New evidence that blueberry (*Vaccinium* spp.) has floral traits that enable pollination at night

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Abstract

The role of nocturnal pollinators has been globally understudied, particularly in agriculture, but growing evidence suggests that crops often assumed to be pollinated during the day also receive additional pollination by night-active animals. In our study, we assessed six different types of evidence to determine whether blueberry flowers, which are typically associated with diurnal pollinators, could also be pollinated at night. We used observational and experimental approaches commonly used and validated for assessing nocturnal pollination, including variation in stigma receptivity and pollen viability at day and night, nectar standing crop dynamics, temporal effects of pollen germination, pollinator exclusion experiments and the presence of pollen on the bodies of potential nocturnal pollinators (moths). Stigma receptivity and pollen viability were similar between our day and night sample points. We found no negative effects of time of pollination on pollen germination. Nectar availability was strongly associated with time of day, and although nectar was available both day and night, nectar volume was highest at night. Fruit set was highest in flowers with unrestricted access to pollinators, but we are unable to determine the relative importance of nocturnal versus diurnal pollination as these treatments gave the same result as our control. Moths caught in blueberry orchards carried pollen from 25 plant species, with blueberry pollen being the fifth most abundant. Our results show that blueberry flowers have floral traits that allow pollination at night as well as during the day, and that moths, along with other nocturnal insects, may be contributing to this. However, the importance of nocturnal pollination relative to diurnal pollination remains unknown.

Keywords Flowers · Moth · Nocturnal pollinators · Non-bee pollinators · Plant–pollinator interactions

Introduction

Animals, both vertebrate and invertebrate, are responsible for the pollination of between 78 and 94% of all wild flowering plants and, depending on interpretation, roughly 75% of the leading global food crop species or one third of all food crops (Ollerton et al. 2011; Willcox et al. 2017). The European honey bee (*Apis mellifera*) is considered the most important crop pollinator globally, and honey bee colonies are often brought into orchards to maximise pollination (Eeraerts et al. 2023). However, research has shown that diversifying pollination to include other bee and non-bee species enhances fruit and seed set in crops more than increasing visitation by honey bees alone (Garibaldi et al. 2013; Rader et al. 2016). A considerable amount of research has thus been conducted on non-honey bee species as crop pollinators, e.g. wild bees native to a given region (Brittain et al. 2013; Garibaldi et al. 2013; Esquivel et al. 2021). However, extensive knowledge gaps remain given the wide diversity of possible plants and pollinators involved (Garibaldi et al. 2013; Rader et al. 2016, 2020).

Nocturnal pollination has been reported in 30% of angiosperm families, but remains critically understudied, particularly in cropping systems (Macgregor et al. 2014; Borges et al. 2016; Buxton et al. 2018). Moths (Lepidoptera) are the

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most speciose order of nocturnal flower visiting insects and are considered pollinators for at least 289 plant species, but their relative importance to specific plants compared with other pollinators and also larger scale contributions to ecosystem function and food production is unclear (Macgregor et al. 2014; Rader et al. 2020). Moths (and other nocturnal pollinators) have been shown to pollinate or visit flowers of many crop plants (Buxton et al. 2022), including apples (*Malus domesticus*; Robertson et al. 2021), avocado (*Persea americana*; Buxton et al. 2021) and multiple gourd species (Cucurbitaceae; Lu et al. 2021). However, the limited number of studies assessing nocturnal pollination in crops hinders the development of strategies for growers to manage all pollinators to optimise pollination (Rader et al. 2020).

Blueberry (*Vaccinium* spp., Ericaceae) is highly reliant on pollinators for fruit set and fruit quality. Blueberries are typically considered to be pollinated by bees, with managed honey bee and bumble bee (*Bombus* spp.) colonies often brought into orchards in an attempt to boost pollination (Eeraerts et al. 2023). Bumble bees are particularly effective additions to blueberry orchards given their ability to buzz-pollinate, for which the flowers are well adapted (Javorek et al. 2002). Multiple wild unmanaged bee species are also effective blueberry pollinators, with single visits to virgin flowers resulting in > 40% fruit set, which improves with increasing visitation (Sampson and Cane 2000; Javorek et al. 2002). However, blueberries can also be pollinated at night, with moths, beetles (Coleoptera) and flies (Diptera) being reported as the most likely contenders (Cutler et al. 2012; Manning and Cutler 2013; Noone et al. 2022). The floral traits of blueberry flowers—a white corolla (in the human vision spectrum) that is elongated with a restricted aperture—have traditionally been associated with pollination by moths (Newstrom and Robertson 2005). Floral trait matching with the traits of pollinators (i.e. floral syndromes) is often used to suggest potential pollinators, but the accuracy of floral syndromes remains heavily debated within the literature (e.g. Ollerton et al. 2007, 2009).

In this study, we evaluate evidence for the involvement of nocturnal insects, particularly moths, in the pollination of blueberries planted as crops outside the plant's native range. Many different methods can be employed to assess pollinator effectiveness, each with unique trade-offs depending on the context and each with varying degrees of confidence ('strength' in Buxton et al. 2018, 2022). For example, pollinator visitation rates do not provide any information on pollen transfer and are extremely difficult to measure at night, whereas measuring pollen deposition from single visits is more conclusive but also time consuming, and pollinators may alter their behaviour in the presence of researchers (Willcox et al. 2017).This study used six different variables to consider the potential role of nocturnal pollinators in blueberry pollination: timing of stigma receptivity and

pollen viability, variation in nectar availability, effect of pollination timing on pollen germination, pollinator exclusion experiments and the presence of pollen on moths caught in blueberry orchards. While some information already exists on blueberry floral traits such as stigma receptivity and pollen viability (e.g. Brevis et al. 2006; Lang and Parrie 1992), these studies did not address any differences in these traits between day and night leaving the potential roles of diurnal vs. nocturnal pollination open to consideration. While assessing variation in floral traits and pollination success over the day and night may provide circumstantial evidence on their own, when taken as a whole, they can provide a stronger understanding of how blueberries might benefit from both diurnal and/or nocturnal pollinators. Here, we investigate the following questions: (1) Do stigma receptivity and pollen viability differ for blueberry between morning and evening timepoints? (2) How does nectar availability vary from early morning through to late evening? (3) How does the timing of pollination affect pollen tube growth rates? (4) How does fruit set and fruit weight differ among flowers that were pollinated only at night and only during the day? (5) What pollen grains are carried by moths that are caught in blueberry orchards?

Methods

Four blueberry cultivars, including both rabbiteye (*Vaccinium virgatum*; 'Maru', 'Centurion' and 'Centra Blue') and northern highbush (*Vaccinium corymbosum*; 'Reka') cultivars were assessed in September and October 2021 at the Ruakura Research Station in Hamilton, Waikato, Aotearoa New Zealand, for floral traits consistent with nocturnal pollinators contributing to pollination. The floral features assessed were day vs night differences in nectar availability (also known as nectar standing crop), pollen viability and stigma receptivity. These assessments were complemented with a pollinator exclusion experiment (conducted on a separate commercial orchard) and hand-cross pollination tests to determine if the timing of pollination could affect fertilisation success. Moths were also caught over the blueberry flowering period in 2019 and 2020 in four orchards across the Waikato region, New Zealand, to quantify pollen grains on moth bodies.

