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POLLINATION ECOLOGY AND FLORAL VISITOR SPECTRUM OF TURTLEHEAD (*CHELONE GLABRA* L.; PLANTAGINACEAE)

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Abstract—Many flowering plants engage in mutualistic interactions with animals in order to sexually reproduce, exchanging food rewards such as nectar and pollen for the service of pollen transfer between flowers. Floral reward variation strongly influences visitation patterns of both pollinating mutualists and non-mutualist consumers, with consequences for both male and female components of plant reproductive success. Despite the importance of pollination to ecological systems, the pollination ecology of many plants is poorly known. At seven sites over three years, we studied the mating system, floral visitors and pollen limitation of turtlehead (*Chelone glabra* L.), an eastern North America wetland herb. We found that the plant is autogamous, but requires pollinator visitation to set seed. *C. glabra* flowers are protandrous, with floral rewards that vary between male and female sex phases. We found diurnal variation in reward presentation that was a function of both floral phenology and consumer behaviour. *Bombus vagans* Smith, the most common visitor to *C. glabra* flowers, removed a large fraction of available pollen (> 36%) in single visits to newly opened flowers, and compared to other flower visitors, passively transported more pollen on flights between flowers and deposited more to conspecific stigmas, suggesting it was the most effective pollinator. The solitary bee *Hylaeus annulatus* L. made frequent visits to flowers, but contributed little to pollination due to morphological mismatch and because it avoided male-phase flowers. Despite high bee visitation rates, flowers were pollen limited for seed production, possibly indicating a negative effect of non-pollinating flower visitors on plant reproductive success.

Keywords: Nectar, Pollen, Pollen thievery, Pollen limitation, Protandry, Buzz pollination

INTRODUCTION

Evolutionary ecologists are fundamentally interested in factors that govern the abundance, distribution, and evolution of species. Species interactions such as mutualism, predation and competition are ubiquitous in nature, and partner traits often contribute to outcomes of these interactions, with consequences for species distribution, evolution (Thompson 1999), reproduction and ecosystem services provisioning (Garibaldi et al. 2015). For angiosperms, interactions with pollinators are critically important to fitness, with the majority of species benefiting from transfer of pollen within and between flowers by animal pollinators (Ollerton et al. 2011). Deficits in pollinator visitation can limit host plant reproduction (Ashman et al. 2004; Knight et al. 2005) and structure plant population dynamics (Biesmeijer et al. 2006; Lundgren et al. 2015), and interactions between plants and pollinators provide some of the best known examples of evolution and co-evolution by natural selection (Fenster et al. 2004). Nectar and pollen rewards and spatiotemporal variation in their presentation can have strong effects on the community of pollinators visiting flowers (Pleasants 1983; Thomson et al. 2000), and that pollinator community can host species that range from effective pollinators to those that transfer little pollen among flowers and plants (Hargreaves et al. 2012). Despite the wealth of knowledge on the ecology and evolution of plant-pollinator interactions, there are many flowering plant species for which the floral ecology and floral visitor spectrum remain understudied. Nonetheless, natural history studies of a plant's pollination biology can provide key insights into the importance of pollination mutualisms for a species' ecology and conservation.

The purpose of this study was to investigate the mating system and floral visitor spectrum of a protandrous plant reported to be pollinator dependent, *Chelone glabra* L. (Plantaginaceae; hereafter *Chelone*). We chose to study the pollination ecology of *Chelone* because it is a common flowering plant in wetlands in eastern North America and its floral rewards may be important in maintaining bee health (Richardson, Adler, et al. 2015). One prior study reported that *Chelone* is self-compatible and dependent on pollination by bumble bees, but presented only qualitative observational data (Cooperrider 1967). Our goal was to provide quantitative insight into the mating system and floral ecology of *Chelone* to put questions about the plant's floral rewards and floral visitors into a relevant natural history context. We addressed four questions: 1) How do nectar and pollen reward presentation change from male to female sex

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phases, and what is the diurnal impact of insect foraging on nectar and pollen availability? 2) Who are the primary floral visitors to *Chelone* flowers, and what is the relative effectiveness of each flower visitor at transferring pollen from anthers to stigmas? 3) Can nectar and pollen foragers distinguish between male- and female-phase flowers, and does distinguishing among these flower types affect their likelihood of transferring pollen? 4) Are plants pollen limited for fruit and seed production? We predicted that, similar to other protandrous plants, pollen would function as the principal attractant for pollinators during male phase (Bertin & Newman 1993), allowing for pollen collecting visitors that specialize on male-phase flowers and thus may not transfer pollen to female-phase stigmas. We predicted that nectar and pollen harvesting bumble bees would act as pollinators because of an apparent morphological fit between worker caste bees and floral architecture (Cooperrider 1967), but that smaller non-bumble bee visitors would act as pollen thieves (Inouye 1980), consuming pollen but potentially missing contact with stigmas while doing so. Finally, given the potential for non-mutualist pollen removal by bees, we predicted that *Chelone* seed production would be pollen limited, with pollen-augmented flowers producing more seeds than those experiencing natural floral visitation rates (Ashman et al. 2004). Taken together, this research provides a comprehensive analysis of the floral ecology and floral visitor spectrum of a pollinator-dependent plant.

MATERIALS AND METHODS

Study system

Chelone glabra is a perennial herb native to eastern North America, occurring in circumneutral seepage swamps, marshes and anthropogenic wetlands (Gleason & Cronquist 1991; Nelson 2012). It commonly forms dense, near monotypic stands in open marshes (range: approx. 1-50 stems per m^2). Despite clonal vegetative reproduction, *Chelone* genets can often be distinguished because of spatial separation between plants. Individual plants produce numerous stems (range: 1-20+ stems per plant), most terminating in a single racemose inflorescence. Inflorescences initiate 14.4 ± 0.9 SE (range: 6-28) flower buds, of which only 10.6 ± 1.0 SE open in a given flowering season (the others failing to complete development). The sympetalous, white- to rose-colored flowers average 3.0 ± 0.2 SE cm in length (range: 2.6.5-3.5 cm), with 47% of the length comprised of a prominent upper hood and lower standard petal. Flowers are zygomorphic, and the distal margins of upper and lower petal segments typically touch, creating a constricted flower entrance. Each day, 2.1 ± 0.1 SE flowers per stem are open simultaneously (range: 1-6 flowers). Flowers each have four anthers recessed within the hooded upper corolla lip in pairs that are nearly confluent until forced apart by large bees. Pollen sacs dehisce via longitudinal slits and are covered with a dense layer of woolly hairs (Straw 1966). A short staminode is also present. *Chelone* flowers are strongly protandrous. While in male phase (approx. 1 d), flowers have a short, recessed style and anthers that dehisce pollen, often onto the dorsum of foraging bees. While in female phase (approx. 2 d), the style is elongated and recurved approx. 180°, placing the stigmatic surface near the corolla entrance and distal to anthers (L. L. Richardson, pers. obs.). Nectar is secreted by a hypogynous disk of nectary tissue, and production begins at anthesis (Straw 1966). *Chelone* is reported to be self-compatible, but because flowers are protandrous, pollinators are required to carry self-pollen between anthers and stigmas of flowers on the same plant (Cooperrider 1967). Bumble bees are reported to be the primary visitors to *Chelone* flowers (Pennell 1935; Cooperrider 1967; Heinrich 1975; Williams et al. 2014), but the degree to which they are pollinators and whether other bees contribute to *Chelone* pollination or act as pollen thieves is unknown. Bumble bees forage for both nectar and pollen, often sonicating or 'buzzing' *Chelone* flowers to shake pollen from the anthers (Heinrich 2004).

