**Title:** Priority effects in berries: The identity of the initial flower visitor is important for determining fruit weight.

**Authors:** Liam Kendall1,2, Jamie R. Stavert1, Vesna Gagic2, Mark Hall1,3 and Romina Rader1

1. School of Environmental and Rural Sciences, University of New England, Armidale, NSW 2351, Australia
2. CSIRO Agriculture, GPO Box 2583, Brisbane, QLD 4001, Australia
3. Western Sydney University

**Abstract**

1. Flowers are visited by many different taxa of varying efficiencies. While visitation rate is often associated with fruit production and quality, we know little about the mechanisms underlying differences in efficiency. The impact of visit order, or ‘priority effects’ have yet to be tested on pollinator communities that visit pollinator-dependent fruit production .
2. We measured floral visitation sequences (1-20 visits) from either single-species (honeybee *Apis mellifera* or native stingless bee *Tetragonula carbonaria*) or mixed-compositional visits (a mixture of visits from both species) and the resulting fruit weight in two mass-flowering crops, blueberry (*Vaccinium corymbosum*) and raspberry (*Rubus idealis*). We investigated the importance of priority effects by examining how species identity and duration of the initial pollinator visit on fruit weight.
3. Increased visitation by honeybees resulted in greater fruit weight for blueberry, whereas increased visitation from mixed species and stingless bees resulted in greater fruit weight in raspberry. The identity of the initial visitor was important for determining fruit weight in blueberry. We detected a strong priority effect in blueberry, whereby fruit produced from flowers that received their first visit from a stingless bee were initially 79% heavier than those that had a honeybee visit first.However, in raspberry, the duration of the initial visit, irrespective of species, in addition to visitation rate had an additive on fruit weight.
4. The order of the visit sequence to flowers by taxa differing in efficiency has consequences for plant reproduction. Our results suggest that even in highly modified crop systems, priority effects may influence plant reproduction. Understanding the importance of priority effects in addition to traditional measures of pollinator efficiency is an important step forward in plant pollinator community ecology.

**Introduction**

Worldwide, insect pollinators significantly contribute to biodiversity and the provision of ecosystem services within agricultural systems. Despite the recognition that wild and managed pollinators play a globally significant role in crop pollination, there is less widespread appreciation that not all pollinator species or pollinator communities are equally effective. Maximally effective pollination results from a complex assortment of factors that influence the pollination process before and after pollen deposition (REF Karron paper; Willcox et al. 2017). The effectiveness of individual pollinators is determined by the combined outcomes of the amount, quality and timing of the pollen transferred which is strongly related to pollinator foraging behaviour, morphology and density (e.g. grooming, floral preferences, functional traits, mobility and abundance (Rademaker, M.C.J., et al., Funct. Ecol. 11, 554-563, (1997); Requier, F., et al., Ecol. Appl. 25, 881-890, (2015); Hoehn, P., et al., P. Roy. Soc. B., 275, 2283-2291, Rader, R. et al., Div. Dist., 17(3), 519-529, (2011)Sáez, A., et al., J. Appl. Ecol., 51, 1603-1612, (2014). Variation in the identity and spatial and temporal structuring of plant (Cussans, J., et al., PLoS ONE, 5, e11753, (2010) and pollinator communities Rader, R., et al., J. Appl. Ecol., 49, 126-134, (2012) further affect effective pollination (Flanagan, R.J., et al., Oikos, 120, 200-207, (2010); Kunin, W.E., Ecology, 74, 2145-2160, (1993), impacting pollen availability (Hargreaves, A.L., et al., Biol. Rev. 84, 259-276, (2009); Chalcoff, V.R., et al., Oikos, 121, 471-480, (2012) and post-pollination factors, such as pollen-pistil and/or pollen-pollen interactions on the stigma Morales, C.L. & Traveset, A., Crit. Rev. Plant Sci. 27, 221-238, (2008). Pollination failure can thus result from problems at any or all of these stages of pollen transfer Wilcock, C. & Neiland, R., Trends Plant Sci. 7, 270-277, (2002).