Stigma receptivity

To assess stigma receptivity, 4 open flowers from 5 bushes for each of the 4 cultivars (20 flowers per cultivar per time point, 160 flowers total) were collected at 10 a.m. and 8 p.m. (mid-morning and sunset) on 5 October 2021. Collected flowers were young (without visible signs of ageing) and were randomly selected, with each flower being

considered an individual replicate. Stigma receptivity was assessed using cotton swabs dipped in a solution of Peroxtesmo KO (one square of Peroxtesmo KO soaked in 1 mL of deionised water for 24 h). This Peroxtesmo KO solution tests for enzyme activity on the stigmatic surface and a positive reaction (the solution turning blue) is an indicator of stigma receptivity (Dafni and Maués 1998). Each of the stigmas were individually rubbed against the Peroxtesmo KO soaked cotton. If a green/blue reaction occurred on the cotton swab within 30 s, this was taken as indication of the stigma being receptive, and the absence of a reaction indicated a lack of receptivity. The receptivity assessments were conducted within 1 h of flower collection.

Pollen viability

To assess pollen viability, 4 open flowers from 5 bushes of each of the 4 cultivars (20 flowers per cultivar per time point, 160 flowers total) were collected at 10 a.m. and 8 p.m. (mid-morning and sunset) on 5 October 2021. Collected flowers were young (without visible signs of ageing) and were randomly selected, with each flower being considered an individual replicate. While collected at the same time, different flowers were used for the pollen viability and stigma receptivity assays. Pollen was applied to pollen germination plates (recipe described in Lang and Parrie 1992) by holding the flower over a germination plate and using sharp forceps to scrape the sides of the flower, creating vibrations which caused copious amounts of pollen to fall onto the plate. Pollen was left to germinate at 21 °C for 11–12 h before being placed in a − 20 °C freezer to halt germination. One week later, the plates were moved to 4 °C in a domestic refrigerator to thaw and were stored there before assessment. To determine the percentage of viable pollen tetrads, up to 100 tetrads were assessed under a Nikon SMZ18 dissecting microscope by visually scanning the plate in an up and down motion, going from left to right and moving the field of view as necessary until 100 tetrads were counted. Viability was expressed as the percentage of pollen tetrads with at least one pollen tube that had grown longer than it was wide (Lang and Parrie 1992).

Nectar standing crop

Nectar standing crop (the amount of nectar available at a given time) was sampled from 40 flowers for each cultivar between 5 and 6 a.m. (sunrise), 10 a.m., 2 p.m., 6–7 p.m. (sunset) and 10 p.m. Sampling times were selected to represent sunrise, mid-morning, mid-afternoon, sunset and night, with the sunset and sunrise times changing due to the start of daylight savings time (NZDT). Eight flowers were randomly selected over 5 bushes per cultivar per time point. Nectar samples were obtained from each cultivar for each

time point on two separate occasions, giving a total of 80 samples per cultivar per time point, 400 samples for each cultivar and 1600 nectar samples in total. Sampling took place on fine days and clear days and nights with minimal wind, with 'Centurion' and 'Maru' sampled on 20 and 21 of September 2021 and 'Centra Blue' and 'Reka' sampled on 27 September and 1 October 2021. Nectar samples were collected from young flowers (without visible signs of ageing) which were randomly selected, with each flower being considered as an individual replicate. Nectar was collected using a 2- to 20-µL Eppendorf Research Plus pipette set to a volume of 15 µL. The pipette tip was inserted into the flower as far as the base of the corolla and extracted nectar was dispensed into a pre-weighed and pre-labelled 1.5-mL Eppendorf tube containing filter paper. This probing process was repeated until no more nectar was removed, which was typically achieved after two to three extractions. Samples were stored at 4 °C in a domestic refrigerator if they could not be weighed immediately. Each tube was then re-weighed at room temperature to quantify nectar extracted. The lids of the Eppendorf tubes were then opened, and the samples were dried at 22 °C for 1 h before being weighed again to measure the remaining sugars to determine if any differences in nectar weights were due to water content or sugar content. All nectar samples were weighed on an Ohaus Adventurer AX423 scale, which comes with an inherent scale error of 0.001 g. Of the nectar samples, 11.12% returned a negative value and 20.25% of the sugar samples returned a negative value; these were adjusted to zero under the assumption that the negative values appeared because of the inherent scale weighing error.

We also made casual observations of flower visitation while collecting nectar samples, to determine if nocturnally active insects were foraging on blueberry flowers. This was not done following a formal sampling regime but was instead performed opportunistically while we were in blueberry orchards at night. As these opportunistic observations were not formally a part of our sampling regime, no statistical analyses are available, however, our observations are included in the results and in the discussion.

Pollen germination for day versus night

Flower buds were bagged on two cultivars, 'Maru' (three bags per bush, four bushes) and 'Centra Blue' (three bags per bush, three bushes), and left for 1 week to allow flowers to open. Pollen was collected at 7 a.m. and 7 p.m. on 5 October 2021 from the four cultivars used in this study by holding the flower over a Petri dish and using sharp forceps to scrape the sides of the flowers, creating vibrations which caused a large amount of pollen to fall from the flowers and onto the dish. The pollen from the four cultivar was mixed into one composite sample to avoid potential problems with

pollen incompatibility and kept in an incubator set at 22 °C for 12 h. Pollen was applied to virgin stigmas of our two target cultivars at 7 p.m. on 5 October and 7 a.m. on 6 October using a fine tip paintbrush. Pollen applied at 7 p.m. was exposed to an immediate period of cooling over the night, and pollen applied at 7 a.m. was exposed to an immediate period of warming during the day. Bags were reapplied after hand pollinations to prevent additional pollen deposition by insects. Stigmas were excised 12, 24 or 36 h post pollen application and stored in 70% ethanol at −20 °C. Due to insufficient bagged flowers, only two of the three 36-h replicates were available for the cultivar 'Maru'.

Each excised stigma was placed in sodium hydroxide (4 g NaOH in 100 ml deionised water) for 1 h to soften the stigma and then rinsed twice in deionised water. The stigma was dried on a paper towel and placed on a microscope slide, and a drop of 1% aniline blue stain was added to stain pollen tubes (Martin 1959). A cover slip was applied, and the stigma gently flattened with forceps. Slides were examined under fluorescence using a Nikon DSIR microscope with fluorescence filter cube 200–400 mm. The number of pollen tetrads fluorescing on the stigma and the number of pollen tubes growing to 50 μm down the style was counted.

Pollinator exclusion experiments

Pollinator exclusion experiments were conducted over 3 days and 3 nights on a commercially operated blueberry orchard on 6–9 October 2021 (within the second half of the flowering period) in Hamilton, New Zealand, on one cultivar, 'Centra Blue'. Wax-coated paper bags were placed over terminal branches, where no flowers had yet opened, on 10 bushes with the following treatments and replicates:

- Flowers exposed during the night only (night pollination)—two replicate bags per bush.
- Flowers exposed during the day only (day pollination)—two replicate bags per bush.
- Flowers exposed during both the day and the night (open pollination) – one replicate per bush (received no bagging).
- Flowers with no exposure (no pollination)—one replicate bag per bush.
- Manual handling control (the effect of adding and removing bags)—one replicate bag per bush.

Bags were removed from day pollination flowers at 5 a.m. (sunrise) and reapplied at 7 p.m. (sunset) to allow day active insects the opportunity to visit the flowers. Bags were removed from night pollination flowers at 7 p.m. and reapplied at 5 a.m. to allow nocturnally active insects the opportunity to visit the flowers. After three consecutive days and nights of the flowers being exposed to pollinators,

the number of open flowers in each treatment was counted (flower numbers varied within each bagged replicate), and the bags were reapplied to all flowers. Any flowers that opened after bags were reapplied would have received no floral visitation or manual handling from the bags and are presumed to have not set fruit. Our 'open pollination' treatment did not have a wax-coated paper bag applied to the flowers and serves as a treatment where pollinators had unrestricted access to the flowers. Flowers within the 'no pollination' control were bagged prior to their opening, and these flowers had no exposure to any pollinators. The final treatment was a 'manual handling control' to test the potential effect of removing and adding the bags to the flowers. For this, bags were removed from the flowers and then immediately reapplied, once in the morning and again that same evening, with one replicate on each of the 10 bushes. All bags were removed from the day only, night only, handling control and no pollination treatments on 19 October once flowering had ceased. All berries were harvested on 18 January 2022, counted, and percentage fruit set was obtained by determining the number of flowers which successfully developed into fruit. Berries were also weighed, to determine if fruit weight varied amongst the different treatments.

