Herbivory can cause shifts in pollinator foraging behavior by inducing changes in plant traits that influence pollinator attraction (Lehtilä and Strauss, 1997; Kessler et al., 2011; Barber et al., 2012). In this way, herbivory may alter the quantity of pollen deposited on the stigma by influencing, for instance, pollinator visitation rate (Lehtilä and Strauss, 1997; Ghyselen et al., 2015; Russell-Mercier and Sargent, 2015) or duration of flower visits (Barber et al., 2012). Additionally, herbivory may also impact the quality of deposited pollen by affecting the number of flowers visited sequentially on a plant (Steets et al., 2006; Ghyselen et al., 2015; Russell-Mercier and Sargent, 2015), thereby affecting the proportion of self pollen delivered to the stigma through geitonogamous pollination.

In many plant species, the proportion of self vs. outcross pollen deposited on the stigma can have an important impact on pollen tube growth. For instance, in species characterized by a self-incompatible breeding system, the pistil actively constrains pollen germination or pollen tube growth of self pollen (Herrero and Hormaza, 1996). Also in self-compatible plant species, pollen tube growth of self pollen can be hampered, and the presence of self pollen on the stigma may even constrain pollen tube growth of outcross pollen in that same pistil (Cruzan, 1990). During growth, pollen tubes depend on the resources available in the style, and as such, the pistil can affect pollen tube growth by varying the timing and amount of nutritional support (Herrero and Hormaza, 1996). It can thus also
be expected that the nutritional capacity of the recipient plant and factors that influence this capacity, such as herbivory, may affect pollen tube growth.

If the number of pollen grains growing in the style exceeds the number of ovules available in the ovary, male gametophytes may compete for successful fertilization (Herrera, 2002). The haploid genotypes of pollen are expressed during growth toward the ovule (Becker et al., 2003). Hence, pollen tube competition can promote fertilization by genetically superior or compatible fathers, either through direct competition between the growing pollen tubes or through female choice by which growth of pollen tubes is regulated. The intensity of pollen competition is often connected to offspring quality (Skogsmyr and Lankinen, 2002). Due to this process, even in self-pollinated plants pollen competition may promote offspring of better quality by allowing discrimination against pollen carrying recessive deleterious alleles that are also found in the mother plant (Lankinen and Armbruster, 2007).

Also, at the stage where the fittest and/or fastest pollen tubes reach the ovary, selection can act independently of pollen competition before fertilization can take place. This selection can, for instance, occur via late acting ovarian self-incompatibility, where ovules that would otherwise have participated in fertilization are disabled during growth of self pollen tubes in the style (Sage et al., 2006). After fertilization, seeds develop until they are fully mature, though some developing seeds may be aborted for several reasons. Selfed zygotes can, for instance, be aborted due to early-acting inbreeding depression (Owen et al., 2007). But also embryos of lower quality derived from outcross fertilization can be selectively aborted (Marshall and Ellstrand, 1988). As a consequence, plants can preferentially mature seeds of flowers in which the intensity of pollen competition was highest to enhance fitness of its resulting offspring the most (Niesenbaum and Casper, 1994). When, however, resources become constrained for the maternal plant, seed abortion rates tend to increase (Marshall and Ellstrand, 1988; Niesenbaum, 1996). Nonetheless, the selectivity based on pollen tube numbers does not necessarily increase with resource scarcity because other factors like positional patterns in resource acquisition might also determine seed abortion (Niesenbaum, 1996).

