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FLORAL LONGEVITY, NECTAR PRODUCTION, ANTHER DEHISCENCE, AND STIGMA RECEPTIVITY IN HASKAP (*LONICERA CAERULEA*)

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Abstract—Haskap (*Lonicera caerulea* L.) is a temperate fruiting shrub grown commercially in northern regions of Europe, Asia, and North America. Haskap is self-incompatible and requires insect pollinators in order to set fruit; however, very little is currently known about its floral biology or pollinator specializations, particularly in North American cultivars. Here, we examine floral longevity, nectar dynamics, the timing of anther dehiscence, and stigma receptivity in flowers of greenhouse-grown 'Tundra', a Haskap cultivar developed and grown in Saskatchewan, Canada. Anthesis lasted 83.3 ± 25.9 hours (mean ± SD) in un-pollinated flowers; pollination caused early senescence within 34.3 ± 15.2 hours after pollination. Nectar was present from the onset of anthesis, and nectar volume peaked at 9-16 hours after opening. Nectar volume was maintained throughout anthesis and was not resorbed prior to abscission of the corolla from the ovary, and nectar removed during anthesis was replenished to the original volume. The stigma showed a reaction to hydrogen peroxide while still in the bud stage, suggesting it is receptive even before the flower opens. Early stigma receptivity, nectar production, and anther dehiscence maximize opportunities to be successfully pollinated, along with high floral longevity and pollination-triggered senescence. These results suggest that Haskap flowers utilize a generalist, rather than a specialized, pollination strategy. Observations that some flowers open in the evening or were already open in the morning suggest that nocturnal pollinators such as moths may be important, in addition to known diurnal pollinators.

Keywords: Haskap, Lonicera caerulea, floral traits, nectar, pollen, anthesis

INTRODUCTION

Haskap (*Lonicera caerulea* L.: Caprifoliaceae), also known commercially as blue honeysuckle or honeyberry, is an early flowering (April-May), temperate fruiting shrub native to northern parts of Europe, Asia and North America. Its tart blue berry-like fruit may have potential health benefits (Svarcova et al. 2007), and it is growing in popularity as a commercial crop, particularly in North America. Existing research on Haskap cultivation has focused largely on the shape, taste and harvestability of this fruit. However, despite Haskap being self-incompatible (Bors 2008) and requiring insect pollinators in order to set fruit (Bozek 2012), there has been little focus on its pollination biology. Studies of Haskap cultivars grown in Poland have found that it produces abundant pollen and nectar that is favoured by honey bees (*Apis mellifera*) and bumble bees (*Bombus* spp.) (Hymenoptera: Apidae), as well as a variety of solitary bees (Bożek & Wieniarska 2006; Bożek 2007). Similar pollinator guilds appear to visit North American cultivars, and field studies have suggested that bumble bees may be one of the most important pollinators for this cold-adapted crop (Frier et al. 2016). However, there is still little known about the full diversity of floral visitors of Haskap and their relative value to pollination, including the potential for nocturnal pollinators like moths, which are known to be important for other *Lonicera* species (Miyake & Yahara 1998). Additionally, although floral structure and fruit development is rather unique in Haskap (i.e. the fruit develops from the ovaries of both flowers in the inflorescence, which are enclosed by a cupule; Fig. 1), little is known about its specific floral traits or reproductive strategies.

Nectar and pollen are the most common pollinator rewards produced by flowers, and many pollinators, especially bees, rely entirely on these resources in one or all of their life stages (Proctor 1996; Armbruster 2012). Therefore, the nutritive composition, abundance, and availability of these rewards are often indicative of the pollinator guilds associated with the flowers (Fenster & Armbruster 2004). This may be especially true of nectar, which is produced specifically to attract pollinators (unlike pollen, where the reward function is secondary), and its production appears to adapt more quickly to different pollinator guilds than other floral traits (Ackermann & Weigend 2006). Many flowers are found to have distinct patterns of nectar production, nutritive composition, and resorption rates that may be correlated to the abundance, behaviour, and physiology of their associated pollinators (Galetto & Bernardello 1993, 1996; Galetto et al. 1997; Witt et al. 1999; Nepi et al. 2001; Fenster & Armbruster 2004; Wolff et al. 2006; Agostini et al. 2011; Kulloli et al. 2011; Amorim et al. 2013). This reflects the concept of pollination syndromes, whereby a suite of phenotypic floral traits are thought to correlate to the primary functional