Pollen on moth bodies

Moths were caught during the 2019 blueberry flowering period using portable automated Heath moth light traps (with actinic bulbs which emit ultraviolet light) in three blueberry orchards in the Waikato region, and one blueberry orchard in Central Otago, New Zealand. Two of these orchards had no restrictions to flower access, while the other two were fully netted with bird netting. Two traps were used in each orchard, placed in the same location each trapping night and the traps were run in all orchards concurrently. Moths were caught weekly or bi-weekly over the flowering period on clear and calm nights and when access to the orchards was permitted. However, due to orchard management practices such as mowing, some orchards could not be accessed on every trapping night. Every moth trap was cleared each morning, and moths euthanised at −20 °C.

Each moth was swabbed with a 3-mm^3 cube of gelatine fuchsin by rubbing the cube along the antennae, dabbing each eye and each side of the proboscis, two dabs on the thorax and three dabs on the abdomen to ensure as much of the ventral side of moth bodies came into contact with the fuchsin cube. One cube was used to swab one entire moth individual, then the cube was placed on a slide, melted and examined under a microscope at ×40 magnification for the presence of pollen. Blueberry pollen tetrads were counted and identified with the use of a reference pollen slide. We are confident in our identification of the pollen as Ericaceae, and that it is most likely to be from blueberry (*Vaccinium*

spp.). Other native Ericaceae species that may occur within flight range of the orchards do not flower at the same time, so the only other source of Ericaceaous pollen would be from ornamental plants in gardens. Given the dominance at a landscape level of blueberry plants, we are confident that any misidentification would be insignificant. Other pollen grains were also counted and identified where possible, but without reference material the species for many of these pollen grains remain unknown.

Statistical analyses

No analyses were conducted for pollen on moth bodies as we report on count data; however, all other analyses were performed in R version 4.2.1.

Stigma receptivity

To assess the effect of time of day on stigma receptivity, a binomial mixed-effects logistic regression was used on the full data set, including all four cultivars. The random effect was for unique bushes within cultivars as there were not enough cultivar or time points to include cultivar as a fixed effect in the full model. To test for individual cultivar effects, standard logistic regression was performed separately for the 'Centra Blue' and 'Centurion' data, with unique bush as the random effect. For all models, each stigma was treated as a separate data point. Separate analyses were not performed for the 'Maru' and 'Reka' data because all their stigmas were receptive at each time point for many of the bushes sampled (i.e. there was not enough variance for the model). Analyses were performed using the glmer function in the lme4 package (Bates et al. 2015).

Pollen viability

To assess the effect of time of day on pollen viability, a negative binomial mixed-effects regression model was used. A Poisson distribution model was tested initially but was not deemed appropriate as the variance was much larger than the mean (over seven times larger). The outcome was the percentage of pollen tetrads per flower that were not viable, rounded to the nearest whole percentage (as the function used gives warnings for decimal values). Time of day, with levels at 10 a.m. and 8 p.m., was the only predictor variable with the unique bush included as a random effect. The model was run on each cultivar individually, using the glmer.nb function in the lme4 package (Bates et al. 2015).

Nectar standing crop

A linear mixed-effects model was used to determine if nectar weight and sugar weight (run as separate models) differed

significantly among the five different times of the day. Both nectar and sugar weight data were right skewed and so were natural log transformed after adding 0.0005 to all values (as values of zero cannot be logged), resulting in data becoming more symmetrical. This transformed variable was used as the outcome variable and time of day was included as a categorical variable. Random intercepts were included for each unique bush. Residuals were checked to ensure their distribution was approximately normal. The overall significance of the time variable was assessed with a type III ANOVA using Satterthwaite's method for the denominator degrees of freedom. If the time was found to be significant, pairwise comparisons of the estimated means were investigated (using the emmeans function, Lenth 2024), adjusting the *p* values for multiple comparisons using Tukey's method for a family of five estimates. To determine if the effect of time was different for different cultivars, the same model as above was used with the addition of the cultivar main effect and a time-by-cultivar interaction. These analyses were all performed using the lmer function in the lmerTest package (Kuznetsova et al. 2017).

Pollen germination day versus night

We used a negative binomial model to investigate the effects of day vs. night hand pollination on the number of pollen grains that produced pollen tubes of at least 50 μm in length after 12-, 24- and 36-h post-pollination. The model used the number of pollen tubes that grew to 50 μm as the outcome with cultivar and the interaction between pollination time (day/night) and time of measurement (12, 24 and 36 h after pollination) as predictor variables. The inclusion of the interaction allowed us to test whether the pattern of pollen tube growth over time was different for stigmas that were pollinated at different times of day. The total number of pollen tetrads on the stigma was included as an offset in the model, which meant that we were, in effect, modelling the proportion of pollen tubes that grew to 50 μm. Residuals were checked to ensure their distribution was approximately normal. The glm.nb function in the MASS package (Venables and Ripley 2002) was used to fit the model. The data were grouped by bush (multiple flowers from the same bush), so a mixed negative binomial model was fitted initially with random intercepts for unique bushes. However, as the variance of the random intercepts was found to be very close to zero, a standard negative binomial model was sufficient. Pairwise comparisons were made using the emmeans function to interpret the model.

Pollinator exclusion experiments

To assess pollination treatments on fruit set, fruit set (number of flowers which set fruit given as a percentage) was

first adjusted by + 0.5 (as values of zero cannot be logged) and then log transformed (using the natural log). A linear mixed-effects model was applied using the lmer function of the R package lmerTest that included random intercepts for unique bushes. The overall significance of the treatment term was assessed with a type III ANOVA and then, if significant, pairwise comparisons of the estimated means were investigated using the emmeans function, with *p* values adjusted for multiple comparisons using the Tukey method for a family of five estimates.

Results

Stigma receptivity and pollen viability

There was no significant difference in stigma receptivity between the 10 a.m. and 8 p.m. sampling times, averaged across all cultivars ($p = 0.999$) or for 'Centra Blue' ($p = 0.705$) or 'Centurion' ($p = 1$) (Table 1; Table S1 in Supplementary Material). No analyses were performed on 'Reka' or 'Maru' as stigma receptivity was 100% in all samples.

There was also no significant difference in pollen viability between the 10 a.m. and 8 p.m. sampling times, averaged across all cultivars ($p = 0.69$). When looking at each cultivar individually, only 'Centra Blue' showed a significant difference in pollen viability between 10 a.m. and 8 p.m. ($p = 0.0012$), with more pollen germinating at 10 a.m. than 8 p.m. (Table 1; Table S2 in Supplementary Material).

Nectar standing crop

Nectar availability was measured at sunrise (5/6 a.m.), 10 a.m., 2 p.m., sunset (6/7 p.m.) and 10 p.m. for four cultivars (Fig. 1). Overall, for all cultivars combined, we found a highly significant effect of time ($p < 0.0001$) on nectar

availability (Table S3 in Supplementary Material), with nectar availability being greatest at sunrise than at any other time of the day and decreasing over the day.

We also found a highly significant effect of cultivar ($p < 0.0001$) and an interaction between cultivar and time on nectar availability ($p < 0.0001$; Fig. 1; Tables S4 and S5 in Supplementary Material). While 'Centurion' and 'Maru' tended to have fairly constant nectar availability across the time points, nectar availability in 'Centra Blue' and 'Reka' was highest at sunrise and lower over the other time points.

