

Do plant ploidy and pollinator tongue length interact to cause low seed yield in red clover?

VERONICA HEDERSTRÖM ^{1,2,†} MAJ RUNDLÖF,³ GÖRAN BIRGERSSON,¹ MATTIAS C. LARSSON,¹
ANNA BALKENIUS,^{1,3} AND ÅSA LANKINEN¹

¹Department of Plant Protection Biology, Swedish University of Agricultural Sciences, Alnarp SE-230 53 Sweden

²Centre for Environmental and Climate Science, Lund University, Lund SE-223 62 Sweden

³Department of Biology, Lund University, Lund SE-223 62 Sweden

Citation: Hederström, V., M. Rundlöf, G. Birgersson, M. C. Larsson, A. Balkenius, and Å. Lankinen. 2021. Do plant ploidy and pollinator tongue length interact to cause low seed yield in red clover? *Ecosphere* 12(3):e03416. 10.1002/ecs2.3416

Abstract. The loss of long-tongued pollinator species and dominance of a few short-tongued generalist species, related to agricultural intensification in recent decades, may have consequences for the quality, quantity, and stability of yields in insect-pollinated crops. Interestingly, the changes in pollinator community coincide with an increase in poor and variable seed yield in red clover, an important crop for green manure and forage production. However, to date we lack a holistic understanding of the factors that drive seed yield in red clover crops. To remedy this, we related plant and pollinator traits to pollinator visitation, behavior, and pollination efficiency in four diploid and five tetraploid red clover cultivars during three years in a common garden setting. Tetraploid cultivars produced 52% fewer seeds and 41% lower seed weight per flower head compared with diploid cultivars. They also had fewer flower heads per plant, larger florets, and lower pollen viability than diploids. Pollinator species with shorter tongues visited diploid cultivars more frequently than tetraploid cultivars. Pollinator species with longer tongues deposited more pollen and showed higher pollination efficiency in terms of seeds produced after single visits. Our results suggest that while both diploid and tetraploid red clover cultivars benefit from the presence of longer-tongued pollinator species, seed yield in tetraploid cultivars may be more sensitive to the loss of these bumble bee species in intensively cultivated agricultural landscapes.

Key words: ploidy; pollinator efficiency; pollinator preference; pollinator tongue length; *Trifolium pratense*.

Received 27 March 2020; accepted 19 October 2020; final version received 8 January 2021. Corresponding Editor: T'ai Roulston.

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† **E-mail:** veronica.hederstrom@cec.lu.se

INTRODUCTION

Many crops and wild plants depend on animal pollination, primarily from insects, for their sexual reproduction (McGregor 1976, Klein et al. 2007, Ollerton et al. 2011). Bees, including bumble bees, are the primary pollinators of many crops that require animal pollination, and they provide more than half of the global crop pollination (Rader et al. 2016). However, declines in pollinators are reported from all over the world

(Biesmeijer et al. 2006, Dupont et al. 2011, Bommarco et al. 2012, Bartomeus et al. 2013, but see Carvalho et al. 2013) and suggested to be caused by agricultural intensification with increase of monocultures, fragmentation, and loss of natural habitats as well as increased use of agrochemicals (Williams and Kremen 2007, Rundlöf et al. 2008, Potts et al. 2010, 2016). The presence of pollinators with different traits (e.g., tongue length and phenology) and their interactions with flower traits can affect pollen transfer

and thus plant seed set (Garibaldi et al. 2015). Hence, crop yield and seed set in many wild plants are enhanced by a diverse pollinator community (Kearns et al. 1998, Fontaine et al. 2006, Biesmeijer et al. 2006, Hoehn et al. 2008), and the decline of pollinating insects, therefore, poses a threat to both wild ecosystems and agricultural production (Potts et al. 2010).

Land-use changes during the 20th century are thought to have caused the decline of some bumble bee species (Kleijn and Raemakers 2008), which may have direct consequences on crop yield. Studies from Denmark and Sweden have demonstrated fundamental shifts in composition and density of bumble bee communities since the first half of the 20th century, with losses of long-tongued species and dominance of a few short-tongued generalist species (Dupont et al. 2011, Bommarco et al. 2012). Bommarco et al. (2012) suggested that the decline in average seed yield and the doubling in yield variation in red clover, *Trifolium pratense* L., seed production during recent years could be attributed to the current dependence on a few dominant short-tongued pollinators.

Due to its high protein content and ability to fix atmospheric nitrogen, red clover is a valuable forage crop and used as green manure (Taylor and Quesenberry 1996, Boller et al. 2010). However, poor and variable seed set is a persistent problem in clover seed production, particularly in tetraploid (4n) red clover cultivars where seed yield may constitute as little as 20–50% of that in diploid (2n) cultivars (Taylor and Quesenberry 1996, Boller et al. 2010, Rundlöf et al. 2018). Moreover, the tetraploid cultivars are often the most favored for use in grass-clover leys due to their persistence and high yield of green biomass (Taylor and Quesenberry 1996, Boller et al. 2010). The dramatic variation in seed yield among years and locations (Taylor and Quesenberry 1996, Rundlöf et al. 2018) is so extensive that seed companies are occasionally facing problems meeting demands (Öhlund, L., Lantmännen and Dahlqvist, T., Sveriges Frö- och Oljeväxtodlare, *personal communication* 2018). In red clover, which is dependent on pollen transfer by insects to set seed (Free 1993, Taylor and Quesenberry 1996), bumble bees are frequent visitors and thought to be the most efficient pollinators (Bohart 1957, Peterson et al. 1960, Nørgaard Holm 1966, Boller et al. 2010). It

has been suggested that primarily long-tongued bumble bees are able to pollinate tetraploid red clover cultivars, because the more common and short-tongued bumble bees in the subgenus *Bombus sensu stricto* are supposedly less efficient due to the deep corollas (Boller et al. 2010), favoring robbing of nectar without pollen transfer (Free 1965, Inouye 1983, but see Maloof and Inouye 2000). Previous studies have compared the relation between pollinator abundance or pollination efficiency and seed set in red clover visited by honey bees and/or bumble bees (Peterson et al. 1960, Plowright and Hartling 1981, Clifford and Scott 1989, Rao and Stephen 2009), or compared diploid and tetraploid plants pollinated by honey bees (Jing 2017) or by only one *Bombus* species (Vanommeslaeghe et al. 2018). In studies that investigated both plant ploidy and pollinator tongue length, only small differences in corolla tube length were found to cause differences in bee species visiting (Nørgaard Holm 1966). Short- and medium-tongued pollinators were more frequent visitors of diploid cultivars, whereas the long-tongued *Bombus hortorum* was more often found on tetraploid cultivars (Hänninen 1962, Nørgaard Holm 1966). Especially, honey bees were much more frequent on diploid cultivars, stated to be caused by the too long corolla tubes of tetraploids (Julén 1954, Nørgaard Holm 1966). In contrast, Bender (1999) observed both short- and long-tongued pollinator species to prefer tetraploid plants whereas medium-tongued species seemed to prefer diploid plants (Bender 1999). To increase our understanding of how pollinators with different traits, such as tongue length, influence pollination efficiency and success in red clover cultivars that differ in ploidy, more detailed studies of the interactions between plants and pollinators are clearly needed.