In summary, there are several stages in the process from pollen deposition to seed maturation when selection on pollen, pollen tube growth, or zygote development can occur. It can be expected that these different aspects of predispersal selection are influenced by herbivory via its effect on the proportion of self pollen deposited on the stigma and/or via its effect on the nutritional capacity of the maternal plant. However, these aspects have rarely been addressed in a context of herbivory (but see Niesenbaum, 1996). In this study, we investigated the impact of root herbivory on the process of fertilization and seed development following variable pollination treatments in the self-compatible monocarpic Cynoglossum officinale L. (Boraginaceae). Root herbivory was caused by the weevil Mogulones cruciger Herbst (Coleoptera: Curculionidae), an important specialist herbivore that feeds on the roots of C. officinale (Prins et al., 1992; Schwarzlaender, 1997). This study system is perfectly suited to the objectives of our study since this root herbivory significantly affects the number of flowers visited sequentially on C. officinale plants (Ghyselen et al., 2015). Besides this effect on geitonogamous pollination, root herbivory also induced changes in plant traits and reduced reproductive output in C. officinale, suggesting a decrease in nutritional capacity of the plant due to root damage (Prins et al., 1992; Ghyselen et al., 2015). To disentangle herbivory effects on fertilization success and seed production due to pollen quality and/or nutritional capacity, we combined open pollinations with self vs. outcross hand pollinations on each of the infested and noninfested plants in an experimental array. We surveyed several stages in the process from pollen deposition to seed maturation to investigate whether root herbivory affects (1) pollen germination and pollen tube attrition, (2) fruit set and seed initiation, abortion and maturation, and (3) the relationship between pollen tube competition intensity on the one hand, and fruit set and seed initiation and maturation on the other.

MATERIALS AND METHODS

Study system—Cynoglossum officinale is a herbaceous, monocarpic perennial that occurs in open, disturbed sites such as sand dunes and range lands. It is self-compatible, but it greatly depends on bees and bumble bees to increase its seed production (de Jong et al., 1990; Vrieling et al., 1999). Quantitative pollen limitation is unlikely in this species since supplemental addition of pollen in naturally pollinated flowers did not enhance seed set in natural and in experimental populations (de Jong and Klinkhamer, 1989; Ghyselen et al., 2015; C. Ghyselen, unpublished data). Furthermore, stigmatic pollen loads often largely exceeded the four ovules available per flower in naturally pollinated experimental plants (Ghyselen et al., 2015), indicating that pollen competition is a frequently occurring event. However, seed abortion is also frequent (Klinkhamer and de Jong, 1987; de Jong and Klinkhamer, 1989), and it can be selective, resulting in offspring of better quality (Melser and Klinkhamer, 2001).

Adult females of the weevil Mogulones cruciger lay their eggs in petioles or in the shoot at the base of plants of C. officinale in the spring. Eggs develop over 15 d, then hatch. The larvae then migrate into the taproot where they feed until they mature after 31 d and leave the root to pupate in the soil in early summer. Larvae, especially the second and third instars from days 9 to 31 of larval development, are the most damaging to the taproot of the host plant (Schwarzlaender, 1997).

Experimental plants and herbivory treatment—In April 2012, we dug out 40 plants from a large C. officinale population located at the Belgian coast (N 51.23926–51.29911, E 2.93347–3.07463). All plants reached the threshold size to flower (de Jong et al., 1986) and were not yet infected by M. cruciger. Twenty individuals were exposed to four adult root weevils per plant for 5–6 days by caging them on each of the focal plants, while the others (N = 20) served as the control group growing under the same conditions. All weevils and cages were removed after the treatments, and plants were placed outside in a weevil-free experimental garden at the Belgian coast. The herbivory treatment was started 3 weeks before the onset of flowering so that larval herbivory lasted throughout the flowering season (mid-May to end June) (de Jong et al., 1990). After seed ripening in July and August, all plants were excavated, and roots were washed and cut open. For each plant, we recorded whether the roots were infested with M. cruciger larvae. Only plants with signs of root herbivory were categorized as infested (N = 16), all other plants were categorized as noninfested (N = 23). In the infested plants, on average 31% of the root tissue was damaged (range 5–65%). One plant was omitted from the sample due to early mortality.
Pollination treatments—In each of the infested and noninfested plants, we designated eight flowers per cyme to one of three pollination treatments (24 flowers per plant): (1) bagged and hand-pollinated with outcross pollen obtained from three other study plants originating from the same population; (2) bagged and hand-pollinated with self pollen, and (3) open pollination by natural pollinators of C. officinalis from the surrounding dune area. For bagging the flowers, a mesh with a mesh size of 1 mm was used. Since flowers were only bagged a few days during anthesis, we assumed that this procedure does not influence fertilization and/or seed development. Eight flowers on one cyme received the same treatment to avoid potential effects of differential resource allocation between flowers of different treatments within the same cyme (Wesseling, 2007). The position of the three treatment cymes varied between the third and the hand-pollinated cyme (counting started from the top cyme of the plant), with the open-pollinated cyme in each plant at a higher position than the hand-pollinated cymes while outcross- and self-pollinated cymes were alternately higher or lower relative to each other. Due to rabbit herbivory, five experimental plants—two non-infested and three infested with M. cruciger larvae—were damaged and preventively omitted from further analyses.