Chelone leaves and other vegetative tissues contain two secondary metabolites that deter generalist herbivores, the iridoid glycosides aucubin and catalpol (Bowers et al. 1993). However, numerous specialized herbivores use the plant as a host, including foliar herbivores (e.g., *Euphydryas phaeton* Drury (Nymphalidae: Lepidoptera) and *Tenthredo grandis* Norton (Tenthredinidae: Hymenoptera) (Bowers et al. 1993)) and predispersal seed predators (e.g., *Phytomyza cheloniae* Spencer (Agromyzidae: Diptera) and *Endothenia hebesana* Walker (Tortricidae: Lepidoptera) (Stamp 1987)). Seed predators are reported to attack nearly a quarter of *Chelone* fruits (Stamp 1987) and thus may influence the degree to which pollination translate into successful seed-bearing fruits. Depending on timing of attack, fates of fruits hosting seed predators include partial or complete consumption of matured seeds, or abortion before seeds mature (L. L. Richardson, pers. obs.).

Field methods

From 2011-2013, we studied the reproductive biology of *Chelone* at seven plant populations spread over 75 km in northern Vermont, USA (Appendix 1).

Phenology

To study individual flower phenology, in 2011 at two populations we followed flowers from anthesis to when corollas dropped, recording floral sex phase three times daily. At each site, we followed 2-5 flowers on each of 8 plants for a total of 33 flowers. To study population flowering phenology, in 2011 and 2012 we randomly selected 20 focal inflorescences ≥ 10 m apart along a linear transect through populations (two populations in 2011; one population in 2012). We censused inflorescences every 2-3 days, recording total number of flower buds, open flowers of each sex phase and developing fruits. We also noted the presence of herbivores and seed predators.

Mating system

We studied the mating system of *Chelone* in 2011. We randomly chose 20 plants at each of two populations, and on each plant assigned inflorescences to one of three treatments: 'outcrossing', 'selfing', and 'unpollinated'. We covered all inflorescences with bags made of wedding veil before flowering began. We visited populations every 2-3 days during the flowering season to apply treatments. For the

outcrossing treatment, we collected fresh pollen from ≥ 5 plants in the population by sonication with an electric toothbrush and mixed it in a small Petri dish. We removed bags and applied this mixture with a clean pinhead to the stigmas of all open flowers before replacing the bag. We verified by microscopy that this method resulted in pollen grains sticking to stigmas (data not shown). For the selfing treatment, we removed bags, collected pollen by sonication from all open flowers on the same inflorescence, then re-applied this pollen to stigmas of the same flowers with a pinhead cleaned in ethanol. Because we visited the inflorescences every 2-3 days, we did not apply treatments to every flower on the inflorescence. Thus, each time we applied the outcrossing and selfing treatments, we marked the sepals of treated flowers with a black marker (Sharpie, Illinois, USA) so that fruits resulting from our treatments could be identified; we manipulated, on average, 52% of the flowers on inflorescences. Flowers of the unpollinated treatment did not receive pollen, but each time we applied the other treatments, we handled these inflorescences similarly, removing and replacing the bags and marking sepals of open flowers. We removed bags after flowering had ceased and collected infructescences when they were nearly mature. We later dissected infructescences, counting total number of fruits matured and number of seeds in each fruit. We also noted presence of predispersal seed predators in these fruits, but did not analyse whether pollination treatments affected attack rates because placing pollinator exclusion bags over inflorescences may have biased seed predator oviposition.

We conducted all statistical analyses (here and below) using JMP (version 11.2; SAS Institute, Inc. 2014) and R statistical software (R Core Team 2015) and when appropriate compared AIC scores among candidate models to select statistical models that best fit the data. We used linear mixed models ('lme4' library for R statistical software; Bates et al. 2014; R Core Team 2015) to analyse seed number per fruit, comparing Akaike Information Criterion (AIC) scores sequentially to select random and fixed effects for a best-fit model (Bolker et al. 2009). We used analysis of variance to compare the best-fit model with reduced models, then calculated chi-square statistics and significance values for the influence of fixed effects. The full model included log-transformed seed number per fruit as the response variable, pollination treatment and presence of pre-dispersal seed predators as fixed effects, and plant individual and population and individual nested within population as random effects. We used Tukey HSD post-hoc tests to identify statistically significant differences among pollination treatments. We excluded from this analysis fruits where pre-dispersal seed predators made seed counts impossible, but included attacked fruits where accurate counts were possible. Due to high rates of pre-dispersal seed predator attack (36% of fruits collected) and fruit abortion, we were unable to accurately calculate per cent of fruits maturing seed as a function of pollination treatments.

Flower visitors

From 2011-2013, we made collections of *Chelone* floral visitors at seven populations throughout the flowering season. In timed collections, we randomly moved through patches of flowering *Chelone*, collecting by net as many foraging insects as we could in a 30-minute period. Collection efforts occurred throughout daylight hours on days when bees were foraging, and we also made 3 hrs of collections between 2100-2400 hrs to look for nocturnal visitors. We made additional haphazard collections of flower-visiting insects not observed during standardized collecting events, and made observations of bee visits to flowers, noting nectar and pollen collecting behaviour and making sound recordings with a digital voice recorder (Sony, USA) to document any potential bee sonication of flowers. We pinned and identified all collected insects, except that the genus *Lasioglossum* was identified only to morpho-species and flies, sawflies and wasps were identified only to Order (Mitchell 1960, 1962; Michener et al. 1994; Droege et al. 2014; Williams et al. 2014). We used basic summary statistics to compare flower visit frequencies among insects we collected at flowers. We calculated the proportion of collecting events during which we collected each species and the relative abundance of each in the overall collection.

To assess flower visit frequency, at two populations in 2011 we made nine timed observations of bee visits to inflorescences when flower visitors were active. We watched 24-122 flowers at a time, recording every bee visit to flowers during a 60-90 minute period of time. In this work we recorded visits by *Bombus vagans* (Apidae), *Hylaeus annulatus* (Colletidae), *Lasioglossum* species (Halictidae), and several Lepidoptera and Diptera. We lump the *Lasioglossum* into a single taxon for analysis (here and below) because they could not be identified on the wing. To compare flower visit duration among visitor species, we watched individual bees as they foraged on *Chelone*, recording transitions between flowers and flower visit duration with a FileMaker Go database on an Ipad tablet computer (Apple, Inc; Filemaker Pro 12.0).