Polyploidy in plants is frequently reported to modify some main set of floral traits, such as flower morphology, color, phenology, floral scent, quality, and amount of floral rewards (nectar and pollen) and number of flowers, all which are mediating the communication between plants and pollinators (Rezende et al. 2020). Plant traits have also been suggested to cause low seed set in tetraploid red clover cultivars. A former consensus regarding pollination deficiency in tetraploid clover due to the longer corolla tubes (Julén 1954, Wexelsen and Vestad 1954, Boller et al. 2010) has

been contradicted by several studies (Starling et al. 1950, Monks et al. 2010, Vleugels et al., 2015, 2016a, b). For example, Vleugels et al. (2016a) found no correlation between corolla tube length and seed yield; instead, they identified the cause for lower seed yield in tetraploid cultivars to be determined by lower number of ripe flower heads per plant during harvest and less seeds per flower head. Similarly, in a comparison between ten tetraploid cultivars, Amdahl et al. (2016, 2017) suggested that high seed yield per flower head was the most important predictor of high seed yield. These traits may be linked with pollination success, but could also be determined by other factors. For instance, pollinator reward traits, such as nectar production, can be higher in tetraploids than in diploids (McGregor 1976), which contradicts that this factor is a cause of low seed set in tetraploid cultivars. Other suggested reasons for low seed set in tetraploid red clover cultivars are lower pollen viability (Kendall 1967, Buyukkartal 2003, Grebenisan and Savatti 2011) and increased rates of irregularities and anomalies during meiosis (Mackiewicz 1965, Buyukkartal 2008, Vleugels et al. 2019).

Here we focus on both plant and pollinator traits to understand how traits vary among red clover cultivars and pollinator species and how these traits are related to seed production. During the summers of 2010, 2014 and 2015 we used four diploid and five tetraploid red clover cultivars in a common garden to test how cultivars vary in seed production and seed weight under open pollination, and in seven plant traits believed to influence seed production either directly or through pollination success (flower head abundance, florets per flower head, floret size, color, nectar volume, pollen germination, and pollen tube growth rate). We also tested if pollinator preference and behavior (number of visited florets, visitation time) and pollination efficiency (pollen deposition on stigma and seed production after a single pollinator visit) for eight pollinator species were explained by interactions between clover ploidy (or cultivar) and pollinator tongue length (or species).

MATERIAL AND METHODS

In early spring of 2010, 2014, and 2015, plants of the four diploid and five tetraploid red clover

cultivars, also differing in phenology (Table 1), were dug up from fields in southern Sweden (Svalöv, Skåne) and northern Sweden (Lännäs, Västernorrland). The plants were planted in a common garden at the Swedish University of Agricultural Sciences (SLU) in Alnarp, Skåne, before onset of flowering. In 2010 and 2014, we planted 45–50 plants per cultivar, while in 2015, we planted 20–25 plants per cultivar, in neighboring plots (Appendix S1: Fig. S1). We chose to replicate the plots over several years rather than within years (note, however, that not all cultivars are included every year; Table 1). Estimates of plant traits and of pollinator preference and performance were conducted during the red clover flowering period (see Appendix S1: Table S1 for observation units for each conducted examination and trait during different years). Mean (SD) temperature and sum rain during the concerning years and months were as follows: June 15 (0.6)°C, 33 (14.4) mm, July 19 (1.7)°C, 37 (23.1) mm, and August 17 (0.6)°C, 125 (43.3) mm (SMHI 2019). Care was taken to perform observations on all cultivars at every observation occasion, but with different starting order (i.e., to not get any bias in weather or time of day among cultivars). For single visits of pollinators (see “Estimation of pollinator efficiency, single visits”), we selected plants in the middle of the plot to avoid border effects (Appendix S1: Fig. S1).

Measurements of plant traits and seed production

Flower heads per plant.—Flower head abundance was assessed during each pollinator preference transect walk (described below) in 2014 and 2015,

Table 1. Studied red clover cultivars.

Cultivar	Year	Ploidy	Maturation time
SW Ares	2014, 2015	2n	Medium late
SW RK1107	2010	2n	Medium late
SW RK1151	2014	2n	Medium late
SW Yngve	2010, 2015	2n	Late
SW Dagny	2014	4n	Medium late
SW RK1111	2010	4n	Medium late
SW Vicky	2014, 2015	4n	Medium late
SW Betty	2015	4n	“Late” with early flowering
SW Torun	2010, 2015	4n	Late

Notes: Name, ploidy, and time of maturation of red clover cultivars used during experiments in a common garden in Alnarp in year 2010, 2014, and 2015.

as the number of flowering flower heads (≥ 5 open florets) per plant (Rundlöf et al. 2018) for five plants, evenly distributed, along the transect.

Florets per flower head.—Number of florets per flower head was counted from 100 heads per cultivar in 2010 (ten heads per plant), in 2014 from 40 heads per cultivar (one head per plant), and in 2015 from 20 heads per cultivar (one head per plant).

Floret size.—Measurements A–E (Appendix S1: Fig. S2) were taken over consecutive days at peak flowering in July 2010. Since A–E were all shown to co-vary in 2010 (Appendix S1: Table S2), only measurement B (corolla tube length) was collected in 2014 and 2015 and subsequently used for analyses. In ten plants from each cultivar, measurements were taken on three randomly selected open florets on each of three randomly selected flower heads.

Flower color spectra.—The spectra of the light reflected from the red clover flowers were measured using a calibrated Ocean Optics S2000 spectrophotometer (USB4H08872). Two samples from each clover cultivar in 2014 and 2015 were measured, and the mean values were calculated.

Nectar volume.—Nectar volume was estimated using micro-capillary tubes (1 μL), which were placed into the nectar tube of a fully open floret and moved gently up and down to collect the nectar. The height of nectar in the micro-capillary tube was measured with a ruler to calculate nectar volume. In 2014 and 2015 (but not in 2010), plants were covered with nets to exclude pollinators at least one day in advance. In 2010, measurements were done on eight plants per cultivar, in 2014 on ten plants per cultivar (on three flower heads per plant and three florets per flower head), and in 2015, it was done on six plants per cultivar (on four flower heads per plant and three florets per head; Appendix S1: Table S1). Due to time constraints, nectar data were only collected from cultivars Ares, Betty and Vicky in 2015. All nectar collections were done on the same day or on consecutive days with similar weather.

Pollen germination rate and pollen tube growth rate in vitro.—Pollen was germinated in vitro on Hoekstra medium (Hoekstra and Bruinsma 1975) to measure pollen germination rate and pollen tube growth rate. Germination of pollen from ten plants per cultivar was conducted for all cultivars on the same day at approximately the same time

to minimize environmental impact. Pollen germination was performed once per plant in 2010 and twice per plant in 2015 (a mean per plant was used in the statistical analyses). Each germination event included 20 samples involving all cultivars. Pollen from the same two plants was used as positive controls during each germination event. The germinations were done as follows: Flower heads from each plant were collected in marked paper bags and brought to the laboratory. Five milliliter Hoekstra medium was prepared with 1.2 g sucrose and 50 μg agarose. Pollen from 3 to 5 florets per plant was added to two droplets of medium on a microscopic slide using a pair of tweezers. The microscopic slides were placed on top of wet filter papers in Petri dishes and germinated in a dark chamber at a steady temperature (22–23°C) for one hour. Germination was terminated by adding one drop of 100% glycerol. Samples were examined under a microscope. Germination rate was determined as a percentage for 100 pollen grains (in a typical/representative area) per sample. Pollen tubes $< 0.5 \mu\text{m}$ were not counted as germinated. Rate of pollen tube growth was assessed by measuring the length of ten pollen tubes per sample.

Seed production and seed weight.—In 2015, seed production (here defined as number of seeds per head) and seed weight under open pollination were measured by collecting 20 flower heads (one head per plant) per cultivar after maturation and counting the number of florets and seeds per flower head as well as weighing the seeds. Although red clover flowers contain two ovules per floret, normally only one develops into a seed (McGregor 1976). The number of florets per head, therefore, constitutes the potential number of seeds per head. In 2014, 40 flower heads (one per plant) per cultivar, and in 2010, 100 flower heads (ten per plant) per cultivar, were used to assess seed production under open pollination. No weighing of seeds was performed in 2010 or 2014. During the weighing in 2015, all seeds from one head were weighed together, and the measure was then divided by the number of seeds in the head and multiplied by 1000 to get the unit: seed weight per 1000 seeds.