Across whole communities, competitive or facilitative interactions among species can also increase or decrease fruit set Brittain, C., et al., P. Roy. Soc. B., 280, 1754, (2013); Brosi, B.J.& Briggs, H.M., PNAS, 110, 13044-13048, (2013). A visitation sequence is thus not performed in isolation – encounters with other pollinators can alter this visitation sequence resulting in increased or decreased visitation and/or pollen deposition as a result of interactions with other taxa Brittain, C., et al., P. Roy. Soc. B., 280, 1754, (2013); Brosi, B.J.& Briggs, H.M., PNAS, 110, 13044-13048, (2013). For example, non-Apis bees caused honey bees to move more often between rows of sunflower (*Helianthus annuus* L.), increasing the number of seeds produced per visit Greenleaf, S.S. and C. Kremen, Wild bees enhance honey bees' pollination of hybrid sunflower. Proceedings of the National Academy of Sciences of the United States of America, 2006. 103(37): p. 13890-13895. While several studies have explored how visit frequency and pollen transfer efficiency mediate fruit set and quality (REFS), the order in which the visits, few studies have investigated the importance of the order in which visits occur. Understanding how pollinator species interact with flowers collectively, is a significant knowledge gap. In particular, the mechanisms underlying antagonistic, additive or synergistic multispecies interactions are poorly known with respect to a pollinators visit history and how this might impact plant reproduction.

“Priority effects”, whereby the arrival of one species before another may influence an outcome (e.g. the establishment, growth, or reproduction of species arriving later (Fukami 2015), may influence pollination success as a result of variations among taxa in a community. This is because the sequence of cumulative visits by pollinators to flowers over time will thus result in a particular pollen composition transferred by the pollinator that is beneficial or detrimental to a plants reproduction. This pollen load is the direct product of the flowers available to the pollinators at a given time, mediated by pollinator behaviour, such as grooming and floral selection Holmquist, K.G., R.J. Mitchell, and J.D. Karron, Influence of pollinator grooming on pollen-mediated gene dispersal in Mimulus ringens (Phrymaceae). Plant Species Biology, 2012. 27(1): p. 77-85; Feinsinger, P., H.M. Tiebout, and B.E. Young, Do Tropical Bird-Pollinated Plants Exhibit Density-Dependent Interactions? Field Experiments. Ecology, 1991. 72(6): p. 1953-1963).

Given the stigma receptivity of most flowers is generally limited to a few hours to a few days and delays in the arrival of conspecific pollen may result in a shift in strategy to self-pollination [see reviews by Levin, D.A., Mating system shifts on the trailing edge. Annals of Botany, 2011. Eckert, C.G., CONTRIBUTIONS OF AUTOGAMY AND GEITONOGAMY TO SELF-FERTILIZATION IN A MASS-FLOWERING, CLONAL PLANT. Ecology, 2000. 81(2): p. 532-542.], a visit by a particular pollinator before another, may enhance or reduce the probability of a successful pollination event, due to differences in effectiveness. For example, in a study of the impacts of bumblebee grooming behaviour on the gene dispersal of a perennial herb Mimulus ingens, Holmquist et al. demonstrated that more than 95% of the seeds were sired by the last three visits in a pollinators visit sequence. Evaluating the order in which pollinators visit a flower will facilitate a much greater mechanistic understanding of the factors influencing pollen transport.

Here, we examine the extent to which multiple pollinator flower visits by different pollinator influence the fruit weight of two commercially important crop species (blueberry: *Vaccinium* *corymbosum* and raspberry: *Rubus idaeus*). We test this using two common generalist bee species (honeybees *Apis mellifera,* and native stingless bees *Tetragonula carbonaria*) in either single-species visitation sequences or mixed-species sequences. Then, we test whether floral visitation priority effects are diluted as the number of floral visits increases.

**Materials and Methods**

**Study site**

We conducted this study on the North Coast, New South Wales, Australia on a large-scale 350ha-1 commercial berry farm (29.990232°S, 153.143171°E) across two flowering seasons for blueberry (May 2017 and 2018) and one flowering season for raspberry (February-March 2017).

**Plant species and floral structure**

We focussed on one blueberry species: ‘Southern Highbush’ (SH) (*Vaccinium corymbosum,* cultivar Snowchaser) and one raspberry species: *Rubus idaeus,* cultivar: Driscoll Cardinal. Both blueberry and raspberry flowers are hermaphroditic but differ considerably in shape and display. Blueberry flowers are urceolate, and in open flowers, the anthers remain enclosed within the corolla whereas a single stigma protrudes slightly above the corolla aperture. In contrast, raspberry flowers are dish-shaped, with an open floral display, with ~100 stigmas encircled by ~100 anthers (Driscoll Cardinal Patent).

A figure of each would be nice here I think.