To assess whether bees preferentially visited male- or female-phase flowers, on three dates (August 5, 6, and 7, 2011) we examined each flower in a randomly selected group (N = 77, 80 and 122 flowers) and marked the sex phase on the corolla with black ink. The proportion of flowers in male phase was 0.13, 0.59 and 0.37, respectively. For one hour we observed bee visits to these patches of flowers, recording bee species and sex phase of each visited flower. We calculated proportion of male- and female-phase flowers visited by each bee and compared this to expected proportions if bees foraged randomly in the patch. We recorded visits by *B. vagans, H. annulatus*, and *Lasioglossum* species.

Nectar

We studied nectar volume and sugar concentration in two *Chelone* populations in 2011-2013. To assess nectar available to freely foraging floral visitors (nectar standing crop), we randomly selected inflorescences each from different plants and collected nectar from all open flowers (1-6 flowers on each of 105 plants, N = 201 total flowers) with capillary micropipettes (5 µL size; Drummond Scientific, Broomall, Pennsylvania, USA). Because nectar accumulates in a constricted area of the corolla base, we had to sample flowers destructively, but we were careful not to

introduce phloem sap into samples. We used a refractometer (National Industrial Supply, Temecula, CA, USA) to measure sucrose-equivalent sugars, expressed as % Brix, and converted volume and concentration to calories (net energy expressed as kilocalories) present in each flower (Bolten et al. 1979). We recorded time of day, individual plant identity and flower sex phase. We log-transformed nectar volume and energy to meet assumptions of parametric statistics, and used ANCOVA to analyse nectar volume, sugar concentration and energy, considering in full models as fixed effects time of day (simplified as two categories: 'morning', 0900-1100hrs, and 'afternoon', 1300-1600hrs), date of collection, and flower sex and as random effects plant individual, population and individual nested within population.

We also studied correlation of floral morphology with nectar traits for a portion of those flowers (1-5 flowers on each of 75 plants, $N = 159$ total flowers) in 2013. These measurements were taken in the morning and afternoon across the two *Chelone* populations. In combination with the nectar traits, we measured corolla length (from the base of the calyx to corolla opening), lower petal length (from the corolla opening to distal tip) and maximum corolla width (i.e., horizontal distance across the corolla opening) with digital callipers to the nearest 0.01 mm. We then used a multivariate analysis to test for correlations between floral morphology and nectar traits, splitting the dataset by morning and afternoon collections and excluding seven multivariate outliers identified by Mahalonobis distances. We combined the data across the two *Chelone* populations for correlation analysis; analyses within populations showed similar qualitative patterns (data not shown).

Pollen

We studied pollen production and removal patterns by different floral visitors in 2011-2012 at three populations. We placed wedding veil bags over expanded flower buds to exclude visitors. We returned to plants 24 hr later to remove bags from newly opened, unvisited flowers. We collected anther sacs from these flowers after application of three types of treatments: single visits from pollen- and nectar-foraging bees ($N = 138$ flowers); multiple visits from bees over time periods of varying lengths (1-8 hours; $N = 123$ flowers); and unvisited controls ($N = 135$ flowers). We did not record plant identity, but most samples came from different individuals. After single visits we made field identifications of bees to the lowest taxonomic level possible and recorded behavioural observations, including whether bees had audibly sonicated the flower. We also assessed pollen available to foraging bees by collecting open, unmanipulated flowers ($N = 60$ flowers; hereafter "open" flowers).

We excised anthers from flowers with forceps and allowed them to air dry for two weeks in open Eppendorf tubes. We added 1,500 µL of 70% EtOH to dry samples and sonicated them in a water bath for 60 minutes. We then homogenized samples by vortexing and removed 225 µL to a clean container, which we diluted to 1,500 µL with additional EtOH. After vortexing, we removed 3 µL aliquots to a haemocytometer slide and counted all pollen grains at 10× magnification under a dissecting microscope. We made four counts of each sample, computed an average and multiplied to obtain an estimate of total pollen grains present per flower.

We used linear models to compare log-transformed pollen counts from flowers at three sites between unvisited flowers, flowers visited once by foraging insects, and flowers open to insect visitation. We made pollen collections after single visits from 5 bee species; we present a statistical comparison of the two most common visitors (*H. annulatus* and *B. vagans*), and qualitatively summarize results from a smaller number of replicates from the other three species. We tested a full model that included treatment (unvisited, open and single visit flowers) as a fixed effect, and date and plant population as random effects. We used Tukey HSD post-hoc tests to compare means among treatments and among plant populations. To analyse depletion of pollen over time from male-phase flowers, we regressed anther pollen counts against time since anthesis, and compared linear and non-linear models to describe the data.

To investigate pollen transport by floral visitors, at three populations in 2011 we collected free-foraging bees into clean, cyanide kill jars as they left *Chelone* flowers, making note of whether they sonicated the last flower they visited. We immediately pulled bees from kill jars with forceps, removed and discarded hind legs and associated pollen loads (Michener 2000), and rubbed their dorsal sides on a microscope slide with approx. 1 cm^2 of fuchsin gel (Kearns & Inouye 1993) for 10 seconds. We added a cover slip and heated the slide until the gel melted, staining and fixing the pollen. We used a compound microscope at 40× magnification to make 5 pollen counts of randomly selected fields on each slide, distinguishing conspecific vs. heterospecific pollen using a pollen reference library. Pollen was dispersed approx. uniformly across the slides, so we then calculated mean number of *Chelone* and heterospecific pollen grains per microscope view as an index of the full sample present on the slide. We used ANOVA to compare pollen transport by bee species.

We also studied a component of female plant reproduction, pollen deposition to stigmas by different floral visitors, at two populations in 2011. We allowed bees to make single visits to virgin flowers (previously bagged in bud stage), noted whether bees sonicated the flowers, and then collected stigmas with forceps. We also collected stigmas from unvisited flowers as controls for pollen deposited by wind or experimental error. We mounted stigmas in fuchsin gel on microscope slides (Kearns & Inouye 1993), and counted *Chelone* and heterospecific pollen grains with a compound microscope. We present data here for unvisited controls ($N = 19$ flowers) and the most common visitor species, *B. vagans* ($N = 26$ flowers). We used ANOVA to assess whether *B. vagans* was an effective floral visitor, comparing pollen receipt by stigmas of flowers visited by *B. vagans* to pollen grains present on stigmas of unvisited control flowers.

Pollen limitation

We studied pollen limitation of plant reproduction at each of two and five populations in 2011 and 2012, respectively. Because *Chelone* may grow as a densely

aggregated clonal plant with many stems, we could not apply treatments at the whole-plant level (Ashman et al. 2004); we instead identified pairs of inflorescences ($N = 20$ pairs per population in each year) we could confirm were the same randomly selected genet, and applied one of two treatments (pollen addition or open control) to each. Every 2-3 days, we collected pollen from ≥ 5 donor plants (as in the *Mating system* methods), then applied it to stigmas of all open flowers of inflorescences in the pollen addition treatment, marking the sepals of each flower we treated with black ink. We paired this with an open control treatment in which we handled and marked open flowers, but did not add any pollen. By visiting inflorescences every 2-3 days, we treated on average 52% of the flowers on inflorescences. Both treatments were open to natural floral visitation. We later collected infructescences, dissecting those fruits we had treated and marked to count mature seeds and assess seed predator damage.