Pollinator preference, behavior, and efficiency

Observations of pollinators were performed between 9:00 and 18:00 and only on days with

warm weather ($>17^{\circ}\text{C}$), sun and no strong winds (≤ 4 Beaufort), or rain.

Pollinator preference and species presence.—Pollinator preference, measured as pollinator abundance per cultivar, was monitored by transect walks (modified from Pollard 1975, 1977) along the red clover cultivars in 2014 (June 26–July 28) and 2015 (August 3–19) during red clover bloom. During the 85 transect walks (15 on each of four cultivars, two diploids and two tetraploids, in 2014, and five on each of five cultivars, two diploids, and three tetraploids, in 2015), the observer walked slowly along the 10×1 m transect and noted all flower-visiting bumble bees, honey bees, and solitary bees to species/species group. The observer made sure not to observe other bees while handling one that was noted, and during observation time to always move slowly forward without looking back. The same observer performed all observations in 2014 and another observer all observations in 2015. In 2014, total observation time plus handling time was ~ 3 h/cultivar, and in 2015, it was ~ 0.5 h/cultivar, with a fixed observation time of 2 min/10 m in 2015. A bee was considered visiting a flower when it stuck its head or tongue in to get pollen or nectar. We also noted if flower visitors were legitimately visiting flowers or robbing nectar. All bees, except bumble bee queens and honey bees, were caught and put in separate tubes for later identification. Bumble bees were identified to species level, except for *Bombus terrestris*, *Bombus lucorum*, *Bombus cryptarum*, and *Bombus magnus*, which were instead grouped as *B. terrestris* because of difficulty to separate the species morphologically (Murray et al. 2008). All non-*Apis/Bombus* bee species were grouped as solitary bees.

For analyses, pollinator species were divided into three tongue length classes following the length division in Goulson et al. (2005) and tongue lengths in Benedek (1973), Goulson et al. (2005), and Balfour et al. (2013; Appendix S1: Table S3): short (<8 mm), medium (8–9 mm), and long (>9 mm) pollinator tongue length.

Visitation time and number of visited florets per flower head.—Pollinator behavior was monitored to evaluate trait related behavior of bumble bees and honey bees by observation of flower handling time. We counted the number of florets per flower head that was visited, and noted the time

spent per head, we also noted if the visit was legitimate or nectar robbing. Plants of each cultivar were observed separately for 30 min per occasion, and behavior of pollinators visiting a flower head was noted. In total, seven observation rounds per cultivar were conducted (July 4–July 29 in 2014 and July 30–August 20 in 2015). During one observation day, all cultivars were observed (with a few exceptions where half the cultivars were observed the consecutive day with similar weather) with observations distributed over the day and the cultivars.

Estimation of pollinator efficiency, single visits.—Single pollinator visits were used to assess pollination efficiency (pollen deposited on the stigma and seed production) of pollinator species. In this set of experiment, two-three cultivars were chosen to represent tetraploid and diploids, respectively, to make it feasible time wise. Eight plants in 2014 and six plants in 2015 of each chosen cultivar were enclosed in separate net bags before onset of flowering. At an observation round, bags were removed from plants of one cultivar at a time and observations of bees pollinating flower heads were made. Only one bee and one flower head could be observed at a time, and the observed pollinator should have been seen visiting two-three flower heads before in order to be included as an observation. Notes were made on bee species, how many individual florets the bee visited on the flower head, and the type of visit (legitimate or robbing).

In one set of single visits, number of pollen grains on the stigmatic surface was estimated in the cultivars Ares (2n) and Vicky (4n) in 2014 and 2015. Two flower heads per pollinator species and cultivar were observed and then collected in separate paper bags and brought to the lab directly after visitation by the pollinator. In the laboratory, pistils were mounted on sticky tabs and number of pollen grains on the stigma was counted under a dissecting microscope.

In another set of single visits, the probability of seed production and number of seeds produced were measured on the cultivars Ares and Vicky in 2014 (June 26–July 22) and Ares, Betty (4n), and Vicky in 2015 (July 30–August 19). The visited flower head was marked, using tape and pencil, on the stem below the flower with the corresponding id-number, and thereafter covered with an individual net bag. Observations were

repeated for 2–4 plants of each cultivar at a time. Individual net bags were left on the flower head until it was completely wilted and the upper part of the stem had turned brown. The flower head was thereafter collected and stored individually in a marked paper bag, until number of florets and developed seeds were counted for each flower head.

Statistical analyses

Statistical analyses were performed using R, version 3.5.1 (R Core Team 2018). We performed our main analyses with cultivar ploidy and pollinator tongue length group as independent fixed variables (see details of tests, variables, number of observations, N , in Table 2). Note, however, that in the case of nectar volume and single visits, diploids and tetraploids are represented by only one or two cultivars each (2n: Ares; 4n: Betty, Vicky). In another set of analyses, we investigated the effects of cultivar and bee species instead of ploidy and tongue length group (see details in Appendix S2: Table S1).

For all models, we confirmed that covariates were not collinear and that model assumptions were met by checking for distribution of residuals, equal variance between groups, independence, and no overdispersion. To avoid pseudoreplication and ensure independence, the data were either aggregated to an appropriate level or random variables included in the analysis. In cases of differing sample effort, we either added an offset in the model, or performed the analyses on aggregated data. Results for linear mixed models (LMMs) were obtained using Kenward Roger's method and for generalized linear mixed models (GLMMs) using Wald chi-square tests. If an independent variable was significant, post hoc tests, multiple comparisons between groups were computed using estimated marginal means (function `emmeans` in package `emmeans`). Packages used are as follows: `stats`, `lme4` (Bates et al. 2015), `MASS` (Venables and Ripley 2002), `lmerTest` (Kuznetsova et al. 2017), `multcomp` (Hothorn et al. 2008), `multcompView` (Graves et al. 2015), and `emmeans` (Lenth 2018).

RESULTS

We report the results on cultivar ploidy level and tongue length groups in this Results section,

while detailed results for different clover cultivars and bee species can be found in Appendix S2.

Plant traits and seed production

Tetraploid cultivars produced 35% fewer flower heads per plant (Fig. 1a) and had 8% longer corolla tubes (Fig. 1b) compared to diploid cultivars (Table 3). Tetraploids also had 21% lower pollen germination rate (Fig. 1c); however, their pollen tubes grew 16% faster than diploid tubes (Fig. 1d, Table 3). Furthermore, the number of seeds produced per flower head was 52% lower in tetraploid plants during open pollination (Fig. 1e, Table 3). Although the number of seeds per head was positively related to the number of florets per head ($\chi^2_{df=1} = 9.60$, $P = 0.002$), florets per flower head were not related to ploidy (Fig. 1f), neither was nectar volume (Fig. 1g, Table 3).

Seed weight per 1000 seeds in 2015 was 62% higher for tetraploids (Fig. 1h, Table 3). Given that we could not find a difference in number of florets per flower head between tetraploids and diploids (Fig. 1f), but lower number of seeds in tetraploids (Fig. 1e), this result suggests a potential trade-off between seed number and seed weight. However, despite this trade-off, seed weight per flower head in tetraploids was 41% lower than in diploids (Fig. 1i, Table 3).

Flower color spectra were almost the same for flowers from the different ploidy levels. The first peak was in the blue-green area, the second was in the yellow, and then, there were several peaks in the red area of the spectrum (Fig. 2).

Pollinator preference, behavior, and efficiency

Pollinator preference and species presence.—A total of 555 individuals belonging to eight species (or species groups) of bees were observed visiting red clover flowers during the 85 transect walks (15 walks per cultivar in 2014 and five per cultivar in 2015). Of the 555 bees, 62 % had short, 28 % had medium, and 11% had long tongues. Number of observed individuals per species was as follows: *Apis mellifera*: 159, *Bombus pascuorum*: 104, *B. lapidarius*: 66, solitary bees: 63, *B. terrestris*: 54, *B. sylvarum*: 50, *B. hortorum*: 46, and *B. subterraneus*: 13. The bees grouped as solitary bees consisted of *Andrena* spp. and *Melitta* spp.