**Floral visitation**

For both blueberry and raspberry, we placed organza bags (mesh size < 0.01mm) over developing shoots and tagged developing flowers before opening. We un-bagged flowers upon opening and observed visits from the two dominant pollinator species (European honeybees, *Apis mellifera* and native stingless bees, *Tetragonula carbonaria*). This resulted in sequential visits from honeybees (number of visits range: x-x), stingless bees (number of visits range: x-x) or a mixture of honeybees and stingless bees (number of visits range: x-x). For each pollinator visit, we ~~recorded the foraging behaviour of the individual (i.e., either pollen or nectar foraging) based on the presence or absence of corbiculae (i.e. pollen baskets, comprised of collected pollen, on the hind legs) and~~ recorded visit duration in seconds. Once the flowers had been visited for the target number of visits, each flower was re-bagged for the duration of its flowering to prevent further visitation from pollinators. We picked the fruit upon ripening and measured fruit weight (grams) in both species using an analytical balance (accuracy: 0.00 g) on the same day.

**Data analysis**

**Does a mixture of floral visits from different pollinator species result in greater fruit set and/or heavier fruits than visits from a single species?**

To test whether visits from multiple pollinator species result in greater fruit weight compared with visits from single species we used a LMM with berry weight as the response variable and species visit composition (honeybee, stingless bee, or a mixture of both; categorical) in interaction with the number of visits (continuous) as fixed effects. For the blueberry model, we included the random terms of block crossed with year because visitation data were collected from different plant individuals in different cropping blocks across two growing years. For the raspberry model, we included a random effect term of block because visitation data were collected from different cropping blocks, in one growing season. Finally, we tested whether slopes for fruit weight with an increasing number of pollinator visits were significantly different from zero for each pollinator species using “emmeans” (V.X.X.X, REF et al. XXXX).

**Do interspecific pollinator priority effects mediate fruit weight in different plant species with distinct floral structures?**

To identify pollinator priority effects in blueberry and raspberry, we subsetted each data set to only include fruit weights produced as a result of mixed pollinator visits (minimum two visits to flowers from both honeybees and stingless bees). We then categorised each fruit as a being produced from a flower initially visited by either a honeybee or a stingless bee. With this data structure, we specified linear mixed effect models (LMMs) to estimate fruit weight as a function of the initial floral visitor (honeybee or stingless bee; categorical) in a three-way interaction with the total number of floral visits (continuous) and ratio of honeybee to stingless bee visits (continuous). In the blueberry model, we included two crossed random effect terms (block and year; categorical) because visitation data were collected in the same orchard block across two growing years. In the raspberry model, we included one random effect term (block; categorical) as data were collected across multiple orchard blocks within the same growing year. We then conducted model selection on these models using the *MuMIn* package (v.X.X.X, Ref et al. XXXX) and found that, in both blueberry and raspberry, the best model for predicting fruit weight included the initial floral visitor, number of floral visits and their interaction. Accordingly, we present model-estimates and slope contrasts for these reduced models. To calculate slope contrasts (i.e., whether there was a significant difference in model-estimated slopes for fruit weight according to the identity of the initial visitor) we used emmeans (v.X.X.X, Ref et al. XXXX). Thus, evidence of a pollinator priority effect is indicated by a significant difference in the model-estimated slope for fruit weight as a function of the number of pollinator visits between the two initial visitor categories (i.e., honeybee or stingless bee) or a signficant relationship with the initial visit duration.

We specified all LMMs using the *glmmTMB* package (v.0.2.3, Brooks et al., 2017). For both intra- and interspecific priority effects analyses, we modelled fruit weight with a Gaussian distribution. We assessed model residuals using the *DHARMa* package (v.0.2.2, Hartig 2019). All analyses were conducted in *R* v3.5.1 (R Core Team, 2018).

*~~Intraspecific priority effects~~*

~~We first subsetted our data to single-species compositional visits (categorical; honeybees or stingless bees). We then estimated fruit set or fruit weight as a function of the first floral visitor’s behaviour (categorical; pollen or nectar foraging) in interaction with the proportion of pollen forager visits (continuous) and total number of floral visits (continuous). Again, in blueberry, we included one random terms:~~ *~~year,~~* ~~as visitation data were collected in the block across two years,~~~~and in raspberry, one random term:~~ *~~block,~~* ~~as data were collected across multiple blocks.~~

**Results**

**Does a mixture of floral visits from different pollinator species result in heavier fruits than visits from a single species?**

For blueberry, we found that fruit weight increased with an increasing number of visits from honeybees (slope = 0.136 ± 0.049, *z* = 2.759, *P* = 0.003; Figure 3) and weakly for stingless bees combined with honeybees (slope = 0.022 ± 0.016, *z* = 1.318, *P* = 0.095) but not for stingless bees only (slope = 0.018 ± 0.078, *z* = 0.237, *P* = 0.407). Overall, fresh weight from flowers visited by both honeybees and stingless bees were significantly greater than those visited by honeybees alone (contrast: 0.114 ± 0.0519, *t* = 2.199, *P* = 0.044).