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FIGURE 1. A typical Haskap inflorescence. The downward facing flowers are pale yellow and each has five petals, five stamens and one stigma; the ovaries of both flowers are enclosed by the bracteoles, which have fused into a cupule. The ovaries and bracteoles develop into the Haskap 'berry', actually a multiple fruit.

pollinator guild, and contrasts with the theory that most plants are in fact generalists that are visited by a wide variety of pollinator guilds (i.e., polyphily) (Waser et al. 1996; Ollerton & Watts 2000; Freitas & Sazima 2006; Johnson & Nicolson 2008; Petanidou et al. 2008; Ollerton et al. 2009). In either case, understanding the patterns of nectar production and other intra-floral aspects during anthesis may be especially important for agriculturally significant plants, where growers must decide whether to use managed pollinators, and what species will be most effective. Currently, the nectar dynamics of Haskap are entirely unknown; information on nectar production as well as other important aspects of anthesis will help refine and optimize the pollination strategy for this crop and ultimately help growers to maximize commercial yield.

The purpose of our study was to determine floral longevity, nectar dynamics, anther dehiscence, and stigma receptivity of Haskap flowers to learn more about the plant's reproductive strategy and its associated animal pollinators. Our specific objectives were to determine: (1) how long flowers are open, and whether floral longevity is affected by pollination; (2) when nectar is produced during anthesis, and whether total nectar production is affected by nectar removal; (3) when the anthers dehisce; and (4) how early in anthesis the stigma becomes receptive.

MATERIALS AND METHODS

Study site and plants

All experiments were performed during the month of February, 2015 at the University of Saskatchewan (Saskatoon, Saskatchewan), in a glass research greenhouse with daytime temperatures held at 15°C and nighttime temperatures at 10°C. The Haskap plants were grown under full spectrum lighting between 6 AM and 11 PM, with supplementation by natural light. We used a total of 8 Haskap bushes from the cultivar 'Tundra' for our experimental treatments, and pollen from unrelated plants originating from Japanese germplasm which is compatible with 'Tundra' was used as a pollen source (hereafter referred to as the pollinizer). Each potted plant was seven years old (maintained by the breeding program at the University of Saskatchewan), and kept in cold storage for a period of winter dormancy. Upon introduction to the greenhouse, the plants required approximately 10-14 days to begin flowering. Plants were watered every 2 days. As other researchers were using this space, a commercially purchased colony of *Bombus impatiens* Cresson was introduced to the greenhouse for pollination four days before the completion of our experiments. To prevent visitation in our experiments, flowers that remained to be sampled when the bees were present were covered with pollinator exclusion bags (Delnet® Pollination™ Bags) – this included 25/95 (26.3%) of the flowers used to assess anthesis length, 30/207 (14.4%) of the flowers used to assess nectar and pollen dynamics, and only 1/62 (1.6%) of the flowers used to assess the effect of nectar removal. Visual inspection of the data did not suggest that bagged flowers differed from un-bagged flowers and the data from both were pooled in our analysis.

Length of anthesis

Four experimental treatments were used to determine the duration of flowering in an inflorescence and the effects of pollination on anthesis length: (1) anthers removed, stigma not pollinated; (2) anthers left intact, stigma not pollinated; (3) anthers left intact, stigma hand-pollinated; and (4) anthers removed, stigma hand pollinated. In each case, the treatments were applied to both flowers in the inflorescence. Each potted plant received 3 replicates of each treatment, and the treatments were assigned in a randomized order as inflorescences opened. Anthers were removed immediately after the flowers opened, before they had dehisced, and hand pollination was performed in the morning, after the inflorescences had been open for at least 24 hours. Pollen was collected from a nearby pollinizer by pinching the anthers between fingertips and then dabbed directly on the stigmas of the experimental flowers. The inflorescences were observed once in the morning (9 AM), afternoon (2 PM) and evening (7 PM), and the beginning and end of anthesis were recorded, with the end marked by separation of the corolla of at least one of the flowers from the ovaries.