Number of flower visitors was explained by the number of flowering flower heads per plant

Table 2. Statistical analyses on clover ploidy and pollinator tongue length class.

Testing	Test	Dependent variable	N	Independent fixed variables	Random variables	Family	Link	Comment
Flower heads/plant	GLMM	Number of flowering flower heads/plant (average/cultivar/survey round)	85 survey rounds	Ploidy + year	Round nested within year	Gamma	log	
Florets/flower head	GLMM	Florets/flower head (summed number of florets/plant)	301 plants	Ploidy + year	Cultivar nested within year	NB	log	Offset, to account for different number of heads/plant sampled among years
Floret size	LMM	Floret size (mean corolla tube length/plant)	130 plants	Ploidy + year	Cultivar nested within year			
Nectar volume	LMM	Nectar volume (mean microliter/floret and plant)	90 plants	Ploidy + year	Cultivar nested within year			
Pollen germination	LMM	Mean number of germinated pollen grains/plant	90 plants	Ploidy + year	Cultivar nested within year			
Pollen tube growth rate	GLMM	Mean pollen tube length/plant	90 plants	Ploidy + year	Cultivar nested within year	Gamma	log	
Seed production	GLMM	Summed number of seeds/head and plant	301 plants	Ploidy + year + summed number of florets (standardized)	Cultivar nested within year	NB	log	Offset, to account for different number of heads/plant sampled among years
Seed weight	GLMM	Thousand seed weight	67 (67 heads, 1 head per plant)	Ploidy	Cultivar	Gaussian	log	
Seed weight	LM	Seed weight/flower head	67 (67 heads, 1 head per plant)	Ploidy				Cultivar not added as random factor due to singular fit, result is the same with or without this random factor.
Pollinator preference and species presence	GLMM	Number of observed individuals	680 observations (85 transect walks, 8 potential species)	Pollinator tongue length × ploidy + number of flowering heads/plant + year	Cultivar nested within year + round nested within year	NB	log	
Visitation time	GLMM	Floret handling time	348 bee individuals	Pollinator tongue length × ploidy + year	Cultivar nested within year	Gamma	inverse	
Number of visited florets/flower head	GLMM	Number of visited florets/head	348 bee individuals	Pollinator tongue length × ploidy + year	Cultivar nested within year	Gamma	inverse	
Pollinator efficiency, pollen deposited on stigma during a single visit	LM	Number of pollen grains deposited/floret (mean/head)	36 flower heads	Pollinator tongue length × ploidy + year				Only performed on Ares (2n) and Vicky (4n). <i>Bombus lapidarius</i> (worker) and <i>B. subterraneus</i> were only observed on 2n plants during this assessment and therefore excluded from the analysis
Pollinator efficiency, seed production after a single visit	GLM	Number of seeds/bee individual (mean/head if bee visited several heads)	185 bee individuals	Pollinator tongue length × ploidy + year		NB	log	Only performed on Ares (2n), Vicky and Betty (4n). <i>B. terrestris</i> queen excluded from analysis because we only observed one
Pollinator efficiency, probability of seed production after a single visit	GLM	Seeds (at least one seed) or no seeds	185 bee individuals	Pollinator tongue length + ploidy + year		Binomial	logit	Only performed on Ares (2n), Vicky and Betty (4n). Interaction not included due to Betty only being part of this assessment one year

Notes: Presenting what was tested, which test, dependent variables, independent fixed variables, random variables used, as well as number of observations (N) for analyses performed. For tests on cultivar and pollinator species level, see Appendix S2: Table S1.

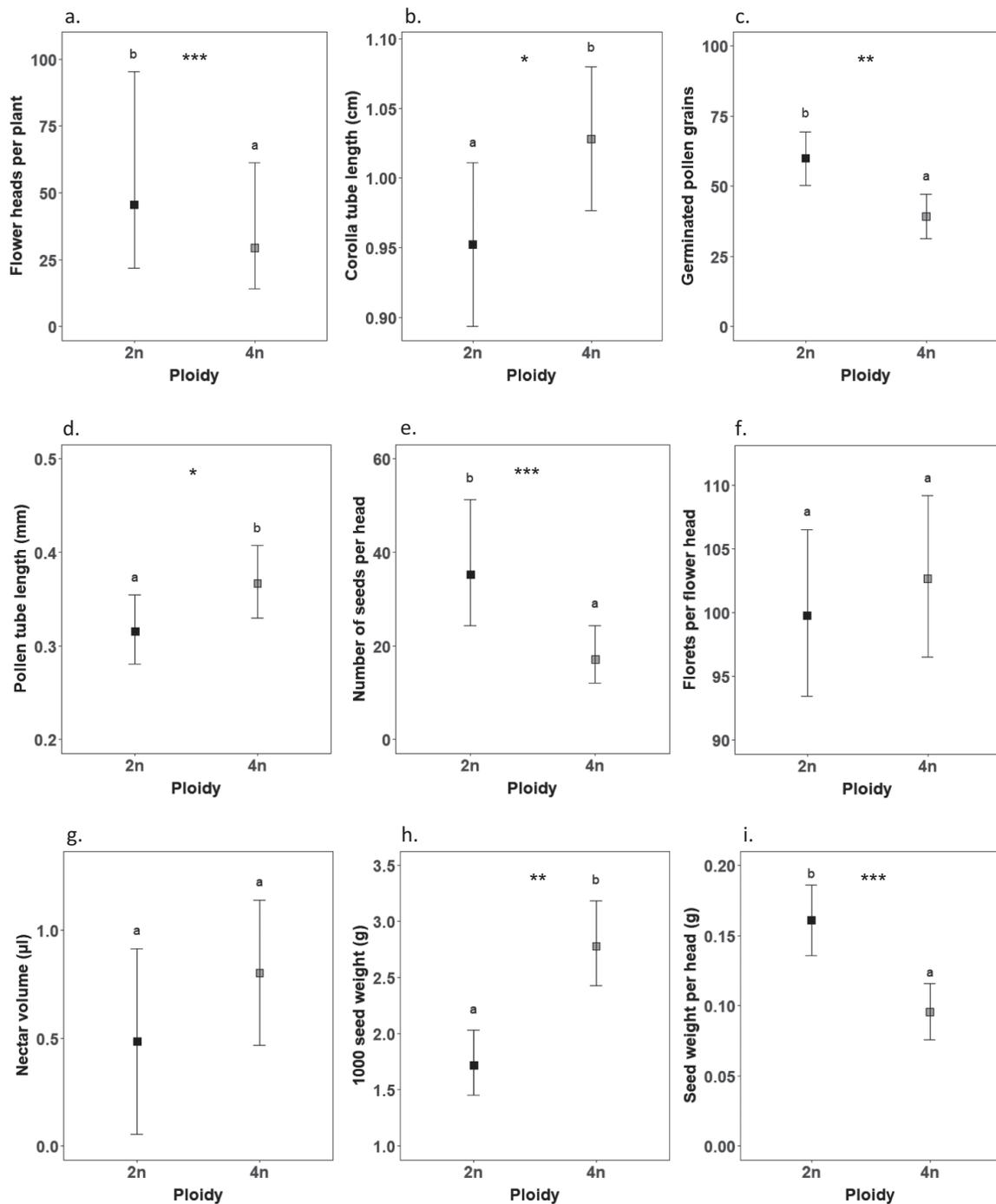


Fig. 1. Plant traits and seed production in relation to red clover ploidy. Estimated marginal means and confidence limits (95%) for (a) flower heads per plant, (b) corolla tube length (cm), (c) pollen germination rate in vitro, (d) pollen tube growth rate in vitro (tube length [mm/h]), (e) seeds per flower head during open pollination, (f) florets per flower head, (g) nectar volume (µL), (h) 1000 seed weight per plant (g), and (i) seed weight per flower head per plant (g), in relation to ploidy (2n, diploid; 4n, tetraploid). Pairwise comparisons of estimated marginal means are indicated by letters. Means sharing a letter are not significantly different. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Table 3. Summary of how plant traits and seed production in red clover are related to ploidy.