**Figure 3.** Weight of blueberry fruit from flowers visited by only honeybees or stingless bees or a mixture of honeybees and stingless bees. Solid lines are the model-estimated fruit weights with an increasing number of pollinator visits and shaded ribbons are the model-estimated confidence intervals. Hollow circles are the actual data.

In contrast, for raspberry, we found that fruit weight increased with an increasing number of visits from stingless bees (slope = 0.178 ± 0.069, *z* = 2.559, *P* = 0.006; Figure 4) and stingless bees combined with honeybees (slope = 0.043 ± 0.016, *z* = 2.657, *P* = 0.0042) but not from honeybees only (slope = 0.026 ± 0.029, *z* = 0.886, *P* = 0.1883).



**Figure 4.** Weight of raspberry fruit from flowers visited by only honeybees or stingless bees or a mixture of honeybees and stingless bees. Solid lines are the model-estimated fruit weights with an increasing number of pollinator visits and shaded ribbons are the model-estimated confidence intervals. Hollow circles are the actual data.

Despite these trends, for both raspberry and blueberry we found no differences between slopes (association between fruit weight and the number of pollinator visits) for fruits produced from stingless bee versus honeybee visits. Similarly, there were no differences between slopes for fruits produced from a mixture of stingless bee and honeybee visits versus visits from either one of these taxa (Table SX).

**Do interspecific pollinator priority effects mediate fruit weight in different plant species with distinct floral structures?**

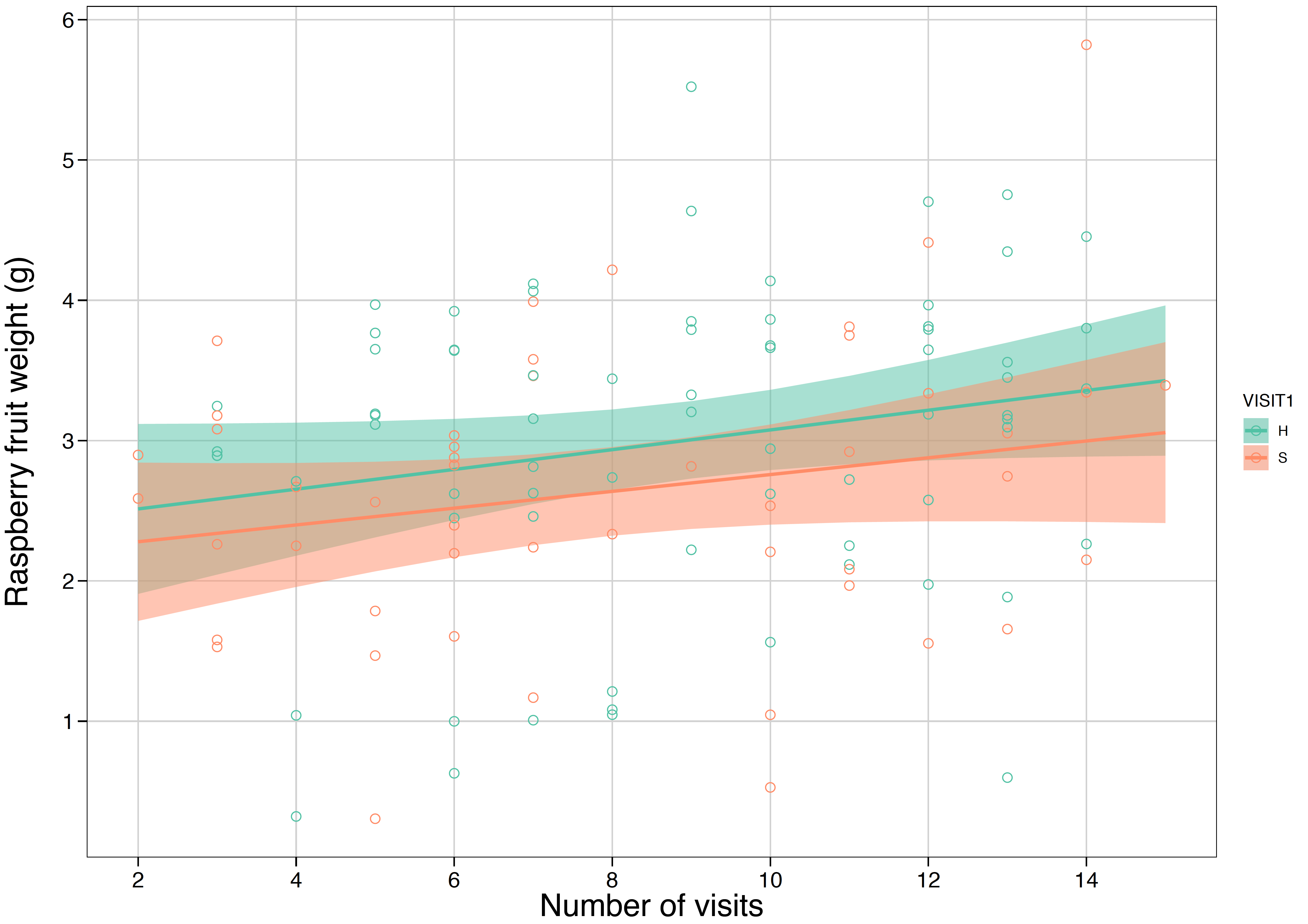
We detected a strong priority effect in blueberry, whereby fruit produced from flowers that received their first visit from a stingless bee were initially 79% heavier than those that had a honeybee visit first (*t =* 2.601 , *P* = 0.009, stingless bee : visit number interaction, *t* = -2.284 *P =* 0.02240) and exhibited significantly different slopes (honeybee – stingless bee slope contrast = 0.071 ± 0.031, *t* = 2.283, *P* = 0.0273; Figure 1). With an increasing number of visits, weight of fruit produced from flowers those that received first received honeybee visits increased significantly (b = 0.051 ± 0.017, t = 2.9, *P* = 0.006), whereas weight of fruit from flowers that first received a stingless bee visit did not increase (b = -0.02 ± 0.026, t = -0.787, *P* = 0.436).