Pollen germination and pollen tube attrition—To investigate herbivory effects on pollen tube growth, we cut off one pistil per pollination treatment just above the ovary in each study plant. Pistils were fixed in 9:1 ethanol–acetic acid and stored in 70% ethanol. They were prepared for epifluorescence microscopy by softening them in 0.5 mol/m 3 NaOH and staining them in 0.05% w/v aniline blue solution in 0.1 M phosphate buffer pH 8.5 (Ghysselen et al., 2015). For each style, we recorded the number of conspecific pollen grains and the numbers of pollen tubes in the stigma and at the base of the style. We calculated pollen germination as the proportion of conspecific pollen grains with pollen tubes that grew into the stigmatic tissue and pollen tube attrition in the style as the proportion of germinated pollen that did not reach the base of the style.

Fruit set and seed initiation, abortion, and maturation—After seed ripening, we counted the number of ovules, filled seeds and aborted seeds in each of the treated flowers. Filled seeds were large and firm, while aborted seeds were smaller and/or shriveled. We considered a flower that produced at least one filled seed as a flower that had set fruit. Proportional fruit set was then calculated as the number of fruits divided by the number of treated flowers. We calculated seed initiation as the proportion of ovules resulting in filled or aborted seeds, seed abortion as the proportion of initiated seeds that aborted, and seed maturation as the proportion of ovules resulting in filled seeds.

Statistical analyses—All analyses were performed in SAS 9.4. (SAS Institute, 2013) using PROC GLIMMIX with estimation of degrees of freedom by the Kenward Rodgers procedure. We corrected for overdispersion where necessary and used a backward selection procedure for the effects of herbivory, pollination treatment, and their interactions with each other and, for the pollen tube competition analyses, with the number of pollen tubes at the stigma. For all analyses, root crown diameter measured before the herbivory treatments, and cyme position and flower position on the cyme were included as covariates to correct for any differences in initial plant size between infested and noninfested plants and to reduce confounding effects of cyme and flower position.

Pollen germination and pollen tube attrition—To assess whether herbivory affected pollen germination and pollen tube attrition depending on pollination treatment, we used generalized linear mixed models. We first analyzed the number of conspecific pollen grains, and because this significantly differed between pollination treatments (Fig. 1A), we included this variable as a covariate in the models for pollen germination and pollen tube attrition. In each model, pollination treatment (open, outcross and self pollination), herbivory (infested vs. noninfested), and the interaction between both factors were included, with plant ID additionally entered as a random grouping variable. For the number of pollen grains on the stigma, a Poisson distribution was specified, and for pollen germination and pollen tube attrition, a binomial distribution was specified.

Results

Pollen germination and pollen tube attrition—The number of conspecific pollen grains on the stigma was not affected by herbivory (F 1,25.86 = 1.44, P = 0.24) nor by its interaction with pollination treatment (F 1,54.69 = 0.71, P = 0.50). Pollination treatment, on the other hand, significantly affected the number of pollen grains deposited on the stigma (F 2,47.88 = 8.31, P = 0.0006), with open pollination resulting in the highest and outcrossing in the lowest stigmatic pollen loads (Fig. 1A).

Pollen germination was not affected by herbivory (F 1,23.67 = 0.01, P = 0.91), nor by pollination treatment (F 2,71.06 = 0.78, P = 0.46; Fig. 1B) or the interaction between both factors (F 2,47.88 = 1.02, P = 0.37).