Dependent variable	Independent fixed variable	F	Chisq	df	P	Estimates (cl) ploidy	
						2n	4n
Flower heads/plant	Year	–	6.08	1	0.014		
	Ploidy	–	28.23	1	<0.001	45.55 (23.84–87.05)	29.44 (15.48–56.01)
Florets/flower head	Year	–	90.54	2	<0.001		
	Ploidy	–	0.46	1	0.50	99.76 (93.87–106.02)	102.64 (97.03–108.57)
Floret size (cm)	Year	1.46	–	2, 5.88	0.31		
	Ploidy	8.63	–	1, 5.43	0.029	0.95 (0.90–1.00)	1.03 (0.99–1.07)
Nectar volume/floret (µL)	Year	0.13	–	2, 4.69	0.88		
	Ploidy	4.11	–	1, 4.06	0.11	0.02 (0.01–0.03)	0.03 (0.02–0.03)
Pollen germination rate (%)	Year	10.69	–	1, 3.87	0.03		
	Ploidy	38.66	–	1, 3.55	0.005	59.91 (52.47–67.35)	39.27 (33.01–45.53)
Pollen tube growth rate (mm /h)	Year	–	468.30	1	<0.001		
	Ploidy	–	4.61	1	0.032	0.32 (0.29–0.35)	0.37 (0.34–0.40)
Seed production/head under open pollination	Year	–	61.77	2	<0.001		
	Ploidy	–	47.36	1	<0.001	35.30 (24.30–51.20)	17.10 (12.10–24.30)
Seed weight/1000 seeds (g)	Year	–	–	–	–		
	Ploidy	25.16	–	1	<0.001	1.72 (1.49–1.99)	2.78 (2.47–3.13)
Seed weight/flower head (g)	Year	–	–	–	–		
	Ploidy	21.62	–	1	<0.001	0.16 (0.14–0.19)	0.10 (0.08–0.12)

Notes: Statistical test results with F or chi-square (Chisq) statistics, degrees of freedom (df), and P-values for plant traits and seed production in relation to year (2010, 2014, 2015) and ploidy (2n, diploid; 4n, tetraploid). Estimates are model-based mean values and 95% confidence limits (cl), and bold numbers indicate significantly larger value.

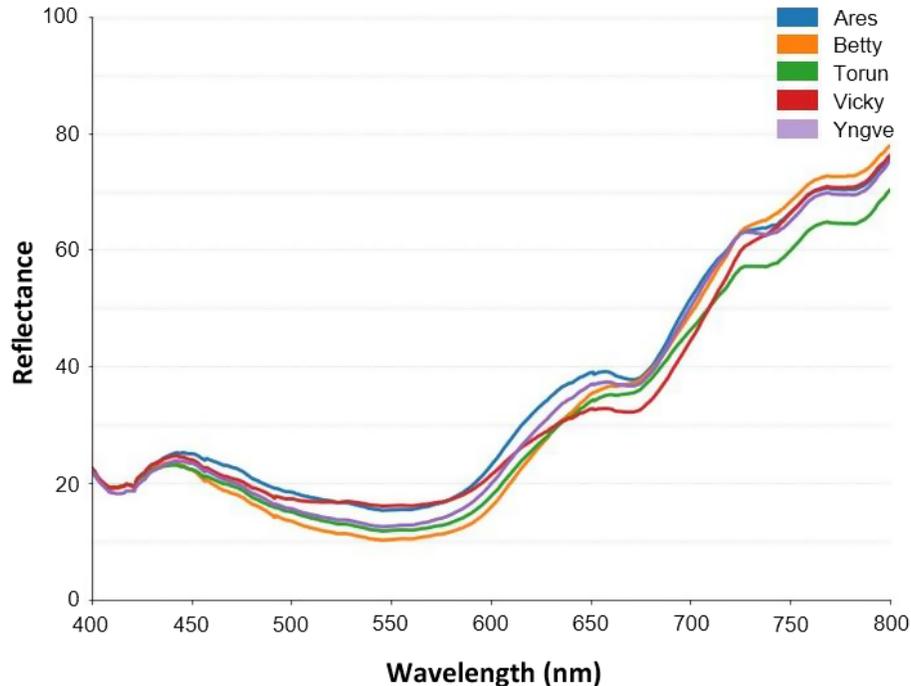


Fig. 2. Flower color in red clover cultivars. Reflection curves for flowers of two diploid (Ares and Yngve) and three tetraploid (Betty, Torun and Vicky) red clover cultivars in 2015.

($\text{chisq}_{df} = 11.92_1$, $P < 0.001$) and an interaction between tongue length class and plant ploidy (Table 4). Both short- and medium-tongued species visited diploid clover more frequently than tetraploid clover, while long-tongued species visited diploid and tetraploid clovers to the same extent (Fig. 3a, Appendix S2: Table S2). Similarly, from the perspective of the plants, diploid clover was more frequently visited by pollinator species with short and medium tongue lengths, while

there was no difference in visitation among tongue length classes in tetraploid clover (Fig. 3a).

Visitation time and number of visited florets per flower head.—We observed the behavior of 384 unique bee individuals during 2014 and 2015. Due to few observations of solitary bees and bumble bee queens, these individuals were excluded from analyses. The remaining 348 individuals belonged to seven bee species or species groups (*A. mellifera*, *B. hortorum*, *B. lapidarius*, *B. pascuorum*, *B. subterraneus*, *B. sylvarum*, and *B. terrestris* group). In total, four of the 384 individuals were observed robbing. All four individuals belonged to the *B. terrestris* group, meaning that 12% of *B. terrestris* individuals were observed robbing (20% of *B. terrestris* on tetraploids, and 5% on diploids, were observed robbing).

Handling time per floret differed among tongue length classes, but was not related to plant ploidy, or the interaction between tongue length and ploidy (Table 4). Pollinator species with shorter tongues spent more time per floret than those with medium long tongues, while long-tongued species did not differ from the other two tongue length classes in handling time (Fig. 3b, Appendix S2: Table S2). Number of visited florets was not related to tongue length class, ploidy, or the interaction between tongue length and ploidy (Table 4, Fig. 3c, Appendix S2: Table S2).

Estimation of pollinator efficiency, single visits.—For the assessment of pollen deposition during single visits, we observed 39 bees, from seven species or species groups, when foraging on in total 39 flower heads (20 on diploid plants and 19 on tetraploid; Appendix S2: Table S3). Since *B. lapidarius* (worker) and *B. subterraneus* were only observed on diploid plants, these were excluded from the analysis and data from 36 heads and 36 bees (7 long-, 15 medium-, and 14 short-tongued) were included. Number of pollen grains deposited on the stigmatic surface after a single pollinator visit was related to pollinator tongue length, but not to ploidy of the three investigated cultivars and there was no interaction between tongue length and cultivar (Table 4, Appendix S2: Table S2). Long-tongued species deposited more pollen grains than species with medium or short tongues (Fig. 4a, Appendix S2: Table S2).

For the assessment of seed production after single visits, we observed 186 individual bees

Table 4. Summary of pollination and pollinator visitation in red clover in relation to plant ploidy and pollinator tongue length.

