from one pot were weighed. Total number of seeds in each pot (TNS) was calculated by dividing TSW by the average weight of an individual seed in each pot.

2.4.6. Data analysis

T-tests were used for comparing two data groups (treatments 5H and 15H) with normal distributions (NFS, FSL and FSH of *P. palustris*). We used ANOVA to test for the main effects of and the interactions between nutrient supply ratio and nutrient supply level on sexual reproduction traits. Since we identified many significant interaction effects between nutrient supply level and N:P supply ratios (see Appendix B), we further explored the difference among all treatment combinations of nutrient supply ratios and nutrient supply levels, using pairwise comparisons with a correction for making multiple comparisons. For traits with small differences ($\leq 40\%$) in sample size between treatments (FFD, NPS, SSL, SSH, SPW, TSW, and TNS of *H. lanatus*), Gabriel's procedure was used for post-hoc testing. When the differences in sample size between treatments were large ($> 40\%$) (FPP and FPI of *H. lanatus*), Hochberg's GT2 was used for post-hoc testing. Both of these tests have good control of Type I errors in multiple comparisons (Field, 2013). In instances of heteroscedasticity or non-normality, data was log- or square root-transformed. In cases where homogeneity of variances was significantly different, Games-Howell procedure was applied, which was the case for SPL of *H. lanatus*. This test performs well when homogeneity of variances can not be assumed, but is more liberal in controlling Type I errors than the other tests used (Field, 2013). Linear regression was used to test the combined effects of SSL and either SPL or SPW on SSH. All tests were performed with SPSS 23.0 (SPSS, Chicago, U.S.A.); figures were also created in SPSS.

3. Results

3.1. *Holcus lanatus*

In general, plants of *H. lanatus* grew healthily with 97% survival.

3.1.1. First flowering date (FFD) and length of the flowering period (FPP and FPI)

Flowering of *H. lanatus* started in the beginning of April; the first treatment to flower was 15H (i.e., N:P supply ratio of 15, high supply level); see Fig. 1a. Nutrient supply level (low supply level (L) versus high supply level (H)) rather than nutrient supply ratio (5, 15, 45) was

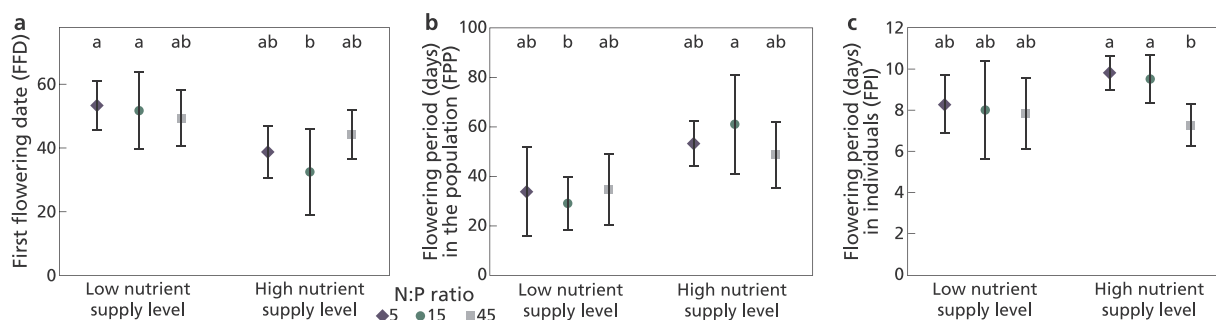


Fig. 1. First flowering date (FFD) and length of the flowering period (FPP and FPI) plotted against the supply ratios and levels of N and P of *H. lanatus*. (a) First flowering date (FFD) in days relative to the date the first flower appeared in each pot; the first date in the experiment on which flowering occurred was taken as 0. (b) Flowering period (days) in the population (FPP) was calculated by subtracting the FFD in a certain pot from the time the last flower in that pot finished flowering. (c) Flowering period (days) in individuals (FPI) was tracked on random individual flowers in each pot by recording the starting time and finishing time of flowering of each individual flower. Data are means \pm SD. Significant differences between treatment combinations are indicated by different letter codes. Values with the same character are not significantly different ($P = 0.05$).

decisive for FFD: plants growing under treatment 15H flowered earlier than those under treatments 5L and 15L (Fig. 1a).