We used a linear mixed model to analyse seed number per fruit. The full model accounted for our paired sampling design by including as random effects individual plant genet (i.e., from which a pair of inflorescences was included in the experiment) and individual nested within plant population. Fixed effects in the model included pollen addition treatment (pollen addition vs. control), plant population and their interaction. We included population as a fixed effect in this analysis because we were interested in asking how these particular populations responded to pollen supplementation and whether the magnitude of pollen limitation varied among them. Due to high rates of predispersal seed predator attack (57-75%; see Results), we were unable to calculate per cent of fruits maturing seed. However, we used a generalized linear mixed model to study whether the frequency of predispersal seed predator attack was dependent on whether we added supplementary pollen to stigmas. We investigated a full model that included as response variable whether a fruit contained evidence of predispersal seed predator attack (presence/absence of frass, larvae or pupae), as a fixed effect pollen addition treatment, and as random effects plant individual and population. To compare pollen limitation of *Chelone* to that reported for other plants, we calculated the Hedges' *g* effect size of the difference between pollen addition and control groups (Gurevitch et al. 2001; Knight, Steets, et al. 2005). An effect size of 0.2 would be considered a small effect of pollen supplementation, 0.5 medium, and >0.8 large (Cohen 1988).

RESULTS

Phenology

The *Chelone* flowering period was 7 July-20 September and averaged approx. 66 days, with a peak of flowering around 5 August across two populations in 2011 and 2012. Individual flowers were open (mean + 1 SD) 3.00 ± 0.70 days, with flowers functionally male during the first day, and in female phase thereafter. We found that anthesis could take place at any time of day, but that most flowers opened at night when pollinators were not active.

Mating system

We found a significant effect of pollination treatment on seed set per fruit ($\chi^2 = 15.57$, $P = 0.0004$; Fig. 1). Post-hoc tests revealed that relative to controls, seed set was increased by 1.5 times by addition of self pollen ($Z = 4.03$, $P = 0.0002$) and 1.4 times by addition of outcross pollen ($Z = 2.90$, $P = 0.01$), but selfing and outcrossing treatments were not significantly different from each other ($Z = 1.27$, $P = 0.41$; Fig. 1).

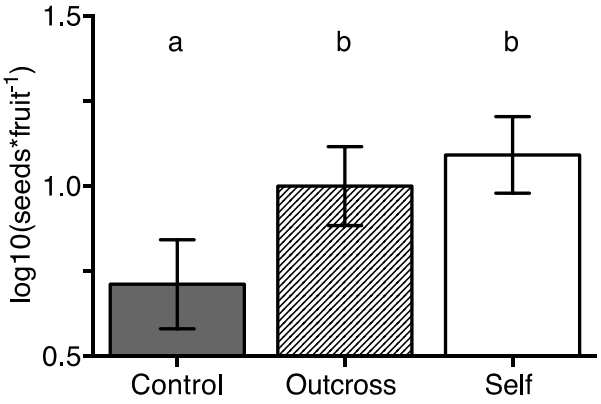

FIGURE 1. Log-transformed numbers of seeds produced per fruit in unpollinated control flowers, flowers that were outcrossed with pollen from other individuals, and flowers that received only self pollen. Data presented are means ± SE, and means that are significantly different are marked with different lower case letters.

Flower visitors

During 14 hours of daytime netting, we collected 18 species of solitary and social bees (Hymenoptera: Apoidea), and small numbers of flies (Diptera), sawflies (Hymenoptera) and wasps (Hymenoptera) foraging for nectar and/ or pollen at *Chelone* flowers (Tab. 1). We made a total of 3 hours of observations of flowers at night (2100-2400 hrs), but did not observe any nectar or pollen collecting visitors. Worker caste bumble bees were the most common visitors (75.4% of visits), and two species, *Bombus impatiens* and *B. vagans*, accounted for 71.6% of all collections. Bumble bees were observed to collect nectar, pollen or both resources from flowers, often audibly sonicating anthers while inside flowers. *B. vagans* workers quickly entered flowers after landing and crawled to the nectaries, making contact with both anthers and stigmas, but *B. impatiens* and other bumble bee species had difficulty forcing their way through the constricted floral entrance, typically inserting only their heads and forelegs, and making less contact with sexual parts of flowers. A variety of solitary bee species, most commonly *Hylaeus annulatus* females (6.8% of all visits), also collected nectar and pollen from flowers, but due to their smaller size, they frequently did not contact stigmas while in flowers. Sound recordings revealed that *H. annulatus* commonly sonicated anthers to release pollen. The three most common foragers varied significantly in length of their flower visits (*B. vagans* < *B. impatiens* < *H. annulatus*; Welch's test: $F_{2,59.8} = 3.68$, $P = 0.03$). *H. annulatus* flower visits were 1.7 times longer (mean = 7.1 seconds; Tukey test: $P = 0.05$) than those of *B. vagans*

Visitor	No. Collections	Fraction of collections present	Relative abundance
Apis mellifera	1	3.6	0.3
Augochlorella aurata	2	7.1	0.5
Bombus bimaculatus	1	3.6	0.3
B. borealis	2	7.1	0.5
B. fervidus	*		
B. griseocollis	*		
B. impatiens	9	28.6	2.4
B. ternarius	2	7.1	0.5
B. terricola	*		
B. vagans	265	100.0	71.6
Halictus rubicundus	1	3.6	0.3
Hylaeus annulatus	25	53.6	6.8
Lasioglossum (4 morphospecies)	41	46.4	11.1
Megachile gemula	2	7.1	0.5
M. inermis	1	3.6	0.3
Sawfly sp.	1	3.6	0.3
Wasp sp.	2	7.1	0.5
Diptera spp.	15	25.0	4.1

TABLE 1. A total of 370 insect specimens, including bees, other hymenoptera and flies, were collected during 28 30-minute observations at *Chelone glabra* flowers from 2011-2012 in seven sites. Asterisks indicate insect species collected outside of standardized collecting events.

(mean = 4.2 seconds), and other comparisons were not significantly different. We recorded pollen collection via sonication by both bumble bees and *H. annulatus*, and noted that for the latter, buzzing was often too quiet to hear without amplification. *B. vagans* workers demonstrated behavioural flexibility, often switching between sonication and passive pollen collection behaviours as they moved among plants. We also observed that *B. vagans* workers readily consumed nectar through holes chewed in corolla tissue by a florivore (*Tenthredo grandis*), and individuals switched between this behavior and 'legitimate' nectar foraging (*sensu* Inouye 1980).