Dependent variable	Independent fixed variable	F	Chisq	df	P
Pollinator preference	Year	–	0.18	1	0.67
	Ploidy	–	4.25	1	0.039
	Tongue length	–	30.13	2	<0.001
	Ploidy × Tongue length	–	11.86	2	0.003
Visitation time/floret/flower head	Year	–	0.71	1	0.40
	Ploidy	–	1.95	1	0.16
	Tongue length	–	14.31	2	<0.001
	Ploidy × Tongue length	–	1.04	2	0.59
Number of visited florets/flower head	Year	–	6.67	1	0.0098
	Ploidy	–	1.89	1	0.17
	Tongue length	–	1.73	2	0.42
	Ploidy × Tongue length	–	1.35	2	0.51
Single visit, pollen grains deposited on stigma	Year	11.40	–	1	0.0021
	Ploidy	0.88	–	1	0.36
	Tongue length	4.87	–	2	0.015
	Ploidy × Tongue length	0.37	–	2	0.69
Single visit, probability of at least one seed	Year	–	3.10	1	0.078
	Ploidy	–	2.37	2	0.31
	Tongue length	–	14.23	2	<0.001
	Ploidy × Tongue length	–	–	–	–
Single visit, seed production	Year	–	57.22	1	<0.001
	Ploidy	–	34.70	2	<0.001
	Tongue length	–	22.25	2	<0.001
	Ploidy × Tongue length	–	8.64	2	0.013

Notes: Statistical test results with F or chi-square (Chisq) statistics, degrees of freedom (df), and P-values for: Pollinator preference, visitation time, and number of florets visited as well as single-visit pollen deposition and single-visit seed production, in relation to year (2014, 2015), ploidy (2n, diploid; 4n, tetraploid) and pollinator tongue length class (S, <8 mm; M, 8–9 mm; L, >9 mm) and the interaction between ploidy and tongue length class. See Appendix S2: Table S2 for estimates. In the case of single visits, 2n and 4n are represented by only one or two cultivars each (2n: Ares; 4n: Betty, Vicky).

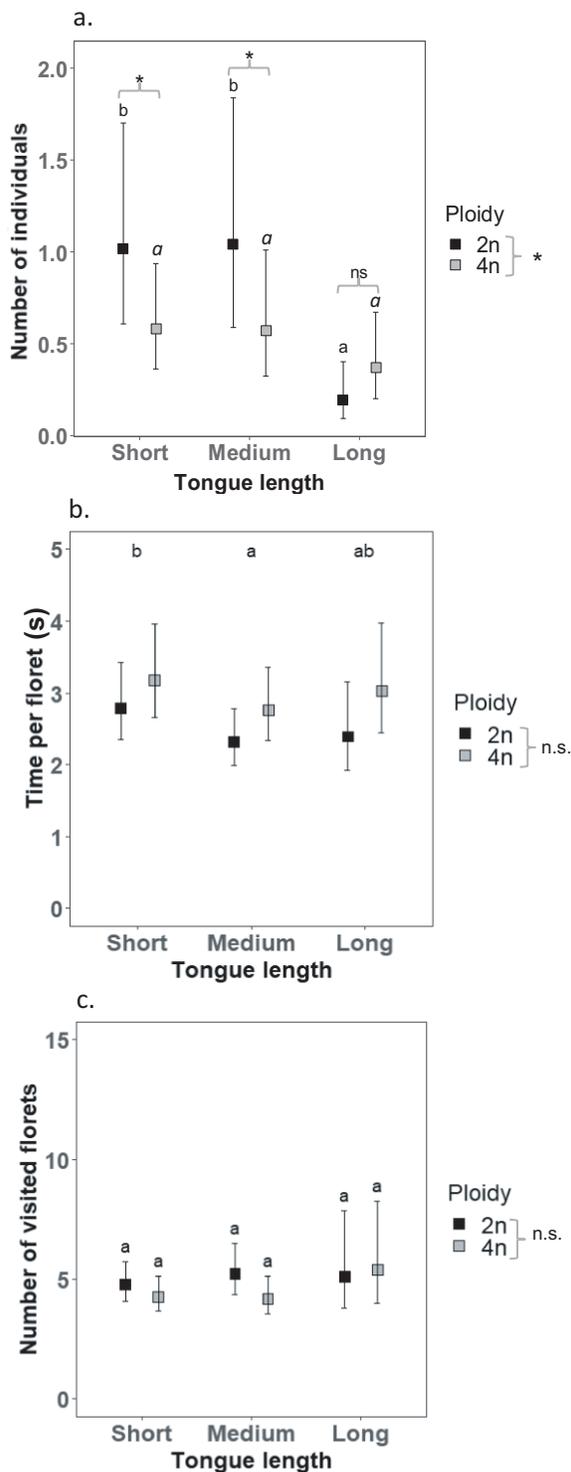


Fig. 3. Pollinator preference and behavior in relation to red clover ploidy and tongue length class. Comparison of estimated marginal means and confidence

when foraging on, in total 285 flower heads, whereof 121 heads were on diploid plants (64 in 2014 and 57 in 2015) and 164 on tetraploid plants (48 in 2014 and 116 in 2015). Out of the 186 bees (which belonged to eight different species/groups), 37 had long tongue, 66 had medium long tongue, and 83 had short tongue (Appendix S2: Table S3). Similar to observations of robbing during transect walks, we only observed robbing on four of the 285 flower heads. The robbing was done only on the cultivar Vicky (4n) by three different bee individuals of the species group *B. terrestris*. In three cases of robbing, no seeds were produced, and in one case, the flower head developed three seeds.

Pollination was unsuccessful (no seeds were found) in 102 of the 285 flower heads. The probability of producing at least one seed depended on tongue length, but not ploidy (Table 4, Appendix S2: Table S2). There was a higher probability of at least one seed being produced after a visit by medium- or long-tongued species compared with after a visit by short-tongued species (Fig. 4b, Appendix S2: Table S2).

Seed production per flower head was explained by an interaction between tongue length and plant ploidy (Table 4). Visits by short- and medium-tongued pollinator species resulted in more seeds in the diploid compared with the tetraploid plants, while there was no difference in seed production between ploidies after a single visit from a long-tongued pollinator species (Fig. 4c, Appendix S2: Table S2). Furthermore, within the tetraploids, a visit from a long-tongued pollinator resulted in more seeds being produced than a visit from a short-tongued pollinator, while a visit from a medium-tongued

(Fig. 3. *Continued*)

limits (95%) for (a) number of observed pollinator visitors from different tongue length classes, (b) time spent per floret for bees of different tongue lengths, and (c) number of visited florets per head between tongue length groups, and clover ploidy (2n, diploid; 4n, tetraploid). Pairwise comparisons of estimated marginal means are indicated by stars, n.s. or letters. Means sharing a letter are not significantly different. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; n.s. $P > 0.05$. In (a), italicized and non-italicized letters indicate separate comparisons.