The effects of nutrient supply level on FPP were similar to those found for FFD, but only for the N:P 15 treatment, i.e., plants growing under treatment 15H had a significantly longer flowering period than plants growing under treatment 15L (Fig. 1b).

However, the effect of nutrient supply ratio rather than nutrient supply level was decisive for FPI: plants growing under treatments 5H and 15H had significantly longer FPI than those under treatment 45H, as well as those under treatments 5L, 15L and 45L (Fig. 1c).

3.1.2. Number of panicle stalks (NPS), seed stalk length (SSL) and height (SSH), as well as seed panicle length (SPL) and weight (SPW)

NPS was significantly higher at the high nutrient supply treatments with the highest numbers at N:P 15 (Fig. 2a). SSL was significantly higher for plants growing under treatment 5H than those under treatment 45H, as well as those under treatments 15L and 45L (Fig. 2b). However, SSH did not vary significantly between different treatments (Fig. 2c).

SPL showed a similar tendency as SSL (Fig. 2b), i.e., plants growing under treatment 5H produced longer seed panicles than those under treatment 45H, as well as those under treatments 15L and 45L (Fig. 3a). Plants produced significantly heavier panicles under treatment 5H than in treatments 45H, 5L, 15L, and 45L (SPW; Fig. 3b).

3.1.3. Seeds: total weight and number produced per pot (TSW and TNS)

At the end of the experiment, the number of seeds collected was 207,861 and their total dry weight was 65.4 g. The average numbers of seeds per pot per treatment were: 1138.84 (5L), 306.27 (15L), 696.61 (45L), 8353.92 (5H), 13,927.00 (15H) and 2113.11 (45H); the average weights of seeds per pot per treatment were: 0.26 g (5L), 0.10 g (15L), 0.18 g (45L), 2.43 g (5H), 4.69 g (15H) and 0.67 g (45H). In

general, nutrient supply had a critical influence on both TSW and TNS in each pot for both N:P 5 and 15, but not for N:P 45. At high nutrient supply level, seed number and weight in treatment 15H were slightly higher than in treatment 5H and about seven times higher than in treatment 45H. The difference between treatments 5H and 45H for TNS was significantly different. Nutrient supply ratio had no effect at low nutrient supply level (Fig. 4).

3.2. *Parnassia palustris*

Although germination success of *P. palustris* was high, only 42% of the seedlings of *P. palustris* survived in the experiment.

3.2.1. First flowering date (FFD)

The earliest first flowering date was in the beginning of June, while the latest first flowering date was in the beginning of August. However, differences between treatments in FFD of *P. palustris* could not be statistically tested because of the low survival of flowering individuals in the experiment.

3.2.2. Number of flower stalks per plant (NFS), flower stalk length (FSL) and height (FSH)

The comparisons of these three sexual reproduction traits of *P. palustris* were restricted between treatment 5H and treatment 15H, due to the high mortality and low bearing flowers and seeds rate of the other treatments. In detail, the number of flower stalks per plant of *P. palustris* (NFS) was significantly higher on plants under treatment 5H than treatment 15H (Table 1). However, there was no significant difference of flower stalks length (FSL) or flower stalks height (FSH) growing on plants under treatment 5H and treatment 15H (Table 1).