Overall, we found a significant effect of time on sugar weights in nectar ($p = 0.0398$, Fig. 2; Table S6 in Supplementary Material). We also found a highly significant effect of cultivar ($p < 0.0001$) and an interaction between cultivar and time on sugar weight ($p < 0.0001$, Table S6 in Supplementary Material). While we saw no effect of time on sugar weights in 'Centra Blue' or 'Centurion', sugar weights were significantly higher at 2 p.m. than at 10 a.m. in 'Maru' and significantly higher at sunrise compared with 2 p.m. and sunset in 'Reka' ($p = < 0.0001, 0.0001,$ and 0.0005 respective, Tables S7 and S8 in Supplementary Material).

We made observations of floral visitations by nocturnal insects during the nectar sampling and found an unknown Culicomorpha species (Diptera) and the New Zealand endemic moth *Scoparia halopis* (Crambidae: Scopariinae) actively foraging on blueberry flowers (Figs. S1 and S2 in Supplementary Material).

Pollen germination day versus night

Overall, we found a significant difference in pollen germination between day and night hand pollination and a significant effect of time since pollination on pollen tube growth ($p < 0.001$; Table S9 in Supplementary Material). We also found a significant cultivar effect, but in both cultivars the stigmas pollinated in the evening grew a higher proportion of pollen tubes to 50 µm than stigmas pollinated during the morning and so the data are presented with the two cultivars combined.

The results of the model show that after 12 h, stigmas pollinated in the morning had 85 times more pollen tubes growing to 50 µm than stigmas pollinated during the evening ($p < 0.0001$; Fig. 3; Tables S9, S10, S11 in Supplementary Material). However, after 24 and 36 h, stigmas pollinated during the evening had 2.84 and 2.11 times more pollen tubes growing to 50 µm than stigmas pollinated in the morning respective ($p < 0.001, 0.001$ respectively; Fig. 3, Tables S9, S10, S11 in Supplementary Material).

Pollinator exclusion experiments

The 'open pollination' treatment showed a significantly higher fruit set than the other treatments, and there was

Table 1 The percentage of receptive stigmas and viable pollen at 10 a.m. and 8 p.m. for four blueberry cultivars

Cultivar	Time	Stigma receptivity (%)	Pollen viability (%)
'Maru'	10 a.m	100	96.65
	8 p.m	95	97.85
'Centurion'	10 a.m	90	97.73
	8 p.m	90	97.15
'Reka'	10 a.m	100	96.67
	8 p.m	100	98.50
'Centra Blue'	10 a.m	75	97.87*
	8 p.m	80	91.90*

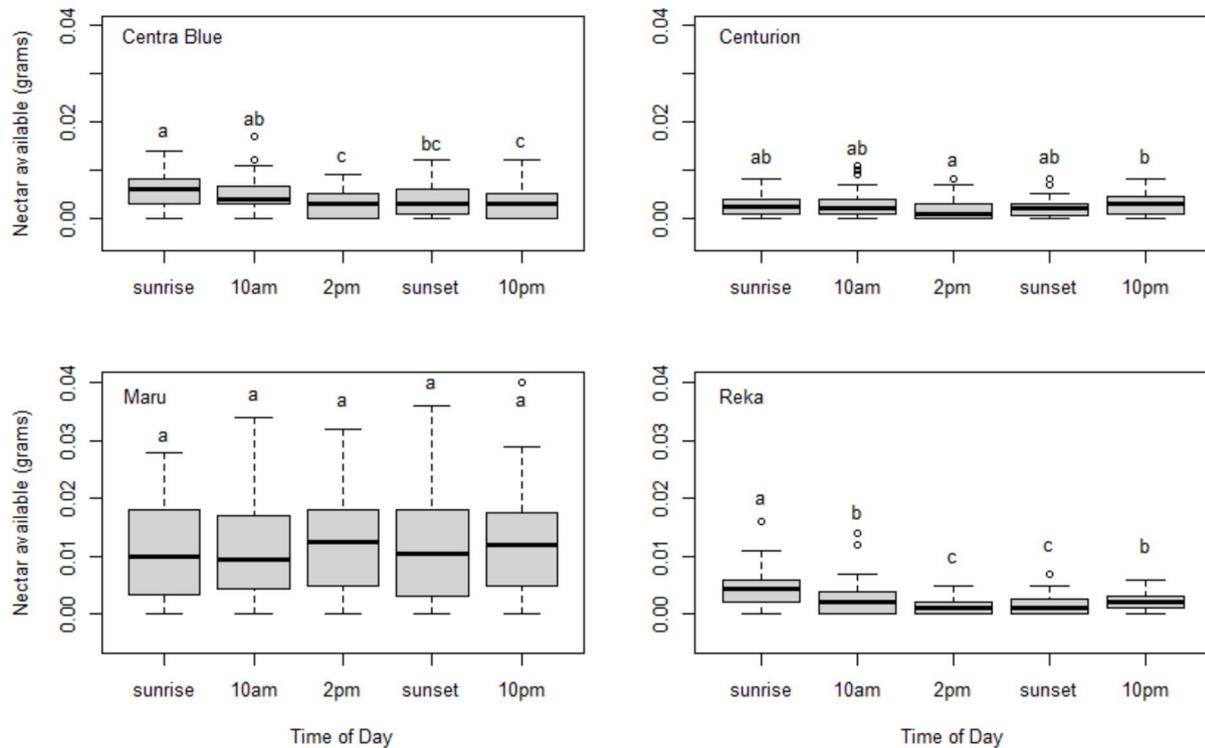*Symbol indicates significant differences ($p < 0.01$)

Fig. 1 Average nectar availability (in grammes) in the four different blueberry cultivars; 'Centra Blue', 'Centurion', 'Maru' and 'Reka'. Letters above boxplots indicate significance as determined by pair-wise comparisons from linear mixed effect models, where shared letters indicate no significant difference. Note that nectar values are only comparable within each cultivar and not across the different cultivars

no significant difference in fruit set for the 'day pollination', 'night pollination' and 'manual handling' treatments (Fig. 4). The 'no pollination' treatment was significantly lower than the day pollination, night pollination and manual handling treatments. See Table S12 in Supplementary Material for full summary statistics.

Berry weights in the 'no pollination' treatment were significantly lower than in the 'day pollination', 'night pollination' and 'manual handling' treatments, but there was no significant difference between the berry weights for 'no pollination' and 'open pollination' (Fig. 5). Berry weights were significantly higher in the 'night pollination' treatment compared with the 'open pollination' treatment, but there was no significant difference in berry weight among the 'day pollination', 'night pollination' and 'manual handling' treatments. See Table S13 in Supplementary Material for full summary statistics.

Pollen on moth bodies

A total of 429 moths were caught over 9 trapping nights in 4 blueberry orchards. The most common moths caught were *Agrotis ipsilon* (Noctuidae) and various *Ichneutica* species (Noctuidae). To assess pollen loads, 198 macro-moths

were swabbed for pollen, of which 19 (9.6%) were found to carry blueberry pollen and 155 (78.3%) had pollen from other plant species. Of the 19 moths carrying blueberry pollen, an average of 4.8 blueberry pollen tetrads were carried, and 8 of the 19 moths were found to carry only blueberry pollen. Blueberry pollen tetrads accounted for 5.2% (100 tetrads) of 1918 pollen grains removed from moths caught in the orchards. Blueberry was the fifth most abundant pollen species recorded, and the fifth highest number of moth individuals carried blueberry pollen. Pollen from 25 different species was recorded on moth bodies, however only 10 plant species were represented by more than 10 grains and only five pollen species were represented by 100 or more pollen grains present.