**Figure 4.** Weight of blueberry fruit from flowers first visited by a honeybee versus those first visited by a stingless bee. Solid lines are the model-estimated fruit weights with an increasing number of pollinator visits and shaded ribbons are the model-estimated confidence intervals. Hollow circles are the actual data.

In raspberry, a strong priority effect, raspberry fruit weight were best explained by an additive relationship between the duration of the first visit and the total number of visits, irrespective of pollinator identity (scaled first visit duration, b = 0.215 ± 0.093, *t* = 2.323, *P* = 0.02; total visit number, b = 0.075 ± 0.027, *t* = 2.745, *P* = 0.006).

~~However, we found no evidence of a pollinator priority effect in raspberry, where fruit weight was similar across the visit number range regardless of first visitor’s identity (honeybee – stingless bee slope contrast = 0.024 ± 0.035,~~ *~~t~~* ~~= 0.699,~~ *~~P~~* ~~= 0.48; Figure 2).~~ The ratio of honeybee to stingless bee visits had no effect on fruit weight for both blueberry and raspberry.



**Figure 4.** Weight of raspberry fruit from flowers first visited by a honeybee versus those first visited by a stingless bee. Solid lines are the model-estimated fruit weights with an increasing number of pollinator visits and shaded ribbons are the model-estimated confidence intervals. Hollow circles are the actual data.

**Discussion**

Honeybees and stingless bees differ in their effectiveness pollinating both blueberries and raspberries, resulting in crop-specific trends between floral visitation patterns and fruit quality. The effectiveness of animals at pollinating different plant species is dependent on the functional traits of both the pollinator (e.g., body size, tongue length) and the plant (e.g., nectar accessibility, corolla depth) and is termed ‘trait matching’. Garibaldi et al. (2015) found that trait matching between plants and pollinators benefits pollination services more than trait diversity through the synergistic influence of pollinator morphology (body size, and behaviour), although it is ultimately mediated by plant mating system and level of self-compatibility, and the inherent need for pollen transport between and within individuals (Minnaer et al. 2019).