The duration (h) of anthesis for each treatment was compared using a Kruskal-Wallis test, followed by multiple comparisons using the Mann-Whitney U test, with p-values adjusted using the Bonferroni correction. In addition, we

calculated the percentage of flowers (including flowers used in nectar and anther analysis) that were first observed to be open in the morning, versus the afternoon or evening. All statistical analysis was performed in R (R Core Team 2014).

Nectar, pollen, and stigma dynamics

In order to determine nectar and pollen dynamics, we measured nectar volume (pooled from both flowers) and recorded the number of dehisced anthers per inflorescence over the lifespan of the flowers. Sampling was done three times per day, at 9 AM, 2 PM, and 7 PM. Each inflorescence was only sampled once – once it had opened, it was randomly assigned a temporal period (9 AM, 2 PM, or 7 PM on the first, second, or third day of anthesis) in which to be sampled, and three flowers were assigned to each period per bush (9 periods, with 3 replicates, for a total of 27 flowers per bush). However, because flowers were observed to open throughout the day, flowers sampled at the same time of day may have been open for different lengths of time. To account for this, we analyzed the data by distributing the inflorescences into discrete 8 hour intervals (0-8hrs after the onset of anthesis, 9-16hrs, …, 65-72hrs) based on their actual length of anthesis at the time of sampling.

Nectar sampling was performed by inserting a 5µl micro-capillary tube into the bottom of the corolla to draw up the nectar, and the nectar from both flowers in the inflorescence was collected into a single tube. The total nectar volume (V_n) was calculated by measuring how much of the tube was filled, and then applying the formula:

$$V_n = \frac{5\mu l}{L_c} * L_n$$

L_c is the length of the capillary tube until the 5 µl mark, and L_n is the length of the tube filled by the nectar sample.

To test for the effect of nectar removal on nectar production, three inflorescences were randomly selected from each bush. Starting at the beginning of anthesis, we removed the nectar from each of the inflorescences at 7 PM each day after opening, and recorded the volume of nectar as above. This was repeated daily until the end of anthesis.

Nectar production and anther dehiscence were analyzed using a Kruskal-Wallis test with the sampling interval as the predictor variable and nectar volume or number of dehisced anthers as the response, followed by multiple comparisons using the Mann-Whitney U test, with the *P*-values adjusted using the Bonferroni correction. The effect of nectar removal on total nectar production was analyzed using the same method as nectar production and anther dehiscence, with sampling day as the predictor and nectar volume as the response. To compare total nectar production from multiply-sampled flowers to single-sampled flowers, we added the total amount of nectar produced by each multiply-sampled inflorescence, using only inflorescences that were sampled 3 times total, to get the average nectar produced over the lifetime of the inflorescence. This was compared to the discrete 8-hour interval from single-sampled flowers that had the highest average maximum volume of nectar (i.e. the volume of nectar produced by a flower that is not visited), using a Mann-Whitney U test.

To determine when the stigmas become receptive with respect to stage of anthesis, we removed the stigmas from 3 inflorescences at different stages of opening, beginning with inflorescences that were still tightly closed. The stigmas were removed from each flower using forceps, and the styles were clipped towards the base as far down as possible. We ensured there were no pollen grains on the stigma using a hand lens, and then held the stigma in a 3% hydrogen peroxide solution and watched for the formation of bubbles on the stigma surface, which would indicate the presence of peroxidases (Dafni et al. 2005). This was repeated at progressively more advanced stages of anthesis until the stigmas showed receptivity. As old stigmas can react with hydrogen peroxide even though they are no longer receptive (Dafni & Maues 1998), only the beginning of receptivity was identified.

RESULTS

Length of anthesis

Anthesis began at various times throughout the day; however, the majority [i.e., 60.7% (162/267)] were first observed to be open in the morning (it is possible some of them opened earlier, during the night – see discussion), versus 18.3% in the afternoon, and 21.0% in the evening. Anthesis length differed between treatments ($H = 26.9$, df = 3, $P < 0.001$; Fig. 2), with pollinated inflorescences flowering for a shorter duration [59.5 ± 16.5 h (mean ± SD)] than un-pollinated inflorescences (83.4 ± 25.9 h). There was no effect of emasculation on length of anthesis. Following hand-pollination, anthesis lasted another 34.3 ± 15.2 hours.