When we investigated the frequency of flower visits to patches, one bee species, *B. vagans*, made 91.0% of visits during observations. The majority of other visits were made by *Lasioglossum* spp. (4.7%) and *H. annulatus* (3.1%). Each flower received 2.85 ± 0.51 SE bee visits per hour averaged across floral sex phases. However, individual bee foraging patterns with respect to flower sex phase did vary, and some bees visited one sex phase more often than expected by chance (range of proportional divergence from expected proportion of male flower visits of 0.5: -0.87 to 0.59). Overall, *H. annulatus* visited male-phase flowers significantly less often than expected ($t_{16} = -2.76$, $P = 0.01$), whereas neither *B. vagans* ($t_{50} = -0.55$, $P = 0.59$) nor *Lasioglossum* ($t_{11} = 0.49$, $P = 0.63$) preferentially visited flowers based on sex phase.

Nectar

Standing crop nectar volume, sugar concentration and energy content were each best described by models including as fixed effects time of day, flower sex phase and a time*sex interaction. Flowers open to insect visitation contained 1.69 ± 0.17 SE μL nectar. We found that nectar standing crop volume was 2.4 times higher in the morning than the afternoon ($F_{1,142} = 17.63$, $P < 0.0001$) and 1.7 times higher in female- than in male-phase flowers ($F_{1,142} = 6.71$, $P = 0.01$), and there was a significant interaction between time of day and flower sex phase ($F_{1,142} = 5.28$, $P = 0.02$; Fig. 2a). Concentration of *Chelone* nectar sucrose-equivalent sugars was 34.38 ± 1.17 SE % (range: 9.5-64.5%). Nectar sugars were 1.46 times more concentrated in flowers sampled in the afternoon ($F_{1,84} = 35.19$, $P < 0.0001$; Fig. 2b), but other effects were not statistically significant ($F_{1,84} < 2.87$, $P > 0.09$). Standing crop nectar contained 3.76 × 10-3 ± 3.48 × 10-4 kcal energy. Caloric reward of female-phase flowers was 168% higher than that of male-phase flowers ($F_{1,82} = 7.97$, $P = 0.006$; Fig. 2c), but other effects were not statistically significant ($F_{1,29} < 2.39$, $P > 0.13$).

We found that nectar standing crop was positively correlated with some measures of flower length (in the morning, petal length, and in afternoon, both corolla and petal length; Tab. 2). Nectar sugar concentration was not associated with other floral traits in the morning, but was negatively correlated with afternoon volume and petal length. Caloric reward of nectar was positively correlated with volume in both morning and afternoon samples. Caloric reward in morning samples was also positively correlated with corolla width and petal length; in afternoon samples nectar energy content was correlated positively with corolla length and negatively with sugar concentration. There was no correlation between corolla length and width either in morning or afternoon flowers. However, there was positive allometry between corolla and petal lengths in morning but not in afternoon (Tab. 2).

Pollen

Across populations, anthers of unvisited flowers contained 1.30 ± 0.08 × 105 pollen grains. The best-fit model of pollen present in flowers included treatment

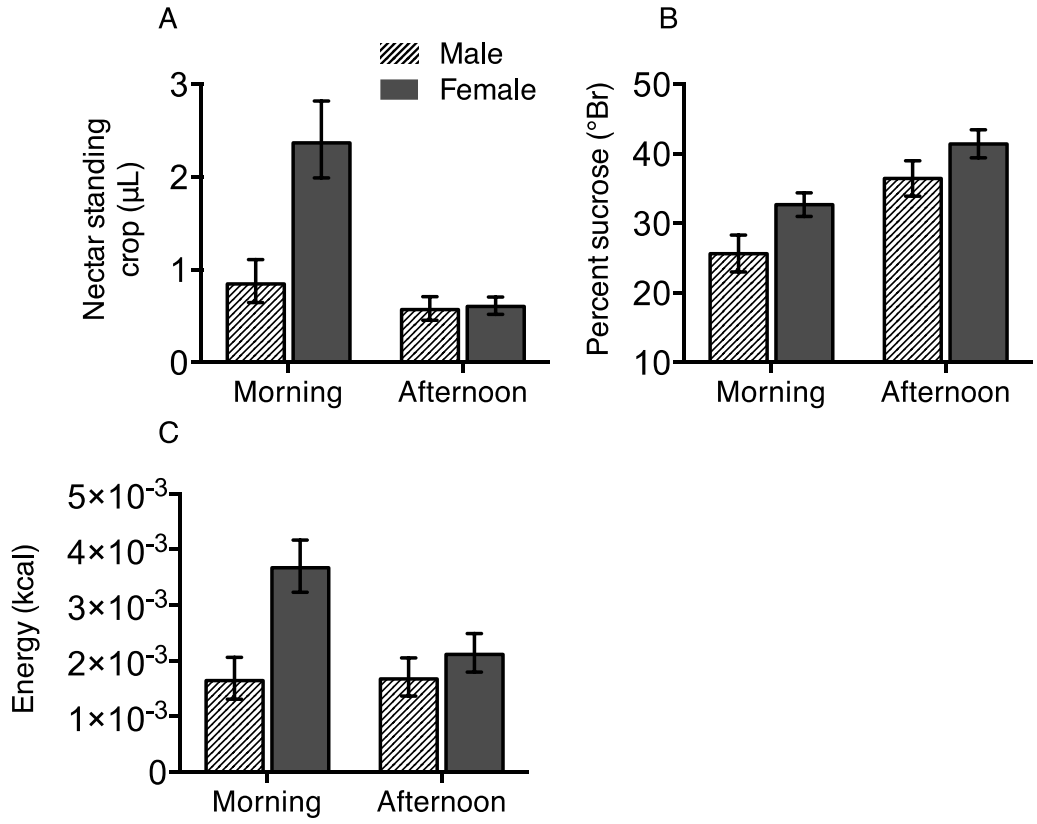

FIGURE 2. Least square means ± SE of A) nectar standing crop (µL), B) sugar concentration (Brix) and C) energy content (kilocalories) of male- and female-phase flowers sampled in the morning (at daily outset of flower visitation) and afternoon (≥ 5 hrs after floral visitation had begun).

TABLE 2. Correlations among flower morphology and nectar traits. Values are Pearson product-moment correlation coefficients. Bold values are statistically significant at $P \leq 0.05$ (*), $P \leq 0.001$ (**) and $P \leq 0.0001$ (***).