Discussion

We applied six methods commonly used to infer pollination modes and found evidence in five of these to suggest that blueberries can be pollinated at night. Neither stigma receptivity nor pollen viability differ significantly at two time-points (day and night) in the four blueberry cultivars representing rabbiteye and northern highbush species. Nectar
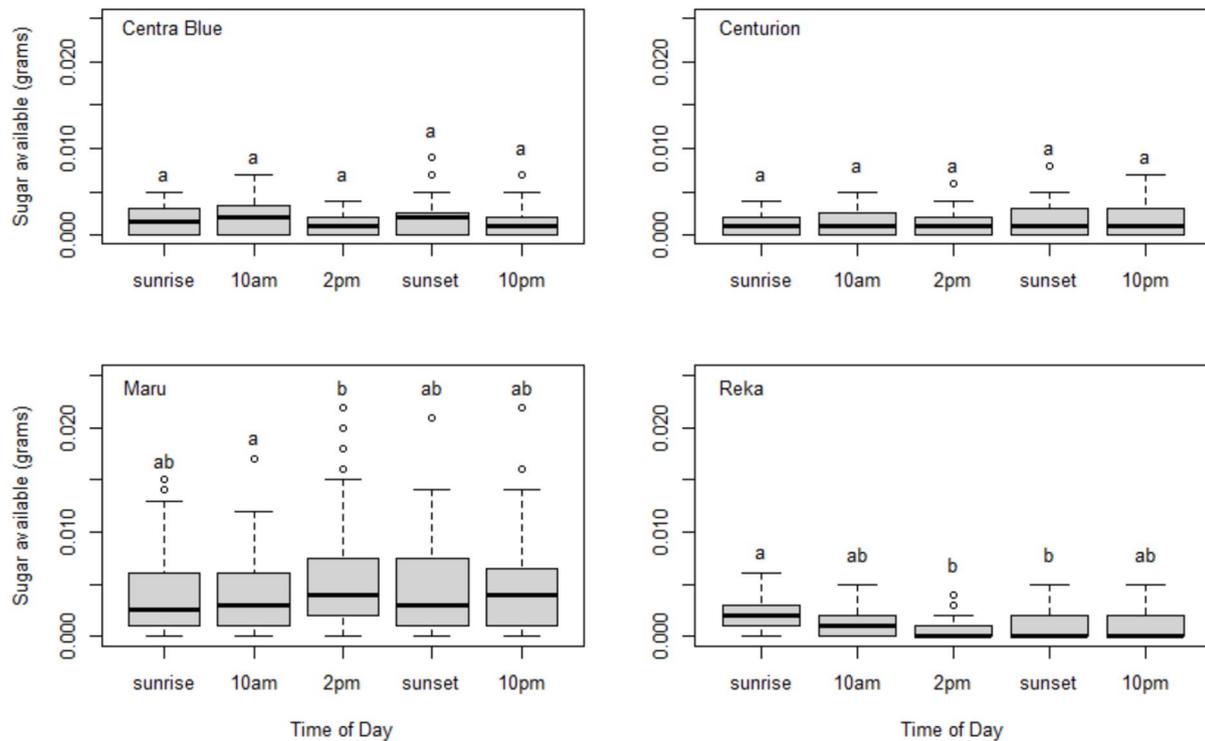
Fig. 2 Average sugar availability (weight in grammes) in the four different blueberry cultivars; 'Centra Blue', 'Centurion', 'Maru' and 'Reka'. Letters above boxplots indicate significance as determined by pairwise comparisons from negative binomial linear mixed effect models, where shared letters indicate no significant difference. Note that sugar values are only comparable within each cultivar, and not across the different cultivars

and sugar dynamics were complex and cultivar specific, but nectar is available during the night as well as during the day. Pollen tube growth rates differed significantly between two timepoints (day and night), with an apparently higher growth rate in flowers pollinated in the evening. We were unable to determine if fruit set and fruit weight differed among our pollination treatments as an unknown level of self-pollination occurred within our treatments; however, flowers with unrestricted access to pollinators set the highest number of fruit. We also showed that moths caught in blueberry orchards carry blueberry (and other) pollen, although often in low quantities. Together, these five approaches indicate blueberry flowers could utilise both day and night pollination.

Stigma receptivity and pollen viability

For successful pollination, viable pollen must be transported to a conspecific stigma when the stigma is receptive (King et al. 2013; Popic et al. 2013). We found stigmas were receptive and pollen tetrads were viable both in the morning and the evening, suggesting that this would allow blueberry flowers to be pollinated just as effectively at night as they are during the day. Blueberry stigma receptivity has been shown to decrease with increasing flower age, and fruit set is optimum if pollination occurs within 5 days of flower opening, but it is still possible after 8 days (Brevis et al. 2006). The flowers used in our study were young with no visible signs of flower ageing, which may explain why our stigma receptivity values were so high. The pollen viability values were also high in our samples, but similar to that reported by Lang and Parrie (1992), who applied pollen from five different cultivars to germination plates following the same protocol and reported pollen viability from 90.5 to 96.3%. Our stigma receptivity and pollen viability results are within the range reported by other diurnal-only studies (Lang and Parrie 1992; Brevis et al. 2006), indicating that the night and daytime levels we observed are adequate for pollination.

Nectar standing crop

We sampled nectar at five different times of the day (sunrise, 10 a.m., 2 p.m., sunset and 10 p.m.) in four blueberry cultivars to assess whether the time of day may have an influence on nectar availability. Overall, we found a significant effect of time of day on nectar availability, with more nectar
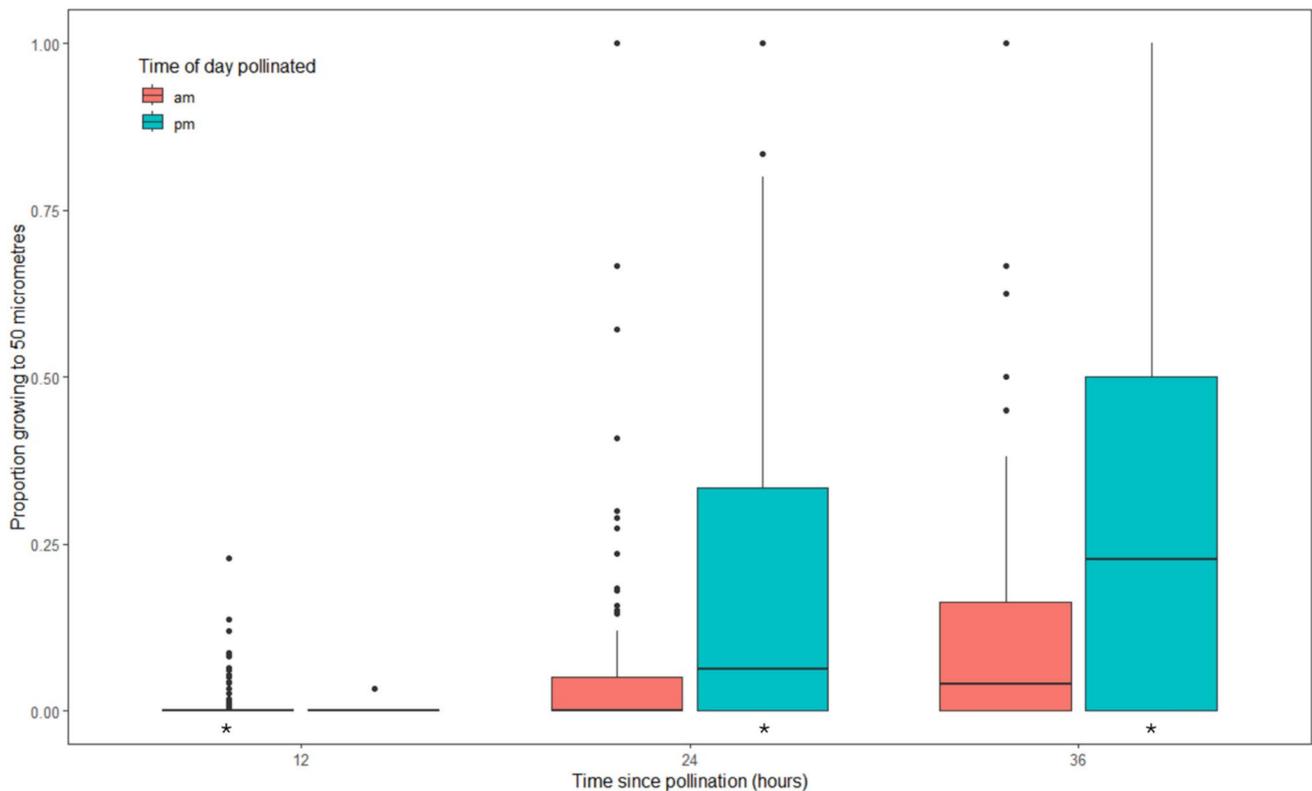
Fig. 3 Proportion of pollen tubes growing to 50 μm down the style 12, 24 and 36 h after hand pollinations done during the morning (a.m.) or the evening (p.m.). The proportion of pollen tubes growing for the morning and evening samples have been standardised for the number of pollen tetrads deposited on the surface of the stigma and the data were averaged across the cultivars. An asterisk within a measurement period indicates there was a significant difference in pollen tube growth ($p < 0.05$), with the asterisk beneath the bar that was significantly greater