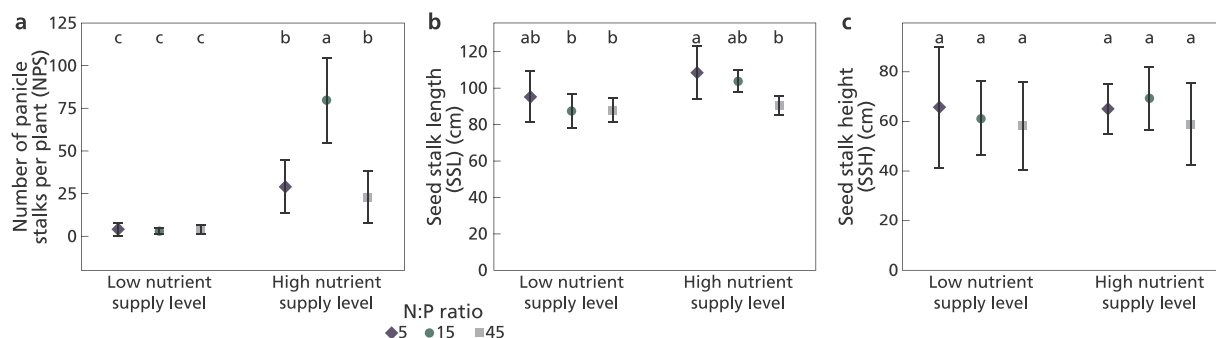


Fig. 2. Number of panicle stalks (NPS) (a), seed stalk length (SSL) (b), and seed stalk height (SSH) (c) of *H. lanatus*. Data are means \pm SD. Values indicated by the same character are not significantly different ($P = 0.05$).

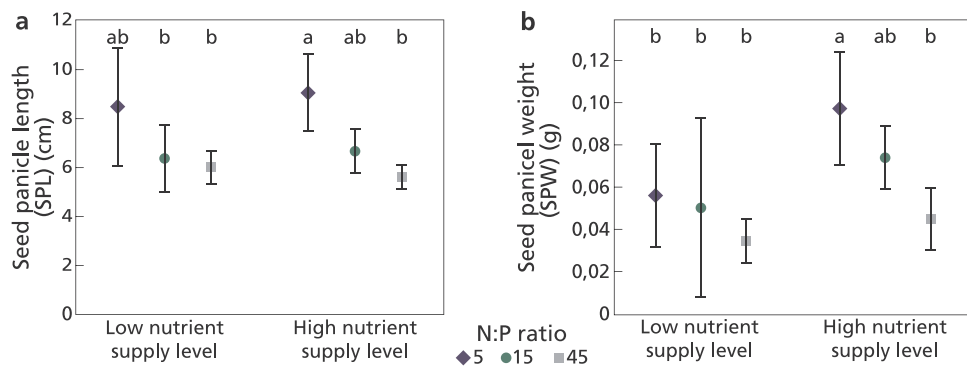


Fig. 3. Seed panicle length (SPL) (a) and seed panicle weight (SPW) (b) of *H. lanatus*. Data are means \pm SD. Values indicated by the same character are not significantly different ($P = 0.05$).

3.2.3. Seed production

At the end of the experiment, seeds of *P. palustris* could only be collected from plants growing under treatment 5H, since in the other treatments plants did not flower or produced ovaries that died before seeds developed. As it was not possible to compare seed production among different treatments, we do not report the number and weight of *P. palustris* seeds.

4. Discussion

In this experiment, we measured the response of traits that can be regarded as a set of proxies for the investment in sexual reproduction and dispersal capacity to fertilization treatments varying in N:P supply ratios (5, 15, and 45, respectively indicating low relative N-supply, co-limited N and P supply and low relative P-supply) and nutrient supply levels (low and high). We did so for a common species (*Holcus lanatus*) and an endangered species (*Parnassia palustris*) that were grown from seeds collected from two sites where relative P-availability was low.

4.1. Nutrient supply level-dependent effects of N:P supply ratio on investment in sexual reproduction

The results of this study showed that N:P supply ratio affected plant investment in sexual reproduction differently at low and high nutrient supply levels: N:P supply ratio affected reproduction traits at the high nutrient supply level markedly, but rarely had an effect at the low supply level. Since the results for *P. palustris* were very limited due to high mortality and extremely low flowering, we restrict this analysis to *H. lanatus*.

At the high nutrient supply level, *H. lanatus* invested less in sexual reproduction at the high N:P supply ratios indicative of low relative P-supply: the flowering period of individual flowers was shorter (Fig. 1c), and there were fewer and shorter flower stalks (Fig. 2a, b), lighter and

Table 1

P. palustris flower stalks: number per plant (NFS), length (FSL) and height (FSH).

	NFS	FSL	FSH
5L	0.07 \pm 0.13	/	/
15L	0.13 \pm 0.33	39.5	39.5
45L	0	/	/
5H	2.33 \pm 1.55 ^A	29.01 \pm 6.23 ^A	24.16 \pm 11.05 ^A
15H	0.75 \pm 0.79 ^B	22.54 \pm 12.30 ^A	19.25 \pm 5.86 ^A
45H	0	/	/