Nectar, pollen, and stigma dynamics

Nectar was evident as soon as the individual flowers opened and may be produced even before the onset of anthesis. Nectar production peaked after 9-16 hours of anthesis, with maximum average nectar volume reached between 33-40 hours (Kruskal-Wallis test, $X^2 = 41.2$, df = 8, $P < 0.001$; Fig. 3). Nectar volume was maintained throughout anthesis and nectar was not resorbed. We observed that the corollas of the flowers would often abscise from the ovaries with nectar still present.

Anthers began to dehisce almost immediately after flowers opened, and proceeded rapidly over the first 24 hours, with the majority of anthers dehisced after 25-32 hrs (Kruskal-Wallis test, $X^2 = 130.2$, df = 8, $P < 0.001$; Fig. 3).

Due to the 14 hour gap between the last observation of the day and the first observation the next morning, it is possible that the 8 hour bins do not account for all possible error (i.e. flowers might actually belong in the following interval if they opened between 7 PM and 1 AM). Although we believe this to be a minority, we repeated the analysis on a subset of the data where we could be confident of the time

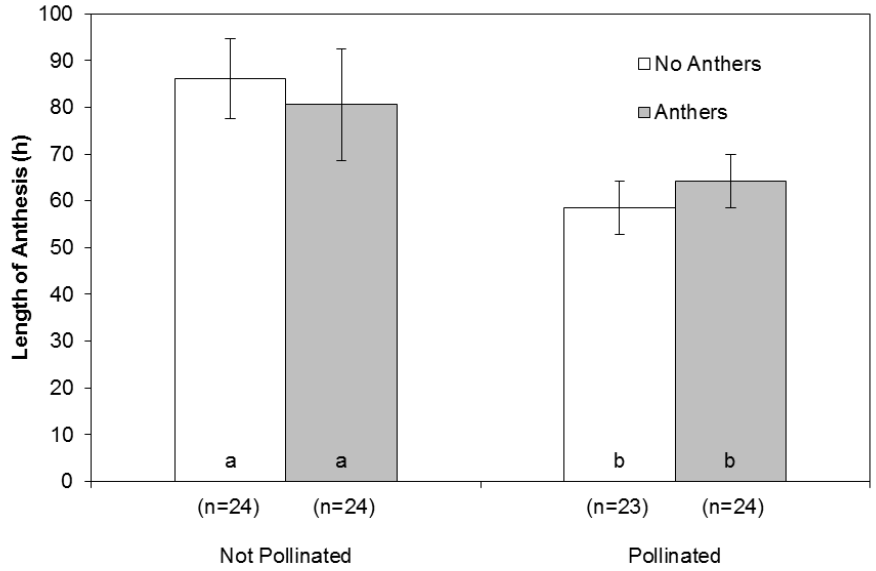

FIGURE 2. The mean length of anthesis (h), from opening of the bud to abscission of the corolla, for un-pollinated and pollinated Haskap inflorescences that had the anthers removed or left intact. Vertical lines represent the 95% confidence interval. Bars with different letters are significantly different according to pairwise comparisons using a Mann-Whitney U test with p-values adjusted for multiple comparisons using the Bonferroni correction.