Blueberry requires buzz-pollination for greater pollen transport. However, both honeybees and stingless bees are unable to buzz-pollinate. Thus, both nectar and pollen collecting honeybees deposit less pollen than buzz-pollinating species (e.g., blueberry bees *Habropoda* sp. and bumble bees *Bombus* sp.; Benjamin and Winfree 2013). Yet, our results suggest that honeybees are able to overcome their inability to buzz-pollinate through increased visitation (longer sequences) as opposed to stingless bees or mixed visits which do not demonstrate a positive relationship between visitation rate and fruit quality. However, we found the opposite in in raspberry, wherein…. This suggests that the quality of individual floral visits (e.g., pollen deposition rates) as well as their order may be as important as the quantity of visits in predicting fruit quality and mediated through interspecific and behavioural priority effects.

The influence of how species interact in floral visitation over time and the collective influence mixed pollinator visitation sequences is little known. In mixed-species visits, priority effects explained a large amount of variation in blueberry fruit quality in this study. This suggests that trait matching may be more important in determining fruit quality in blueberry compared to raspberry, and is likely due to differences in floral structure (see Fig. 1). Although neither species can buzz-pollinate, stingless bees overcome limitation by virtue of their body size (ITD: 1-2mm, Kendall et al. 2019, may as well self-cite myself hahaha), which results in within-flower visitation behaviours. In contrast, honeybees spent significantly less time on flowers, and primarily interacted with corolla opening, using their forelegs or proboscis to collect pollen or nectar. By spending more time in the flower, and directly interacting with the anthers, this may increase the likelihood of firstly, autogamous pollen (from the same flower), and secondly, geitonogamous pollen (same species, different clonal individual), which are beneficial means of pollination given the cultivar’s self-compatibility (REF), leading to greater fruit weight.

Our results also suggest that honeybees facultatively improve later visitation events within a sequence. In contrast, stingless bee priority effects diminish the influence of later visits within a given sequence, which may account for the lack of association between visitation sequence length and fruit quality in sequences where only stingless bees visit. In contrast, previous studies have shown that nectar-robbing Carpenter bees (Xylocopa virginica) have a negative influence on the pollination services provided by honeybees by altering their behaviour to become secondary thieves (Dedej & Delaplane, 2004).

In raspberry, we found that species-level behavioural differences were less important for fruit quality, which is likely because this crop has open-dish shaped flowers. However, initial visit duration, irrespective of species, had an additive effect on fruit quality, in conjunction with total visit number. Increased visit duration may lead to higher likelihood of stigmatic contact, as a function of high nectar availability in the virgin flowers, and given there are ~100 stigmas per flower, this increases the likelihood of more stigmas receiving pollen.

However, although as stingless bees exhibit longer periods in visiting blueberry, the species-level priority effect may be a result of both duration and species identity, although species in interaction with visit number better explained the variance in blueberry weight than duration and visit number.

In laboratory studies, blueberry pollen reacted enzymatically in medium within 90 minutes *in vivo*, with maximum germination occurring within eight hours (Huang and Johnson 1996). In contrast, pollen germination rates are not known for raspberry, although germination is achieved in 20-25 hours in closely-related blackberries (*Rubus* subgen. *Rubus*) (Nybom 1985). As such, the presence of species compositional differences and priority effects in fruit quality are, hypothetically, influenced by the period of time from pollen adhesion to ovule fertilisation, and the likelihood of abiotic (wind or water) as well as subsequent floral visitors dislodging and transporting pollen between and within flowers (Minnaar et al. 2019). Thus, we now require research aiming to identify the physiological processes that drive priority effects in pollination.

Effective management of mass-flowering crops to optimise fruit production requires understanding of the processes that drive the intricate interactions between pollinators and flowers. At the plant scale, flowers differ in pollen limitation, which strongly affects the quality of fruit production (REF, must be true). Thus, behavioural differences between pollinator taxa, and the degree of trait-matching between flowers and pollinators, highlights the benefit of pollinator species in crops and conserving biodiversity in agroecosystems. Although we demonstrated differential trends in species complexes and priority across these two species, overall, both species are beneficially influenced by the presence of multiple pollinator species, due to their differential behaviour and trait matching highlighting the need to conserve natural habitats and biodiversity in ensure continuity pollination services.

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**Author contributions**

LKK and RR conceived the study and designed the experiments. LKK and MH collected data. LKK, JS and VG undertook the data analyses. LK and JS wrote the manuscript and all authors contributed substantially to the final version.

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