being available at sunrise than at any other time of the day. However, this trend appears to be driven by two of the cultivars ('Centra Blue' and 'Reka') because nectar availability in the other two cultivars ('Centurion' and 'Maru') did not significantly differ among the time points measured, indicating that nectar dynamics may be cultivar specific. The nectar dynamics recorded in our study may be influenced by differences in floral morphology; cultivars with wider corolla apertures and/or shorter corolla lengths may be more accessible or more attractive to pollinators, resulting in greater nectar removal. However, we have not assessed this here. The relationship between nectar removal and floral morphology could be a productive area for research for both blueberries and other species.

Nectar production in blueberries has been shown to correlate with insect visitation rates (Jablonski et al. 1985) and increasing visits to flowers also increases the probability of fruit set (Kendall et al. 2020). The pattern we observed of higher nectar volumes at sunrise and lower volumes in the afternoon is likely due to insect activity, particularly honey bees and bumble bees actively foraging on flowers during the day. Higher nectar in the morning could also reflect

poor flower visitation by insects at night. We did not perform any formal insect visitation surveys, however, casual observations made during the nectar sampling showed that nocturnal insects do visit blueberry flowers to forage, e.g. an unknown Culicomorpha species (Diptera) and the New Zealand endemic moth *S. halopis* (Crambidae: Scopariinae) (Figs. S1 and S2 in Supplementary Material). Both Culicidae and various Lepidoptera families, as well as flies and beetles, were recorded in lowbush blueberry orchards at night in similar studies and were suspected of being pollinators (Manning and Cutler 2013; Noone et al. 2022).

We found a significant effect of time of day on the amount of sugar in nectar overall, but this appears to be largely driven by the cultivar 'Reka'. Within each cultivar, we also report on some cultivar-specific effects; for example, sugar weights were significantly higher at sunrise than at 2 p.m. in 'Maru' and sugar weights were significantly lower at sunrise than at 2 p.m. in 'Reka'. Having nectar and sugar availability dynamics that appear to be cultivar specific suggests that nectar production is highly variable across blueberry plants as a whole, so generalisations about these interactions across all blueberry cultivars should be made with care.
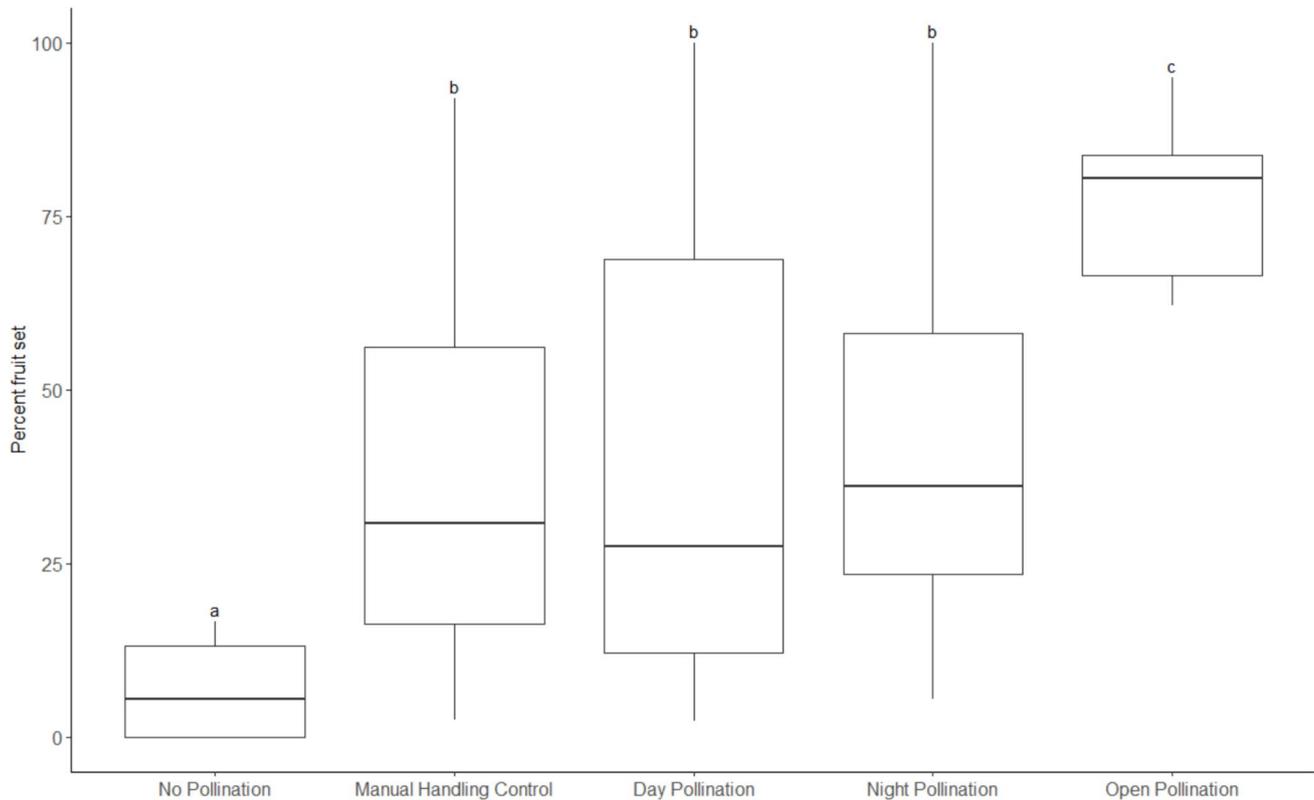
Fig. 4 Fruit set of blueberry flowers from the five different treatments in 'Centra Blue'. Letters above boxplots indicate significance as determined by pairwise comparisons from a linear mixed effect model, where shared letters indicate no significant difference

We attempted to assess nectar recharge rates—the rate at which flowers replenish their nectar after an insect visit—but we did not observe nectar being replenished. It may be that these flowers do not replenish nectar once it is removed, however, our methods were potentially inadequate (the flowers tend to be damaged using our method). It seems likely that blueberry flowers can replenish nectar over time since multiple floral visits increase the probability of fruit set (Kendall et al. 2020), suggesting a fitness benefit and selection on factors such as nectar replenishment that would encourage multiple pollinator visits. To resolve this, we recommend assessing blueberry nectar availability in the absence of pollinators during the day and night.