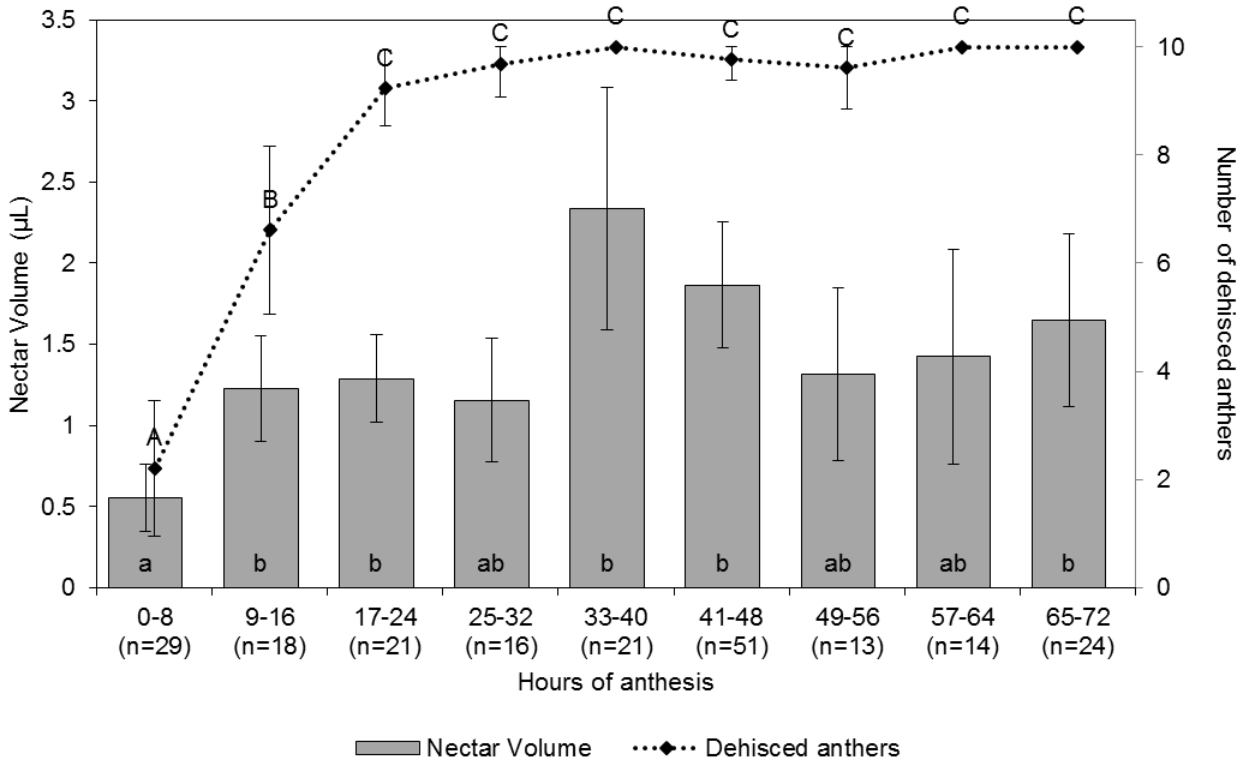

FIGURE 3. The mean amount of nectar (µL) (left Y-axis) and number of dehisced anthers (right Y-Axis) in Haskap (*Lonicera caerulea*) inflorescences per 8 hour interval after the onset of anthesis (X-axis). Vertical lines represent the 95% confidence interval. Plots with different letters are significantly different according to pairwise comparisons using a Mann-Whitney U test with p-values adjusted for multiple comparisons using the Bonferroni correction.

the flower opened – the results of this analysis show the same trend as the entire data set (results not shown).

After removal, nectar was wholly replenished throughout anthesis (Fig. 4). Maximum nectar volume recorded at the end of the second day was significantly higher than on day 1, supporting our previous finding that maximum nectar production occurs during the second day. Total nectar produced by inflorescences with their entire volume twice removed was significantly higher than single-sampled inflorescences (6.1 ± 3.8 µl compared to 2.3 ± 1.7 µl; Mann-Whitney U test, $W = 27$, $P < 0.001$).

Evidence of peroxidase activity on the stigmas was observed in flowers that were still tightly closed, suggesting that the stigma may be receptive even before the onset of anthesis.

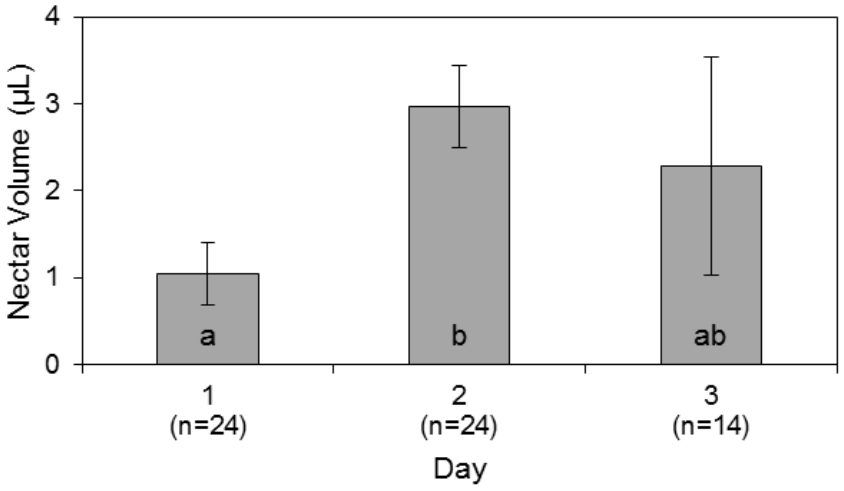

FIGURE 4. The mean amount of nectar (µL) in Haskap (*Lonicera caerulea*) inflorescences that had nectar removed once per day, up to 3 consecutive days. Vertical bars represent the 95% confidence interval. Plots with different letters are significantly different according to pairwise comparisons using a Mann-Whitney U test with p-values adjusted for multiple comparisons using the Bonferroni correction.