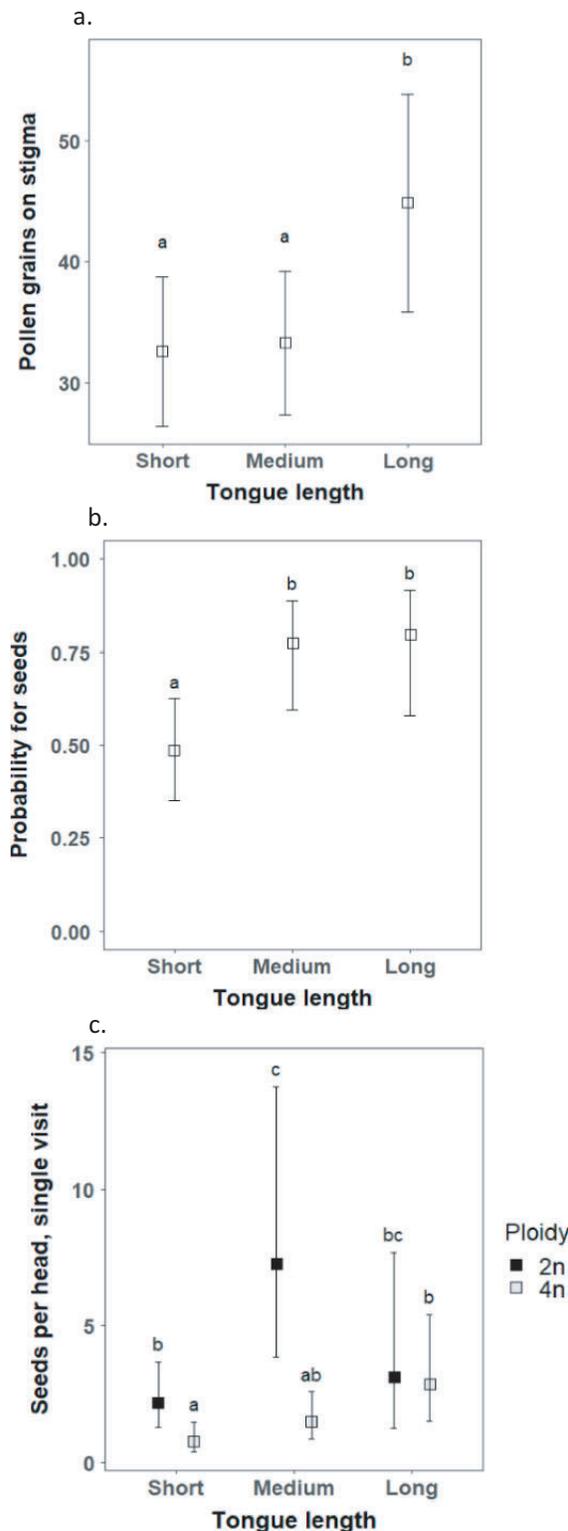


Fig. 4. Pollinator efficiency in red clover in relation to cultivar ploidy and pollinator tongue length.

pollinator resulted in intermediate seed production (Fig. 4c, Appendix S2: Table S2). For the diploid cultivar, higher seed production was observed after a visit from a medium-tongued pollinator compared with a short-tongued pollinator, while seed production after a visit by a long-tongued pollinator resulted in intermediate number of seeds (Fig. 4c, Appendix S2: Table S2).

DISCUSSION

We investigated plant traits and pollinator performance, singly and in combination, as possible causes for low seed production in tetraploid red clover cultivars. We found that the tetraploid cultivars produced 52% fewer seeds per flower head, resulting in a 41% lower seed weight per head, than the diploid cultivars. Tetraploid cultivars also had 35% fewer flower heads per plant, 8% larger florets, and 21% lower pollen viability. Pollinator species with short and medium tongue length showed a preference for diploid cultivars when given a choice, while long-tongued pollinator species did not differentiate flower visits by ploidy level. However, pollinator species with medium or long tongues were more efficient in pollinating diploid and tetraploid red clover, respectively, measured as number of seeds produced per head after a single pollinator visit.

Seed production difference between ploidy levels

Low seed yield in tetraploid red clover has been acknowledged as a persistent problem starting already when tetraploid cultivars were introduced about 70 yr ago (Julén 1954). We found 52% lower seed production, measured as number of seeds per flower head, in the investigated five tetraploid cultivars compared with the

(Fig. 4. *Continued*)

Estimated marginal means and confidence limits (95%) for single visits for (a) pollen deposited on stigma, (b) probability of at least one seed being produced in relation to tongue length, and (c) number of seeds produced per flower head after a visit by pollinators of different tongue lengths, in relation to clover ploidy (2n, Ares; 4n, Betty and Vicky). Pairwise comparisons of estimated marginal means are indicated by letters. Means sharing a letter are not significantly different.

four diploids over the three experimental years. Seed weight was higher in tetraploids in the year this was measured, but could not fully compensate for fewer seeds. In fact, the total seed weight per flower head was 41% lower in tetraploids. These results are in line with what has previously been reported (Taylor and Quisenberry 1996, Boller et al. 2010, Rundlöf et al. 2018). Seed quality was not investigated in this study, but we acknowledge the importance of including this factor in future studies. It should also be noted that our experimental field design with small, unreplicated plots of both diploid and tetraploid clover might not be fully comparable with field conditions. For example, it has been shown that proximity to diploid clover can have a negative effect on seed production in tetraploids as the presence of haploid pollen may hinder germination of diploid pollen (Julén 1950). Another factor that may influence our estimate of seed production is that during the first two years we did not account for the presence of the seed eating weevil *Protapion trifolii*, while we used an insecticide to control weevils the last year. This weevil species is well known to have a strong influence on red clover seed yield in commercial fields (Lundin et al. 2012) and can be more abundant in flower heads in tetraploid red clover fields than in diploid fields (Rundlöf et al. 2018).

Plant and pollination traits

Most studies have suggested that the low seed set in red clover tetraploids is caused by plant traits that either influence seed set directly (e.g., Mackiewicz 1965, Kendall 1967, Buyukkartal 2003, Buyukkartal 2008, Grebenisan and Savatti 2011, Vleugels et al. 2019) or have an indirect effect as a consequence of inferior interactions with pollinators (Nørgaard Holm 1966, Boller et al. 2010). Our tetraploid cultivars differed in both seed production and seed weight, suggesting a possible genetic influence on the reduced seed yield. Measurements of seven plant traits indicated that the tetraploid cultivars had fewer flower heads per plant, larger florets (longer corolla tubes), and lower pollen germination rate. Floral morphology was, in a review (Rezende et al. 2020), the most frequent flower traits reported to be affected by hybridization and polyploidy. In line with our result on floret size

in red clover, the consensus in the review was that polyploid species generally had larger flowers than diploids. While several studies do not regard red clover flower size as important for seed yield (Starling et al. 1950, Monks et al. 2010, Vleugels et al., 2015, 2016a, b), a relationship between seed yield and number of flower heads per plant has been demonstrated in both diploids (Bond and Fyfe 1968, Monks et al. 2010, Vleugels et al. 2014) and tetraploids (Herrmann et al. 2006, Vleugels et al. 2015, 2016a). Vleugels et al. (2015, 2016a) also suggested that number of mature flower heads per plant (and number of seeds per head) could be used as targets for breeding to increase seed yield in tetraploid red clover. They did not, however, consider whether these traits were influencing seed yield as a consequence of insect pollination due to flower attractiveness and pollinator traits. Number of flowers per plant could influence the number of produced seeds per plant, but also influence the attraction of pollinators to the plant (i.e., have an additive effect). When we related pollinator preference to cultivars and ploidy, the number of visiting bees was indeed (besides an interaction between tongue length or pollinator species and ploidy, see section on “Pollinator and pollination traits” below) explained by the number of flowering heads per plant.

We also found lower pollen germination rate in tetraploid cultivars, which is in accordance with previous studies (Grebenisan and Savatti 2011, Vleugels et al. 2019). While we do not currently know how pollen viability is correlated with siring success and seed production in red clover, this trait has potential to cause low seed production, in particular in combination with deposition of small pollen loads. Finally, we could not find any difference between ploidy level for the pollinator attractive trait flower color and no difference for the reward trait, nectar content, indicating that these traits may be of minor importance for explaining low seed yield in tetraploid red clover. Similarly, and in addition, Vanommeslaeghe et al. (2018) found no relation between nectar volume, or sugar content, and seed yield. In future studies, it would be highly informative to learn more about whether the plant traits that differ between ploidy levels are related to pollination success or to other factors.