While we are unable to draw conclusions about nectar availability and its role in pollinator attraction, we do show that nectar is available for nocturnal insects and that nocturnal insects actively visit these flowers, highlighting some areas for further research.

Pollen germination day versus night

Blueberry pollen viability and stigma receptivity did not vary between two time points (day and night), but the environmental conditions that flowers experience between pollen deposition and ovule fertilisation can negatively or positively influence a flower's chance of setting fruit. Some studies on blueberry pollination success have addressed the impact of weather (Tuell and Isaacs 2010) and the timing of pollination across the season (e.g. late season versus early season; Schut et al. 2017). The time of day can also affect floral volatile production and pollinator attraction (Rodriguez-Saona et al. 2011). It is intriguing that we found an effect of time of pollen deposition on pollen germination (and thus likely pollination success)—a phenomenon that may occur in many other flowering plants. Blueberry pollen tubes can take 48–72 h to germinate and reach the base of the style at 24 °C and potentially longer with 'in field' conditions (Lang and Parrie 1992). However, temperature can fluctuate dramatically on a daily basis during the blueberry flowering season (especially in temperate regions such as the Waikato in New Zealand) and temperatures that are too high or too low can hinder pollen tube growth in blueberries and other plants (Marquard 1992; Yang et al. 2019; Walters and Isaacs 2023). Warmer temperatures are often associated with increased pollen tube growth rates, however, when temperatures are too high, pollen tube growth and the longevity of ovules are negatively affected (Vasilakakis and Porlingis 1985; Yang et al. 2019; Roeder et al. 2021). Optimum
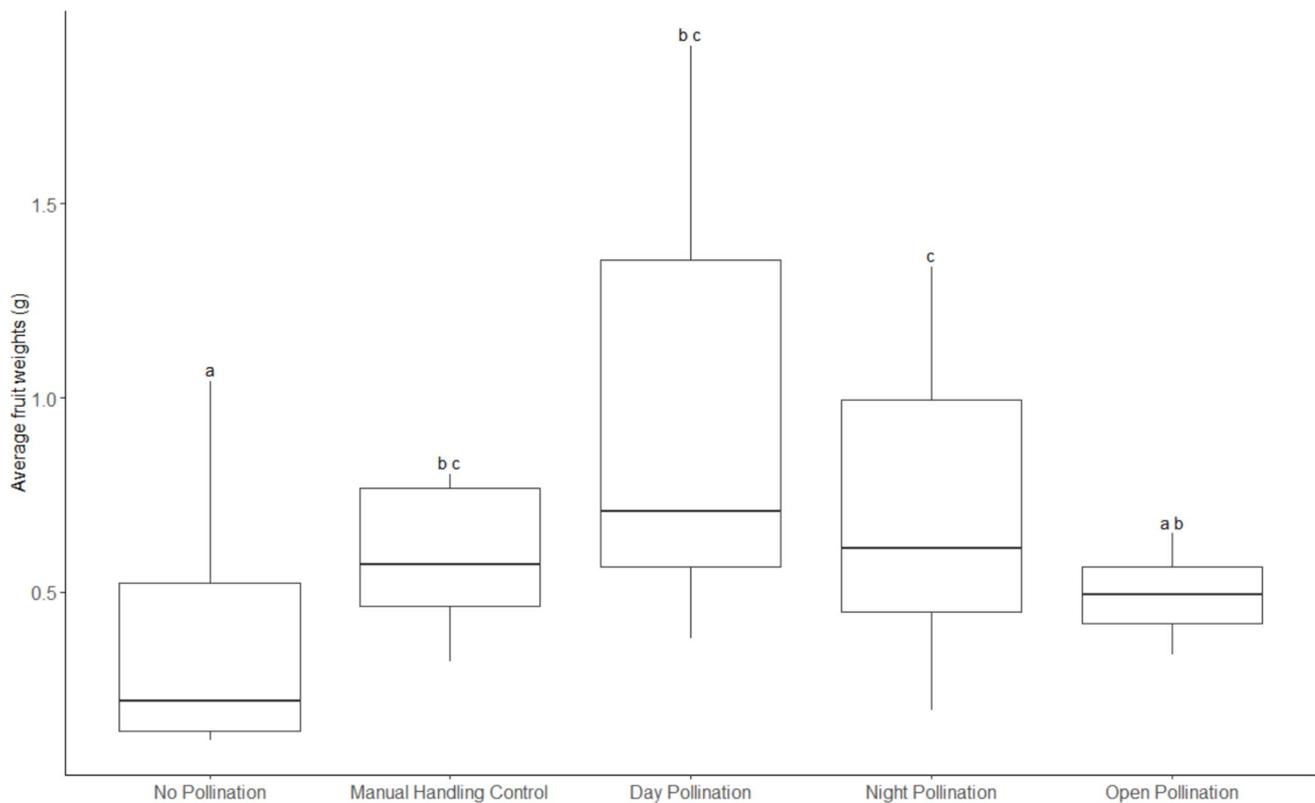
Fig. 5 The average berry weights (in grammes) of all blueberries from flowers from the five different treatments in 'Centra Blue'. Letters above boxplots indicate significance as determined by pairwise comparisons from a linear mixed effect model, where shared letters indicate no significant difference

temperature ranges for pollen germination and pollen tube growth vary among species and are often a reflection of the temperature plants are exposed to in their native ranges (Hedhly et al. 2003). Yang et al. (2019) reported that the optimal temperature for pollen germination in blueberry is 21.4 °C and the optimal temperature for pollen tube growth is 18.4 °C. In our study, when pollen tetrads were deposited at night, the air temperature was 4.7 °C and declining, whereas when the pollen tetrads were deposited at 7 a.m., the air temperature was −1 °C and warming. While these temperatures are far from the optimum reported in Yang et al. (2019), our results suggest that pollen was still able to germinate during the day in that period of warming. Although the blueberry pollen deposited at night was unable to germinate during the initial period of cooling, this 'incubation period' did not reduce later pollen tube growth and appears to have even enhanced pollen tube growth after 24 to 36 h. This provides an interesting avenue for further work. Future trials could consider including additional time points because pollen tube growth rates may differ during morning, mid-day, afternoon and evening pollination. While the optimal time for pollinating blueberry flowers remains unknown, our trial suggests the timing of pollen application is unlikely

to decrease plant reproductive success and berry production, and when considered alongside Yang et al. (2019), suggests that blueberries may be able to adapt to mitigate the effect of low temperature on pollination and fertilisation. As blueberries are grown in a wide range of environments vastly different from their native range, understanding how different temperatures affect fruit production processes (such as pollen tube growth) may be beneficial to improve fruit quality and production, particularly within controlled growing environments.

Pollinator exclusion experiments

Nocturnal pollination in blueberries was first reported by Cutler et al. (2012) for lowbush blueberries in a pollinator exclusion experiment. Our trial on highbush blueberries similarly reinforces the importance of pollination for blueberry plants—for both lowbush and highbush cultivars, as flowers that received no pollination from animals had very little fruit set. However, when determining the relative contribution of nocturnal and diurnal pollinators to fruit set, our pollinator exclusion experiment was unfortunately inconclusive. We found no significant difference in fruit set between our day

and night pollination treatments and our 'manual handling' control.