Pollen tube competition—To test whether plants selectively set fruit and initiated and matured more seeds in flowers with higher intensity of pollen tube competition, we compared fruit set, seed initiation, and maturation in relation to the number of pollen tubes at the stigma. Besides herbivory, pollination treatment, and their interaction, we also incorporated the interaction of the number of pollen tubes with herbivory and with pollination treatment to test whether the selectivity based on pollen tube numbers changes in response to herbivory and to test for differences in the effect of pollen tube competition intensity between outcross-, open-, and self-pollinated flowers, respectively. We used generalized linear mixed models with plant ID as random grouping variable and a binomial distribution.
Fruit set and seed initiation, abortion, and maturation—The proportion of treated flowers that set fruit was higher in noninfested plants compared with infested plants ($F_{1,29.09} = 4.85, P = 0.04$; Fig. 2A). Fruit set did not differ among pollination treatments ($F_{2,190} = 1.76, P = 0.17$), nor was there any significant interaction between pollination treatment and herbivory ($F_{2,176.4} = 2.14, P = 0.12$).

The proportion of initiated seeds tended to be affected by herbivory ($F_{1,30.33} = 2.85, P = 0.10$), though this effect also depended on pollination treatment as indicated by the significant interaction effect between herbivory and pollination treatment ($F_{2,510.4} = 3.56, P = 0.03$; Fig. 2B). Five pairwise comparisons using $\alpha = 0.01$ to correct for multiple comparisons showed that seed initiation in noninfested plants compared with infested plants was higher in outcrossed flowers ($t_{73.16} = 2.20, P = 0.03$) and in open-pollinated flowers ($t_{46.16} = 2.07, P = 0.04$), but did not differ in selfed flowers ($t_{59.76} = -0.27, P = 0.79$). Seed initiation in noninfested plants, on the other hand, was significantly higher in outcrossed compared with selfed flowers ($t_{704.5} = 2.64, P = 0.009$), while it did not differ between selfed and outcrossed flowers in infested plants ($t_{712.1} = 1.07, P = 0.28$). Pollination treatment itself did not influence the proportion of initiated seeds ($F_{2,362.4} = 0.24, P = 0.79$).

The proportion of aborted seeds was neither affected by herbivory ($F_{1,28.81} = 1.91, P = 0.18$; Fig. 2C), nor by pollination treatment ($F_{2,59.38} = 0.24, P = 0.79$), and both factors did not interact significantly ($F_{2,55.04} = 0.08, P = 0.92$).

The proportion of matured seeds was higher in noninfested plants than in infested plants ($F_{1,29.4} = 5.49, P = 0.03$; Fig. 2D). Pollination treatment did not significantly affect the proportion of matured seeds ($F_{2,398.6} = 0.27, P = 0.76$), nor did it interact significantly with herbivory ($F_{2,555.8} = 1.60, P = 0.20$).

Pollen tube competition—The interactions between the intensity of pollen tube competition on the one hand and pollination treatment or herbivory on the other were not of significant importance for fruit set ($F_{2,76} = 0.40, P = 0.67$ and $F_{1,78} = 0.68, P = 0.41$, respectively). Fruit set was also not influenced by the intensity of pollen tube competition ($F_{1,79} = 0.53, P = 0.47$).

The proportion of initiated seeds increased significantly with intensity of pollen tube competition ($F_{1,72.77} = 4.65, P = 0.03$), but this was only the case in infested plants ($\beta = 0.039, SE = 0.019$ compared with $\beta = 0.008, SE = 0.010$ in noninfested plants; $F_{1,58.34} = 2.17, P = 0.15$). The relationship between the intensity of pollen tube competition and the proportion of initiated seeds was not affected by pollination treatment ($F_{2,65.82} = 0.21, P = 0.81$).

The interactions between the intensity of pollen tube competition on the one hand and herbivory or pollination treatment on the other were not of significant importance for seed maturation ($F_{1,78} = 0.67, P = 0.41$ and $F_{2,78} = 0.31, P = 0.73$, respectively). Seed maturation was also not influenced by the intensity of pollen tube competition ($F_{1,79} = 0.03, P = 0.86$).

DISCUSSION

In our study, herbivory did not affect the early stages after pollination, such as pollen germination and pollen tube attrition. However, later stages, like fruit set and seed maturation, were significantly affected by herbivory. Herbivory also influenced the presence of pollen tube competition driven selection on seed initiation.