Values of each trait indicated by different characters are significantly different ($P = 0.05$). Values without \pm SD indicate that only one value was collected from plants under that treatment. / indicates no data available under the treatment.

shorter seed panicles (Fig. 3b), as well as lower seed production (Fig. 4). The differences between N:P 5 (low relative N-supply) and N:P 15 (co-limitation) were always small, except for the number of panicle stalks, with N:P 5 restricting NPS significantly compared to N:P 15. The larger number of stalks in N:P co-limitation than in low relative N-supply in our experiment is in line with results obtained for Meadow Bromegrass, which showed a significant increase of panicle stalk density upon N fertilization (Loeppky and Coulman, 2001). Surprisingly, *H. lanatus* showed considerably higher seed stalk lengths at N:P 5 than at N:P 45, as well as N:P 5 and N:P 45 at low supply level, while no significant differences for seed stalk height were found between any of the treatments. This could be explained by the fact that the stalks bent under the heavier weight of the seed panicles (SPW). This was confirmed by a linear regression, that showed that seed stalk height was not only positively influenced by seed stalk length (SSL), but also negatively by the weight and length of the seed panicles (SPW and SPL; Appendix C). In a word, the lack of N in treatment 5H for *H. lanatus* led to fewer

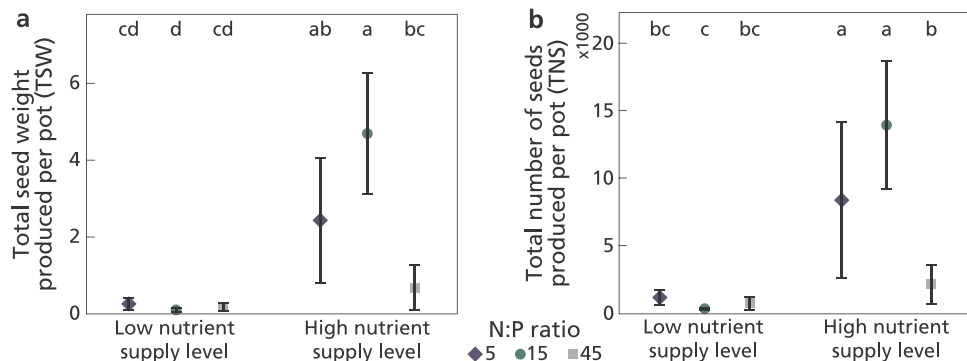


Fig. 4. Total seeds weight (TSW) (a) and number of seeds (TNS) (b) produced per pot of *H. lanatus*. Data are means \pm SD. Values indicated by the same character are not significantly different ($P = 0.05$).

panicles per plant but heavier individual seed panicles.

We did not observe an effect of N:P supply ratio on sexual reproduction traits at low nutrient supply level. A possible explanation for greater investment in sexual reproduction at high nutrient supply could be the dependency of sexual reproduction on plant size, i.e., an economic strategy for plants only to invest in sexual reproduction when they have surpassed a threshold plant size (Bloom, 1985; Schmid et al., 1995). As sexual reproduction organs contain high nutrient concentrations, investing nutrients in sexual reproduction under nutrient-limited conditions may create a high risk for the plant's establishment. The optimal nutrient allocation for plants under nutrient-limited conditions therefore seems to be to first build a stronger vegetative body to increase survival and allow for greater future reproduction (Kozłowski, 1992). Our conclusion challenges a contention made by Fujita et al. (2014) that investment in sexual reproduction of vegetation is mainly related to N:P supply ratio and is independent of nutrient supply level. However, the contrasting result might be explained by the origin of the data. The dataset of Fujita et al. (2014) contained field observations instead of experimental results; if plants are already growing at a particular site, it can be assumed that the nutrient supply level is sufficient to support adequate vegetative growth for investments in sexual reproduction to take place. Furthermore, Fujita et al. worked with average trait values per species and any difference they observed in trait values between sites is therefore the result of species turnover, not of changes in trait expression within species like those we observed in our experiment. Our finding that the effects of N:P supply ratio on investment in sexual reproduction are dependent on nutrient supply level strongly suggests that total nutrient supply should be taken into account when studying the impact of N:P supply ratio on plant sexual reproduction. However, no effect of N:P supply ratio was found on either the first flowering date or the flowering period in the population.