Pollinator and pollination traits

The reduction in diversity and change in community composition of pollinating insects in the agricultural landscape over the past decades may contribute to decreased and more variable seed yield in red clover (Bommarco et al. 2012). This particularly relates to the decrease in pollinators with longer tongues in favor of a few short-tongued species in the Swedish and Danish agricultural landscape (Dupont et al. 2011, Bommarco et al. 2012). In the experimental plots of the current study, which are part of a common garden with high diversity of plant species, we detected eight bee species (or species groups) with the following abundance distribution in tongue length: 62% short-tongued, 28% medium-tongued, and 11% long-tongued. This is notably different from that seen in commercial red clover seed fields in Sweden where 95% of pollinators are short-tongued, mainly consisting of the *B. terrestris* group and *A. mellifera* (Rundlöf et al. 2018). Results from a recent field study in Sweden implied that this dominance of short-tongued pollinators makes it difficult to study the effect of tongue length on red clover seed yield differences related to ploidy, even for the case with added flower strips which had some influence on pollinator distribution (Rundlöf et al. 2018). Investigating foraging preferences of common bumble bee species in our experimental plots, we noted a stronger preference for diploids by both short- and medium-tongued species, but not for long-tongued species. This result is similar to that seen by Hänninen (1962), who observed a preference for tetraploids in *B. hortorum* and a preference for diploids in *B. pascuorum* and *B. distinguendus*. One advantage of our division of tongue lengths into three groups is that we could determine a difference in preference between the medium- and long-tongued groups, which commonly are grouped together as long in other studies (Wermuth and Dupont 2010, Rundlöf et al. 2018). Since our plots were small, it was easy for pollinators to actively select the diploid clover over the tetraploid and indicate pollinator preference given a choice. In commercial clover seed fields, there might not always be a choice. It is possible that short- and medium-tongued pollinators would still prefer tetraploid clover in the case that there are few other floral resources in the surrounding landscape. Yet, the

most common bee species found in commercial red clover fields, *B. terrestris* and *A. mellifera*, are known to forage several km from their nests (Steffan-Dewenter and Kuhn 2003, Osborne et al. 2008) and they may therefore find other choices. It would be valuable to further investigate what causes the observed pollinator preference.

While we found a clear interaction between plant ploidy and pollinator tongue length on pollinator preference, pollinator behavior showed more uniform patterns across plant ploidy. Three out of seven species spent longer time on tetraploids compared with diploids (Appendix S2), but in general short-tongued species spent more time on all florets than medium-tongued species did. Although we did not see a difference in number of florets visited per head, the longer time spent per floret could lead to a reduction in the total number of heads the bee have time to visit. A longer time spent could also lead to higher quality pollination; however, evaluating seed quality was beyond the scope of this study, and we did not find any evidence of better seed production (more seeds) or higher pollen deposit on stigma after visits by short-tongued species. Rather the opposite, with more pollen deposited and a higher likelihood of seed production after a single visit by medium and/or long-tongued species. In contrast to other studies (Hänninen 1962, Bender 1999), we found a rather low robbing rate in our pollinator observations and can therefore not draw any conclusion on the impact of pollinator robbing behavior on the difference in seed production between diploids and tetraploids.

It has long been discussed that the large florets in tetraploid cultivars lead to low pollination efficiency (Julén 1954, Wexelsen and Vestad 1954, Boller et al. 2010). However, few studies have investigated pollinator efficiency in pollinators that differ in tongue length in relation to plant ploidy. Our study revealed that two efficiency traits were independent of plant ploidy. Long-tongued species deposited more pollen on the stigmas of both the tested diploid and tetraploid cultivar after a single visit. We assume that a larger pollen load is an indication of higher pollinator efficiency, but it should be noted that we do not know the relationship between pollen load size and seed production for red clover. In addition, the pollen load assessment is based on only

a few individuals. Moreover, the probability to set at least one seed per flower head was generally higher after a visit by a pollinator with medium or long tongue, compared to a short tongue. The last investigated efficiency trait—number of seeds produced per flower head after a single visit—was instead determined by a combination of plant ploidy and pollinator tongue length. In the tested diploid cultivar, seed production was highest when pollinated by medium-tongued pollinators, while seed production was highest in the tetraploid cultivars when pollinated by long-tongued pollinators, both in comparison with short-tongued pollinators. The higher efficiency for medium- and long-tongued bees in general could be related to weight, size, and/or hairiness pattern of the bee (as the pollen spurts out of the floret, on to the pollinator, when the keel is pressed down by the visiting pollinator). Previous studies have shown that there is a relationship between pollinator tongue length and body size and the pollination function (Garibaldi et al. 2015) and that pollinator hairiness can be a good predictor of pollinator effectiveness (Stavert et al. 2016). This is, however, beyond the scope of this study but should receive attention in future studies. Importantly, the high pollination efficiency of medium- and long-tongued pollinators in red clover pinpoints the significance of conserving these species and to mitigate the changes in bumble bee community evenness for a higher and more stable yield of red clover seeds.

In general, polyploidy in plants can have effect on the interactions between pollinators and plants, and may generate changes in the biology of species (Soltis et al. 2004). Our study adds to the evidence of polyploidy exerting a behavioral change in pollinators toward their plant host (Rezende et al. 2020). With global environmental change threatening the mutualism between organisms (Kearns et al. 1998, Aslan et al. 2013), the importance of governing a diverse, and resilient, pollinator fauna seems evident, both for safeguarding future crop yields and to counteract the threat a declining pollinator fauna poses to wild ecosystems.

CONCLUSIONS

In conclusion, our study on plant traits and pollinator performance in diploid and tetraploid

red clover suggests that there are several problematic aspects that contribute to low seed yield in tetraploid red clover. In line with previous studies, we identify that number of flower heads per plant and floret size differ between cultivars with different ploidy, which may partly contribute to the low seed production in tetraploids. We also found ploidy differences in a third trait, pollen germination rate, which was reduced in tetraploids. This trait has received less attention and may be of importance to study further, in particular in relation to breeding for higher seed production. Even though we currently lack knowledge of whether the investigated plant traits influence seed production either directly or in relation to pollinator performance, our study indicates that pollinator abundance, behavior, and efficiency at least partly contribute to the problem of low seed production in tetraploids. One important influence of pollinators seem to be that short-tongued species prefer to visit diploid clover plants when given a choice. Interestingly, many patterns of pollinator behavior and efficiency were general across plant ploidy rather pointing to the importance of both medium- and long-tongued pollinator species for pollination success. Yet, as tetraploid clover plants have fewer flowers that can be pollinated (due to fewer flower heads per plant), and their pollen grains more often fail to germinate, and the most common pollinator species in the landscape might prefer other flowers when foraging—losing the most efficient pollinators (long- and medium-tongued) seems to be a larger threat to tetraploid than to diploid red clover cultivars. Our work implies that we should combine studies of plant and pollinator traits to gain a deeper understanding about the underlying reasons for the low and variable seed production in tetraploid red clover cultivars. It also points to the importance of actions to conserve and support populations of medium- and long-tongued bumble bee species in agricultural landscapes, as these are important for the future red clover seed production, in both diploid and tetraploid cultivars. If we, in the future, can support these pollinators, and overall a diverse pollinator fauna in the agricultural landscape, at the same time as we breed for tetraploid cultivars with higher seed yield per flower head, we might counter some of the problems with low seed yield for

growers and seed companies. More research focusing on small-scale pollinator–plant interactions, as well as large-scale pollinator–plant–landscape interactions, is needed and of future interest.

ACKNOWLEDGMENTS

We thank Sofia Hydbom, Maria Blasi Romero, and Delphine Lariviere for help with field and laboratory work; Linda Öhlund and Irene Persson at Lantmännen for providing the plants; Adam Flöhr and Jan-Eric Englund for providing valuable statistical consultation; and Olle Anderbrant and Glenn Svensson for providing insightful comments on the study system and the manuscript. The study was funded by PlantLink, The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (Formas), Stiftelsen Lantbruksforskning (with Lantmännen), Sveriges Frö-och Oljeväxtodlare, and The Royal Swedish Academy of Agriculture and Forestry. The funders had no involvement in conducting the study or preparation of the article. Some of the funds financing the project were received together with Linda Öhlund, who is an employee at Lantmännen, a commercial actor with interests in red clover seed production. ML and ÅL have continued this collaboration, and in an ongoing research project, which receives funding from independent sources, the same employee is a co-applicant. The plants included in the study were also given to us by Lantmännen.

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