Our manual handling control was both a self-compatibility treatment that tested for the ability of the cultivars to self-pollinate, and a control to account for the effect of adding and removing bags from flowers. These factors are interrelated because adding and removing bags can agitate blueberry flowers and result in artificial self-pollination. The level of self-compatibility within our study cultivar was unknown, warranting the inclusion of such a control. A similar control was also included in Noone et al. (2022), which they called a 'sham' control. Future studies could resolve this issue by caging entire plants rather than bagging clusters of flowers, as per Cutler et al. (2013). Unfortunately, this was not possible to achieve in our study due to the close proximity of the bushes to each other. While the results from our trial were inconclusive, they do highlight the need for a 'manual handling control' in similar studies.

Nocturnal pollinators and diurnal pollinators contributing fairly equally to the pollination of blueberry is reported by Cutler et al. (2012) and Noone et al. (2022); although in their studies, while nocturnal pollination resulted in reduced fruit set, this was statistically non-significant. However, their studies did not report an additive effect of increased fruit set when flowers are open to both day and night pollinators, which we found. This is likely due to the flowers in our 'open pollination' treatment having unrestricted access to pollinators (i.e. no bags placed on flowers) and thus the flowers had a longer period of time to be visited by pollinators. Both Cutler et al. (2012) and Noone et al. (2022) worked in lowbush blueberry, which has a different form from the rabbiteye type we assessed and used different methods to those employed in our study. The pollinator communities are also likely be very different at the study sites: Kent Island (Canada), Nova Scotia and New Zealand. All these factors may play a role in explaining the differences in results between the trials of Cutler et al. (2012), Noone et al. (2022) and our own.

Average fruit weights did not significantly differ among our 'manual handling control', 'day pollination' or 'night pollination' treatments. We recorded large variability in fruit weights across all the treatments, as was also reported by Cutler et al. (2012). Noone et al. (2022) reported on fruit size instead of fruit weight, but they also found large variations across their treatments. Leaving the berries on the plants for longer to allow them to fully ripen may have reduced this large variation; however, the same argument discussed around the effect of self-pollination from the manual handling of bags on fruit set above also applies to fruit weights here.

We saw an additive effect of nocturnal and diurnal pollination on fruit set (in the 'open pollination' treatment), but this did not occur for fruit weight. This indicates that having day and night pollinators may increase the number of berries produced but may have no effect on fruit quality (when using fruit weight as a measure of fruit quality). There may be a trade-off between fruit number and size, where producing more fruit limits their possible size. Thus, the significantly increased fruit set rate and greater number of berries in the 'open pollination' treatment may limit growth of each berry. In contrast, the lower number of berries in the other treatments may have allowed berries to grow faster and/or larger in response to reduced resource competition, but this remains untested. Another explanation could be that our samples were harvested prior to the harvest of the other berries in the commercial orchard in which this trial was conducted, so many of these smaller berries may have fallen off or grown to maturity if left on the bushes for longer. How this would have affected our results is unknown but is something that could be incorporated into further research. Regardless, we are unable to determine the relative importance of diurnal versus nocturnal pollinators for blueberries in New Zealand based off of these results and highlight this as an area for future research.

Pollen on moth bodies

Measuring the rate of floral visitation and the outcome of single-visit pollen deposition are important ways of assessing how individual taxa contribute to the pollination of plants. The difficulties associated with doing this in the dark mean alternative methods are needed. Detecting the presence and amount of pollen on an individual animals body is particularly useful for identifying associations between plants and nocturnal pollinators (Macgregor et al. 2014). While the presence of pollen on insect bodies is not definitive proof of their role as pollinators for the flowers the pollen originated from, it does demonstrate floral visitation and effect pollen removal. In our study, 10% of the moths we assessed were carrying blueberry pollen and a further 78% of moths were carrying pollen from 24 other plant species. A study conducted in Nova Scotia (Canada) reported that 16–25% of moth individuals caught in blueberry orchards carried blueberry pollen (Manning and Cutler 2013). Individual blueberry flowers require 30–100 pollen tetrads to maximise fruit set and fruit mass and to minimise the time to ripen (DeVetter et al. 2022). In our study, moths that were carrying blueberry pollen carried 4.8 pollen tetrads on average, meaning that flowers would require 6 visits at a minimum by moths to reach the minimum pollen requirements to maximise fruit set. While blueberry pollen represented 5.2% of all the pollen found on moth bodies in our study, this is comparable to honey bees, where blueberry tetrads represented 3–4% of the pollen found on honey bees (Girard et al. 2012). However, honey bees are still efficient pollinators for blueberries because of their high abundance, the location of

pollen on their bodies and their behaviour. Hoffman et al. (2018) demonstrated that pollen is often located on claws, tarsi and legs, and can be transferred to stigmas while honey bees are foraging, grooming and walking over flowers. Since moths are likely less abundant than honeybees, we are cautious to claim that moths may be contributing significantly to the pollination of blueberry flowers. However, despite their lower abundances, moths may be highly effective when they do visit blueberry because pollen has been removed from the proboscis, face and eyes of moths caught in lowbush blueberry orchards (Manning and Cutler 2013). The proboscis comes into direct contact with flowers most often during feeding (Fenster et al. 2004; Macgregor et al. 2019), and so it may well be that this provides accurate, efficient and high-quality pollen transfer for blueberries.

Our results lend support to the argument made by Hahn and Bruhl (2016) that while moths are not considered as primary pollinators in agroecosystems, they may supplement crop pollination by other pollinators and even be pollinators of non-crop plants within the orchard and wider environment. While we detected a low number of pollen tetrads on moths and our pollinator exclusion experiment was inconclusive, the other four floral traits we assessed suggest that blueberry flowers are still able to benefit from and attract nocturnal flower visiting insects. Thus, we believe that the role of moths and other nocturnal insects as blueberry pollinators should not be dismissed. Future work is required to better understand the contribution of nocturnal pollinators to blueberry as well as other crops, and combining assessment of plant traits, pollinator identification and pollination processes, during the day and night, provide a useful multi-disciplinary approach.

Conclusions

Our study demonstrated little difference in stigma receptivity and pollen viability between our day and night sampling points. Nectar availability was strongly associated with time of day, and while nectar was available both during the day and night, there was more available in the early morning. We detected a significant effect of time of day on pollen deposition on pollen tube growth, with greater growth after 36 h in flowers that had been pollinated in the evening rather than the day. However, we consider this difference to be biologically insignificant due to further opportunities for pollen tube growth over subsequent days, and thus, the timing of pollen deposition is not likely to impact on germination success. We also showed that moths carry blueberry pollen, although in low quantities, which is supportive of a possible pollination interaction. We were, however, unable to determine a difference in fruit set rates between flowers pollinated during either the day or the night due to the presumed effect of manual handling resulting in self-pollination. Cutler et al. (2012) provided the first insight that nocturnal pollinators might be important in lowbush blueberry. The evidence that we have gathered builds on this and indicates that highbush and rabbiteye blueberry flowers are also likely able to benefit from both diurnal and nocturnal flower visits, yet further work is required to better understand the contribution of nocturnal pollinators to blueberries. Like Cutler et al (2012), we believe that as efforts are being made to better manage agricultural habitats to promote wild bee pollinators, it may be timely to better understand ways in which we can enhance the contribution of nocturnal pollinators to not just blueberry, but other crops as well.

Author contributions Max Buxton, Anne Gaskett, Janice Lord and David Pattemore conceived the ideas and designed the methodology; Max Buxton collected the data and led the writing of the manuscript. All authors contributed critically to drafts and gave final approval for publication.

Data availability The datasets generated during the current study will be made available on FigShare upon this article being accepted for publication.

Declarations

Conflict of interest The authors have no competing interests to declare.

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