4.2. Low relative P-supply restricts the effect of increasing nutrient supply level on plant investment in sexual reproduction

The results of our experiment also allow for analysis of how trait expressions may change depending on nutrient stoichiometry when overall nutrient supply rises. Same as the discussion above, we restricted this analysis to *H. lanatus* due to the limited results of *P. palustris*.

At N:P 15, increasing the nutrient level significantly advanced the first flowering date (Fig. 1a) and prolonged the flowering period in the population (Fig. 1b), with more seed stalks (Fig. 2a) and higher seed production (Fig. 4a, b). Similarly, in their research on plant vegetative growth, Olde Venterink and Güsewell (2010) showed that at N:P 15 *Alopecurus pratensis* and *Agrostis capillaris* responded most strongly to an increased nutrient supply by producing more biomass. The vegetative growth of *H. lanatus* in our research showed a comparable response on increased nutrient supply (result not shown).

In contrast to these findings at co-limited N:P supply (N:P 15), we observed no influence of increasing nutrient level under low relative P-supply (N:P 45) on any of the sexual reproduction traits except for one (number of panicle stalks per plant (Fig. 2a)). Similarly, increasing nutrient supply levels under low relative N-supply (N:P 5) also had no effect on sexual reproduction traits except for all the four traits directly related to seed output (i.e., number of panicle stalks per plant (Fig. 2a), seed panicle weight (Fig. 3b), total seed weight and number of seeds produced per pot (Fig. 4a, b)). This restrictive effect of deviation from co-limited N:P supply (N:P 5 and N:P 45 compared to N:P 15) may be explained by the phenomenon that adding N (or P) accelerates the uptake of P (or N), whereas N (or P) deprivation restricts the uptake of P (or N) (Gillespie and Pope, 1989; Chaudhary et al., 2008). These relations between N and P can be brought about by a variety of mechanisms. Under natural conditions, the main mechanisms would be the control of P on N microbial fixation and the effect of N on P mineralization (Eisele et al., 1989; Gressel and McColl, 2003). In our

fertilization experiment a possible mechanism could be the shortage of N- or P-related substances needed for growth, such as enzymes (e.g., PO_4^{3-} symporter (Maathuis, 2007), ATP synthase (Oster and Wang, 2000)), nucleic acid, ATP, etc. However, considering the critical role of seed output in sexual reproduction, low relative P-supply might be the critical restriction to the positive response to greater nutrient supply on sexual reproduction rather than low relative N-supply.

4.3. Species-specific responses

Nearly all (97%) the plants of *H. lanatus* grew successfully and produced seed in most treatments, whereas less than half (42%) of the plants of *P. palustris* survived, of which only 10% produced flowers and only two (in the same pot) produced seeds. The pattern of plant survival of *P. palustris* was surprisingly similar to the pattern of sexual reproduction investment of *H. lanatus*: at low nutrient supply level the mortality of *P. palustris* was extremely high and the sexual reproduction investment of *H. lanatus* was markedly worse than at high nutrient supply level. Apparently, both species were seriously hampered by low nutrient supply. High nutrient supply level resulted in better establishment for both species, while at the same time significant differences emerged between N:P supply ratios in investment in sexual reproduction: both the survival of *P. palustris* and the investment of *H. lanatus* in sexual reproduction were good at low N:P supply ratio, whereas at high N:P supply ratio almost all plants of *P. palustris* died and *H. lanatus* invested less in sexual reproduction. However, the effect of pH on the survival of the plants, especially on *P. palustris* was not considered in our research.

5. Conclusions

In summary, our experiment shows that sufficient nutrient supply was the condition for N:P supply ratio to appreciably influence sexual reproduction – i.e., N:P supply ratio had no effect on investment in sexual reproduction at low nutrient supply level but a significant effect at high nutrient supply level. A high N:P supply ratio (indicating a low relative P-supply) led to significantly lower investment in sexual reproduction. On the other hand, low relative P-supply critically limited plant investment in sexual reproduction when nutrient supply level was raised. Based on these findings we highlight the strongly negative influence of low relative P-supply on investment in sexual reproduction, very clearly for the common species and also likely for the endangered species we investigated. However, since we only investigated one common species and one endangered species, we recommend more in-depth empirical studies be carried out on a wider range of species.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.envexpbot.2019.103813>.

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