		Energy	Nectar volume	Nectar sugar	Corolla length	Corolla width
Morning	Energy					
	Nectar volume	**0.963*** **				
	Nectar sugar	0.171	-0.100			
	Corolla length	0.289	0.279	0.038		
	Corolla width	**0.308***	**0.291***	-0.282	0.015	
	Petal length	**0.357***	**0.357***	-0.263	**0.341***	**0.571*** **
Afternoon	Energy					
	Nectar volume	**0.938*** **				
	Nectar sugar	**-0.419****	**-0.704*** **			
	Corolla length	**0.367****	**0.344****	0.165		
	Corolla width	-0.155	0.060	-0.072	-0.070	
	Petal length	0.190	**0.309****	**-0.302***	-0.129	**0.558*** **

(unvisited vs. open vs. single visit flowers) as a fixed effect, and plant population as a random effect. Plant population explained 15.0% of the variance in the overall model, and a Tukey test revealed significant differences in pollen production among populations (Fig. 3a). There were significant differences in pollen grain number among unvisited and open flowers and those that had received single visits from two bee species, *H. annulatus* and *B. vagans* ($F_{3,308.2} = 21.77$, $P < 0.0001$, Tab. 3; Fig. 3b). Comparing pollen remaining in anthers after bee visits to pollen in anthers of unvisited controls, *H. annulatus* ($N = 5$) removed 0.9% of pollen and *B. vagans* ($N = 123$) removed 36.6% of pollen in single visits. Despite this large mean difference in pollen removal, the difference between the two species was not statistically significant in a Tukey HSD post hoc test ($P = 0.62$), possibly due to small sample size for *H. annulatus*. Tukey tests further showed that mean pollen counts of flowers visited by *H. annulatus* were distinguishable from

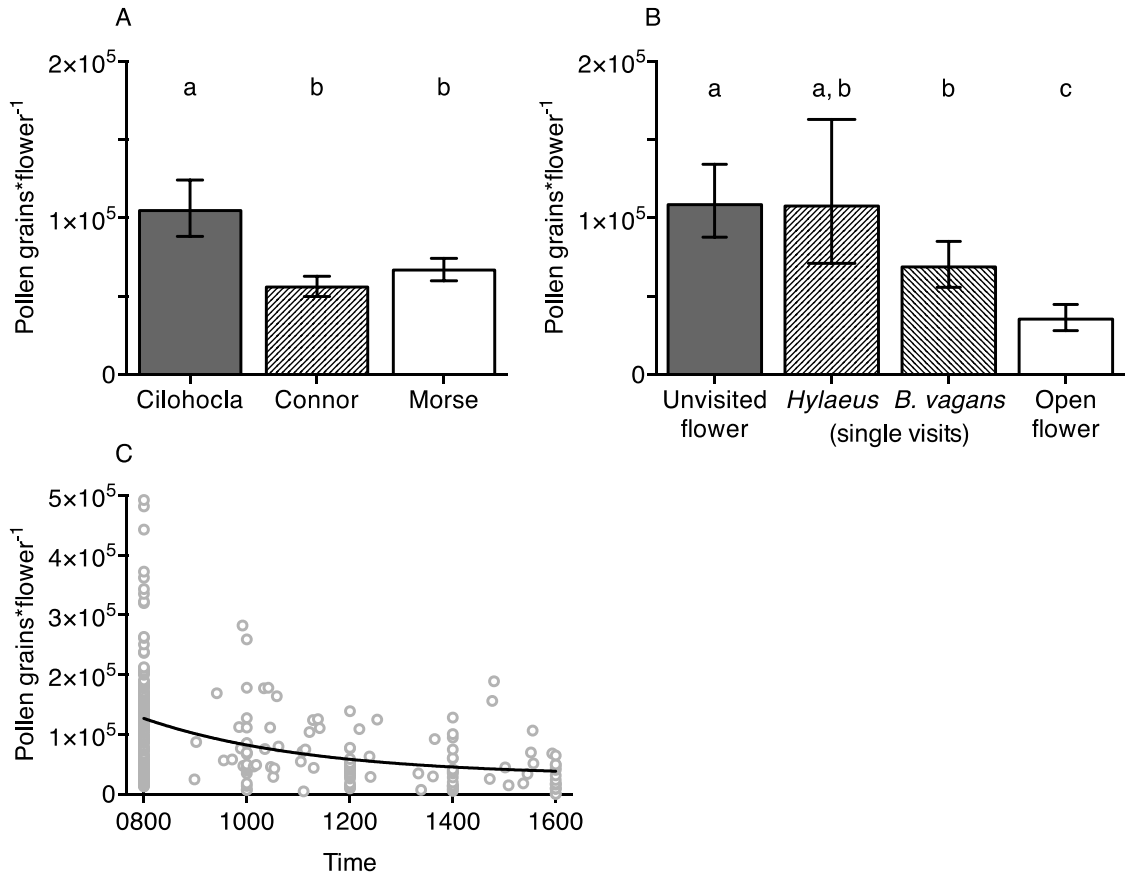

FIGURE 3. A) Back-transformed least square means ± SE pollen grains present in anthers of unvisited, male-phase flowers from three *Chelone glabra* populations; B) Back-transformed least square means ± SE pollen grains present in anthers of unvisited flowers, those that received single visits from *Bombus vagans* or *Hylaeus annulatus*, and unmanipulated flowers open to multiple visits by pollen and nectar foragers; and C) raw numbers of pollen grains remaining in flowers exposed to insect visitation for varying lengths of time. For A) and B), means with different lower case letters are statistically different based on a Tukey HSD post-hoc test ($P < 0.05$).

TABLE 3. Pollen remaining in *Chelone glabra* anthers (mean ± standard error) following single visits by bees compared with pollen in virgin flowers ('unvisited control') and those open to natural visitation by multiple visitors ('open control'). Due to small sample size, visits by *Bombus bimaculatus, B. borealis* and *Lasioglossum* sp. could not be statistically compared to those by *B. vagans* and *Hylaeus annulatus*.

Visitor	Pollen grains per flower		N
	Mean	SE	
Bombus bimaculatus	125,648	45,202	3
B. borealis	61,296	2,573	3
B. vagans	83,943	5,670	125
Hylaeus annulatus	137,333	65,575	5
Lasioglossum sp.	23,929	4,405	2
Open control	40,847	4,515	60
Unvisited control	129,543	8,138	135

those of flowers open to bee visitation ($P = 0.02$) but not unvisited flowers ($P = 1.00$). Pollen counts for *B. vagans* single visits were different from those for open and unvisited flowers (both comparisons: $P < 0.0001$; Fig. 3b).

The amount of pollen remaining in open, male-phase flowers was best modelled by a two-phase exponential decay function, and declined sharply in the first two hours after anthesis ($R^2 = 0.186$; Fig. 3c). Making the assumption that flowers open at night, the model demonstrates that individual flowers contribute little to plant male fitness after the first day they are open, when 85-90% of pollen is predicted to have been removed.

We found that *B. vagans* carried 7.6 and 26.3 times more *Chelone* pollen grains on their thoracic dorsum than *B. impatiens* or *H. annulatus*, respectively (Welch's test: $F_{2,5.18} = 44.55$, $P = 0.0005$). *Chelone* pollen as a fraction of total pollen carried was significantly greater for *B. vagans* (82.9%) than *B. impatiens* (28.9%) or *H. annulatus* (17.3%; $F_{2,18} = 7.68$, $P < 0.004$). There was no difference between *B. vagans* individuals that sonicated flowers and those that did not in numbers or per cent of *Chelone* pollen grains carried ($F < 0.50$, $P > 0.49$).