FIGURE 1 Effect of pollination treatment (outcross, open, and self pollination) in Cynoglossum officinale plants on (A) average number of conspecific pollen grains on the stigma, (B) average proportion of conspecific pollen grains that germinated and resulted in pollen tube growth into the stigmatic tissue, and (C) average proportion of pollen tubes that failed to reach the base of the style. Error bars represent standard errors. Different letters indicate significant differences between pollination treatments ($P_{Tukey adj} < 0.05$).
Prezygotic stage of reproduction—Herbivory did not affect pollen germination or pollen tube attrition in the open-pollinated flowers nor in the hand-pollinated flowers for which quality of the deposited pollen was controlled. Although pollen tubes are dependent on the resources available in the style during their growth (Herrero and Hormaza, 1996), our postulation that herbivory might affect pollen tube growth by decreasing the nutritional capacity of the recipient plant did not hold. Possibly at the time of pollen tube growth, infested plants did not experience that much resource limitation because the damage in the roots was not that extensive yet and did not limit the resources available in the style to support pollen tube growth. In nature, a small proportion of ovipositions already occur in autumn and early spring, but the vast share of ovipositions occurs in April (Schwarzlaender, 1997). Consequently, the amount of root damage at the time of pollen tube growth will in most cases not be larger than in our experiment such that root herbivory by *M. cruciger* may rarely impact early reproductive processes in *C. officinale*.

We did find a significant effect of pollen quality (self vs. outcross) on pollen tube attrition, with outcross-pollinated flowers experiencing 30% more pollen tube attrition than self-pollinated flowers. However, other studies documented contrasting or neutral effects of pollination treatment on pollen tube growth. For instance, in the self-compatible *Erythronium grandiflorum*, fewer pollen tubes reached the base of the style after self pollination (Cruzan, 1990), whereas in the largely self-sterile *Cyrtanthus breviflorus* no difference in growth between self and outcross pollen tubes was observed (Vaughton et al., 2010). Although it is known that *C. officinale* is able to produce seeds after self pollination (Klinkhamer and de Jong, 1987; Vrieling et al., 1999), until now no insights were available on the effects of pollen quality on pollen germination and pollen tube attrition in this species.

The difference in pollen tube attrition between outcross- and self-pollinated flowers that we observed in *C. officinale* might be explained by the composition of the pollen pool. Cruzan (1990), for instance, reported that the attrition of certain pollen tubes may depend upon the source of other pollen tubes growing in the same style in *Erythronium grandiflorum*. Given the observation that in open-pollinated flowers of an experimental *C. officinale* population, selfing rates averaged 0.32, ranging from 0 to 0.70 (Vrieling et al., 1999), it might be possible that pollen tube competition is more important in outcrossed flowers than in open- and self-pollinated flowers. For the latter two, selection of compatible pollen at the stage of pollen germination might be of larger importance (Fig. 1B and 1C). In the open-pollinated flowers and to a lesser extent in the self-pollinated flowers, the stigmatic pollen load was also higher than in outcross-pollinated flowers (Fig. 1A). This higher pollen load could have caused a stigmatic clogging effect, which can impede pollen germination and pollen tube growth of pollen grains (Snow, 1986; Thomson, 1989).

Under such conditions, it can be expected that only the fittest pollen grains germinate. In the outcross-pollinated flowers, on the other hand, selection probably did not yet, or to a much lesser extent, occur at this stage. Another plausible explanation might be that some of the crosses in the outcrossed flowers may have suffered from outbreeding depression, that is reflected in higher pollen tube attrition. The crosses were made with pollen from three other study plants. Because these study plants came from a large population, probably some of the crosses were between plants that would under natural circumstances not cross because the distance between them was too large.

Postzygotic stage of reproduction—Herbivory provoked reductions in the proportions of fruit set and mature seeds over all pollination treatments. All in all, the treated flowers in noninfested plants set 61% more fruits and produced 79% more seeds than those in infested plants (Fig. 2A and 2D). We hypothesize that the impact of root herbivory on reproduction in this plant species is due to a decrease in nutritional capacity because the larvae (1) consume the resources that were stored in the tap root during the years of vegetative growth, and/or (2) damage the transport system within the plant, as was also suggested in the studies of Prins et al. (1992) and Ghyselen et al. (2015).