The best-fit model of pollen deposition to stigmas included treatment (single bee visit vs. unvisited control) as a fixed effect and plant population as a random effect. Stigmas of flowers visited by *B. vagans* had significantly more *Chelone* pollen deposited on them than those that had not received any visits ($F_{1,42.5} = 17.91$, $P < 0.0001$; Fig. 4).

Heterospecific pollen accounted for 2.0% of pollen found on unvisited flower stigmas, and 2.5% of all pollen deposited by *B. vagans* workers.

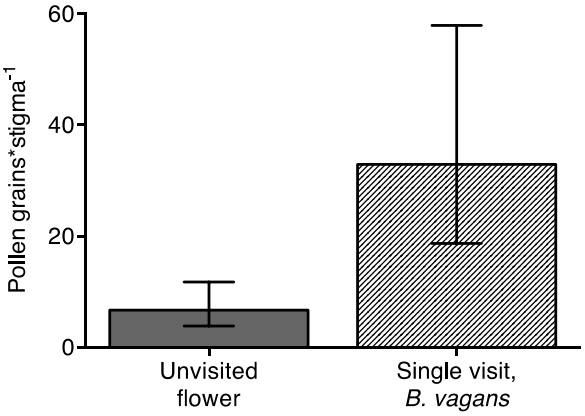

FIGURE 4. Least square means ± SE pollen grains present on stigmas of unvisited flowers and those that had received single visits from *Bombus vagans*.

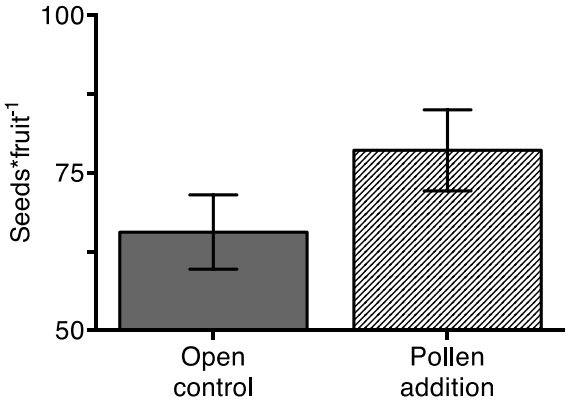

FIGURE 5. Pollen addition to flowers increased seed set per fruit compared to open-pollinated control flowers. Bars are mean ± SE seeds per fruit.

Pollen limitation

In 2011, we lost >75% of all fruits to predispersal seed predators and herbivores; we therefore analyse only 2012 data here. The best-fit model for seed number in this experiment included as fixed effects pollen limitation treatment, plant population and their interaction, and as a random effect, individual plant nested within population. We found that overall, plants were pollen limited for seed set per fruit ($X_5^2 = 16.58$, $P = 0.01$; Fig. 5), with flowers in the pollen supplementation treatment producing 1.20 times more seeds than those in the open pollination treatment. Seed set per fruit varied among populations ($X_8^2 = 16.73$, $P = 0.03$), but the interaction between pollen supplementation and plant population was not statistically significant ($X_4^2 = 9.07$, $P = 0.059$). A Tukey HSD test showed significant pollen limitation in one population (Morse; t = 3.38, $P = 0.03$) but not the other four. The effect size, *d*, of the difference between pollination treatments across the five populations was 0.04 ± 0.09 SE (range: -0.10 ± 0.63 SE in the Valerie population to 0.26 ± 0.31 SE in the Morse population). Of the fruits collected during the 2012 pollen limitation experiment, 59.7% were damaged by pre-dispersal seed predators, but there was no effect of pollen treatment on proportion of fruits attacked ($\chi^2 = 2.34$, $P = 0.13$).

DISCUSSION

Our results support previous qualitative reports that *Chelone* requires insect visitation to set seed and that it is self-compatible (Cooperrider 1967). We recorded visits from approx. 20 insect species to *Chelone* flowers, and show that the most common visitors vary in their ability to vector pollen between flowers. We conclude that *B. vagans* workers, the most common flower visitors, pollinate *Chelone* because they: 1) contacted both anthers and stigmas when they foraged; 2) visited both male- and female-phase flowers; 3) removed by sonication more than a third of all pollen from newly opened flowers; 4) passively carried greater numbers of *Chelone* and fewer numbers of heterospecific pollen grains than other bees when traveling between *Chelone* flowers, and 5) deposited pollen on stigmas while foraging. This result adds to a large body of research showing that some bumble bees can be highly effective pollinators while foraging (Winfree et al. 2007), especially when collecting pollen (Free 1993). By contrast, two other common flower visitors, *B. impatiens* and *H. annulatus*, were less effective pollinators of *Chelone*. Our observations suggest that relatively little pollen is transferred during visits by *B. impatiens* (in which bees do not fully enter flowers) and small, relatively hairless *H. annulatus* females, who often cling to anthers without contacting stigmas. Previous reports show that bumble bee species commonly differ in their value to plants as pollinators (Asada & Ono 1996; Thøstesen & Olesen 1996; King et al. 2013; Strange 2015), as demonstrated here for *B. impatiens* and *B. vagans*. Bumble bees often deposit more pollen in single visits than other flower visitors (e.g., Thomson et al. 2000; Javorek et al. 2002), but there are notable exceptions (e.g., King et al. 2013; Benjamin & Winfree 2014).

We show that *Chelone* is protandrous and that pollinator rewards vary according to sex phase and time since anthesis. When flowers open in male phase, bees may forage for pollen, but little nectar is available. The large initial removal of pollen by *B. vagans* workers we observed (mean: > 36%) falls within the range reported for bumble bees in other pollination systems (Wilson 1995; Thomson & Goodell 2001; Castellanos et al. 2003). In a survey of studies that reported single visit pollen removal measures, bumble bees removed 35.7 ± 19.0 SD % of pollen in virgin flowers (range: 6.7-80.0%; $N = 25$ bee-plant combinations reported in 10 studies; L. L. Richardson and R. E. Irwin, unpublished). This sample includes a range of bumble bee and plant species and collection by both sonication and other methods. Despite this large removal and initial exponential decrease in pollen availability, after an estimated > 20 visits (i.e., at the end of a full day in male phase), anthers still retain approx. 10% of their pollen. Similar exponential decay of pollen availability has been reported in other bumble bee-pollinated plants (Wilson & Thomson 1991). We speculate that the densely hairy anther sacs of *Chelone* flowers slow pollen removal by bees, allowing plants to export male

gametes across a larger number of interactions with pollinators (Harder & Thomson 1989). Our data are consistent with pollen presentation theory, which holds that bee-pollinated plants with high rates of pollen forager visitation should mete out pollen in small doses rather than presenting all pollen at once, as many bird-pollinated plants do (Thomson et al. 2000). Additionally, the occluded anther sacs of *Chelone* may prevent pollen over-exploitation by sonicating bumble bees, who otherwise might remove the majority of pollen in one visit; by corollary, we expect that such an effect increases plant male function by allowing flowers to export pollen following larger numbers of bee visits. Buzz pollinated plants typically feature poricidal anthers (Buchmann 1983) rather than the longitudinally dehiscent type found in *Chelone* (Straw 1966), and buzz pollination is not commonly reported for the Plantaginaceae. Densely woolly anther sacs are also found in one subgenus of *Penstemon* (Dasanthera), to which *Chelone* is closely related. While two *Penstemon* species have been reported to be sonicated by pollinating bees (Cane 2014), they are not in the Dasanthera subgenus. Given our report of *Chelone*'s pollination system, investigating buzz pollination in basal lineages of *Penstemon* which share these anther traits would provide additional evolutionary insight. Moreover, *Chelone* and its floral visitor community may provide a good system in which to test predictions of pollen presentation theory (Thomson et al. 2000).

We report the paradoxical observation that while pollen reward is greatest in newly opened, male-phase flowers, *H. annulatus* females that sonicate anthers to collect pollen preferentially visited older female flowers. Previous research has shown that in plants with temporally separate sex phases such as *Chelone*, pollen harvesters frequently avoid female flowers and so do not pollinate. For example, the dichogamous herb *Impatiens capensis* Meerb. is pollinated by nectar foraging bumble bees that visit flowers in both sex phases, yet pollen foraging honey bees and solitary bees avoid female flowers (Wilson & Thomson 1991). One hypothesis for why *H. annulatus* avoids male flowers, where the pollen reward is greatest, is that this small solitary species may not be large enough to loosen pollen from male phase flowers, and consequently visits older (female) flowers for pollen left after larger bees have repeatedly sonicated anthers. Additionally, these bees could be attracted to the higher nectar rewards we identified in female-phase flowers. Because individuals can restrict their visits to flowers of one sex phase, *H. annulatus* could potentially forage as a pollen or nectar thief (Baker et al. 1971; Inouye 1980), but the extent to which this takes place was beyond the scope of this study. Further research should investigate the cues by which this bee avoids male flowers, and should investigate the effect of *H. annulatus* on *Chelone* pollination success. However, because bee preferences may be affected by ratios of male- and female-phase flowers (Aizen 2001), such work should test *H. annulatus* foraging behavior over a range of flower sex phase ratios.

Similar to other studies of dichogamous plants we found that nectar reward, a function of both secretion and removal, varied according to flower sex phase, and was most abundant in female-phase flowers (Varga et al. 2013). There was strong diurnal variation in nectar volume, concentration and energy reward, and male- and female-phase flowers differed in these traits. Nectar volume declined sharply over the course of the day as foragers removed it, but nectar sugar concentration also dramatically increased, resulting in net energy rewards not predicted by volume alone. There are at least two mechanisms that might account for diurnal changes in *Chelone* sugar concentration. It is possible that flowers manipulate nectar sugar by selective resorption of the liquid in nectar or changes in secretion dynamics (Castellanos et al. 2002; Nepi & Stpiczyńska 2008). Alternately, nectar could passively become more concentrated by evaporation (Corbet et al. 1979). We find limited evidence suggesting evaporation affects nectar volume and concentration in *Chelone*: nectar volume was higher in flowers with greater distance between nectaries and flower opening, regardless of time of day, and nectar sugar concentration was negatively correlated with lip length, but only in afternoon samples, which had been exposed to approx. 5 hours of drying. However, while these associations suggest an influence of floral morphology on evaporation rates, morphology could also influence nectar volume by limiting foragers' access to nectar (Heinrich 2004) or filtering the visitor community, which might alter the nectar microbial community with consequences for the concentration of nectar sugars (Vannette et al. 2013). The interaction between floral morphology and reward quality warrants greater study in this system.

We found that 75% and 57% of fruits were attacked by predispersal seed predators in 2011 and 2012, respectively, 2-3 times that reported for *Chelone* in a previous study (Stamp 1987). Many fruits with evidence of seed predator oviposition (i.e., oviposition scars or larval feeding inside fruits) failed to mature any seeds or associated placenta tissue, suggesting that *Chelone* may abort pollinated fruits after attack. *Chelone* interactions with pollinators must be considered in light of this damage, as well as that caused by other herbivores, including Baltimore checkerspot butterfly larvae (*Euphydryas phaeton*), white-tailed deer (*Odocoileus virginianus* Zimmerman) and white-footed mice (*Peromyscus leucopus* Rafinesque). Like other plant parts, *Chelone* fruits contain the iridoid glycosides aucubin and catalpol (Richardson, Adler, et al. 2015), chemicals known to deter generalist herbivores from consuming the plant's leaves (Bowers et al. 1993). Interestingly, however, many of *Chelone*'s herbivores, including its seed predators, are specialist feeders on plants containing these compounds. Iridoid glycosides have defensive and attractive functions in *Chelone* leaves (Bowers et al. 1993) and floral nectar (Richardson, Bowers, et al. 2015), respectively, but additional work is needed to clarify their role in developing fruits.

In a test of pollen limitation across five plant populations, we found that overall, *Chelone* was pollen limited, but this effect varied among populations and was only statistically significant in one of them. This result is broadly consistent with other research on angiosperm pollen limitation. For example, pollen limitation is commonly reported for plants with spatial or temporal separation of sexes (Ramsey & Vaughton 2000), small, fragmented

populations with restricted gene flow (Knight, Steets, et al. 2005), and situations in which pollinators are harassed by antagonists (Knight, McCoy, et al. 2005), such as the parasitoid flies that hunt bumble bees at *Chelone* (Richardson, Bowers, et al. 2015). While the difference in seed set between means for pollen addition and control treatments was statistically significant, the effect size was small even in the most pollen limited population (Morse) and when compared to those reported in other work (Knight et al. 2006). It was beyond the scope of this study to determine why *Chelone*'s degree of pollen limitation was relatively low. One possibility is that our methods, in which we treated a subset of flowers on single inflorescences rather than all flowers on an individual plant, affected our estimate of pollen limitation. Some plant species can reallocate resources away from flowers that receive insufficient pollen, which may exaggerate measurements of pollen limitation of seed set (Knight et al. 2006). However, we expect this would have led to relatively high, not low estimates of pollen limitation. Additional pollen limitation experiments where all flowers on a plant are treated will be necessary to clarify the extent to which *Chelone* is pollen limited at the whole-plant level.

In conclusion, we document that *Chelone glabra* is self-compatible but requires insect visitation to set seed. We observed visits by a suite of nectar and pollen foraging insects that vary in their effectiveness as pollinators. We report evidence that the phenology of *Chelone* sex phase transition and reward presentation influence pollen transfer between male and female flowers; yet, one common floral visitor takes advantage of protandry to specialize on female-phase flowers. We show that despite high rates of floral visitation, *Chelone* is pollen limited for seed production. However, the outcomes of plant-insect interactions at flowers must be evaluated in light of the high rates of predispersal seed predation we observed. Our work demonstrates how interactions with mutualist and antagonist flower visitors combine to influence plant reproduction, and we project that outcomes of these processes have consequences for population dynamics of this wetland-dominant herb.

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APPENDICES

Additional supporting information may be found in the online version of this article:

APPENDIX I. Field sites where *Chelone glabra* research was conducted, 2011-2013.

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