The number of initiated seeds tended to be affected by herbivory, though this effect depended on pollination treatment. In outcross-
open-pollinated flowers, a lower number of seeds were initiated in infested plants compared with noninfested plants, while selfed flowers did not show such a difference (Fig. 2B). Probably herbivory affected the number of initiated seeds by limiting the resources available for seed production. Although we do not know whether the ovules in which no seeds were initiated were actually fertilized, the omnipresence of pollen tubes in the styles of flowers that did not initiate any seeds (C. Ghyselen, personal observation), in combination with earlier indications that there is more abortion than meets the eye in C. officinale (de Jong and Klinkhamer, 1989), suggests that these ovules were fertilized and aborted at a very early stage of zygote development.

The lower number of initiated seeds in self-pollinated flowers compared with outcross-pollinated flowers in noninfested plants might point to selective abortion of some of the selfed seeds owing to early-acting inbreeding depression (Husband and Schemske, 1996). In open-pollinated flowers of noninfested plants we found an intermediate proportional seed initiation compared with outcross- and self-pollinated flowers of noninfested plants, which probably indicates the presence of a mix of outcross and self pollen in these flowers. In the infested plants, we did not find an obvious intermediate proportional seed initiation in open-pollinated flowers. Though we expect a lower proportion of self-pollen deposition onto those stigmas compared with open-pollinated flowers of noninfested plants, our results do not unequivocally support this hypothesis, possibly because the negative effect of herbivory through a decreased nutritional capacity overrides an effect of pollen quality.

An important note is that in this study we compared flowers that were hand-pollinated with either only outcross or only self pollen, while open-pollinated flowers of C. officinale most probably receive a mixture of both (Vrielings et al., 1999). In some other self-compatible species, outcross-pollen tubes may reach ovules more quickly than self-pollen tubes, thereby reducing self fertilization in flowers with a mixture of both types of pollen (Hessing, 1989; Aizen et al., 1990). In the self-compatible Erythronium grandiflorum significantly more seeds were sired by outcross pollen following simultaneous application of equal amounts of self and outcross pollen due to mechanisms operating after ovule fertilization (Rigney et al., 1993). To further investigate a possible effect of herbivory on pollen quality, that is, the proportion of self vs. outcross pollen that arrives on the stigma, and its consequences for seed initiation, the use of genetic markers is needed to determine the parentage in flowers that received a certain proportion of self vs. outcross pollen and in open-pollinated flowers in response to herbivory in C. officinale.

Seed abortion was not affected by herbivory in our experiment, but there was a trend toward more aborted seeds in infested compared with noninfested plants (Fig. 2C). Since the quality of deposited pollen did not influence seed abortion, again a decreased nutritional capacity causing higher seed abortion in infested plants is the most plausible explanation for this observation. Abortion rates indeed tend to increase when resources become more constrained due to herbivory or other causes (Marshall and Ellstrand, 1988; Niesenbaum, 1996).

**Pollen tube competition**—In an earlier study on C. officinale, we found that the mean number of pollen tubes growing in the style was almost eight times higher than the number of ovules in open-pollinated flowers (Ghyselen et al., 2015), indicating a large potential for pollen tube competition. Cynoglossum officinale can selectively abort seeds, resulting in offspring of better quality (Melser and Klinkhamer, 2001), and the intensity of pollen competition is generally positively linked to offspring quality (Skogsmyr and Lankinen, 2002). Here we found a positive relationship between the number of pollen tubes and seed initiation success, indicating that zygotes of lower quality are preferentially aborted, though only in infested plants. Our results thus empirically confirm the hypothesis that the degree of competition driven selection at the level of pollen tube growth may increase when resources become more limited due to herbivory (Niesenbaum, 1996).

**CONCLUSION**

In this study, herbivory did not affect early stages in the process from pollination to seed maturation, but it did impact later stages such as fruit set, seed initiation, and maturation and selection on zygote development driven by pollen tube competition. Our findings suggest that the impact of herbivory on pollen quality, as previously documented (Ghyselen et al., 2015), was not responsible for the observed differences in fruit and seed production. Instead, decreased nutritional capacity of the mother plant from root herbivory most probably caused higher zygote and fruit abortion and is presumed to be primarily responsible for the observed reduction in female reproductive success. Our study stresses the importance of assessing the impact of herbivory at different stages, from pollination to seed maturation, to better understand the potential cause(s) of herbivory effects on final reproductive output.

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**LITERATURE CITED**