DISCUSSION

The timing of commencement of anthesis in Haskap is staggered; flowers open throughout the day, and likely during the night as well. The majority of flowers were first observed to be open by 9 AM; since observations were not made overnight, it is possible that many of the flowers observed at this time actually opened earlier, as anthesis appeared quite advanced in some of these flowers. Although this should be confirmed by future studies, it could indicate that nocturnal insects such as moths may be important contributors to Haskap pollination, in addition to diurnal bees and flies. This is further supported by our observation that roughly 20% of Haskap flowers also open in the evening.

The average duration of anthesis of the un-pollinated flowers is 83 hours (3.5 days; Fig. 2); however, pollination will significantly shorten this, triggering senescence of the flowers on an average of 34 hours following the pollination event. Our findings are slightly shorter than those of Bożek & Wieniarska (2006) who found that flowers of *Lonicera caerulea* var. *kamtschatica* lived 4-5 days, but they also noted that flowers excluded from pollinators were longer lived. This extended floral period may result in more opportunities for visitation by insects and increased pollination success, while senescence in response to pollination reduces the occurrence of repeat visits to flowers that are already pollinated, and may also reduce damage to the flowers, which can reduce seed set (Young 1988; Burquez & Corbet 1991). Our observation that emasculation has no effect on floral longevity in Haskap suggests it is completely self-incompatible, and self-pollination does not shorten the lifespan of the flower, nor does it result in significant stigma clogging that may prevent cross pollination.

However, although we did not quantify actual levels of self-pollination in this study, field studies have shown significant self-pollination of the stigma (Frier et al. 2016). It is possible that in greenhouse conditions very little self-pollination actually occurs, and in more realistic settings (with vigorous disruption by wind and handling by insect visitors) these factors may be more significant. This is made more likely by the fact that the anthers dehisce almost immediately following the onset of anthesis (Fig. 3), leaving little or no prior opportunity for cross-pollination. This strategy could maximize the chance that pollen is picked up by a floral visitor, but it also could increase the chance of self-pollen interfering with cross-pollination of the stigma and reducing reproductive success (Bertin & Sullivan 1988; Galen et al. 1989; Waser & Price 1991; Broyles & Wyatt 1993; Barrett 2002), especially because the anthers and stigma exist in very close proximity to one another. If this type of stigma clogging is common, this could suggest some competition or trade-off between male and female reproductive success in Haskap. It may be worthwhile to explore differences in style length among Haskap cultivars, as well as compared to wild varieties, as longer styles may be less likely to experience self-pollination.

Nectar production begins as soon as the flowers open, perhaps even slightly before, during the bud stage. We have observed bumble bees visiting Haskap flowers before they are entirely open, and our results suggest that the stigma is receptive at this stage as well. Early nectar production and stigma receptivity may have evolved to take advantage of these early visitors and increase the chance of successful pollination. Nectar production peaks between 8-16 hours of anthesis and is maintained throughout the lifetime of the inflorescence. Considerable variation in the amount of nectar is consistent with findings that variability in nectar production is correlated with large floral displays (Biernaskie & Cartar 2004), as risk-averse pollinators pay shorter visits to a single bush when nectar is variable (Biernaskie et al. 2002). As Haskap produces many flowers simultaneously and is self-incompatible, this strategy would help decrease the instance of geitonogamy and promote cross-pollination.

We found no evidence of nectar resorption, and the corolla abscised from the ovaries with the nectar load intact. However, we only analyzed nectar dynamics in un-pollinated flowers; in some flowers, reabsorption is triggered by

pollination, presumably to reuse the energy resources in berry development (Luyt & Johnson 2002). However, Burquez and Corbet (1991) suggest that if the nectary is lost when the corolla dehisces, as in *Lonicera*, reabsorption is unlikely. Additionally, the entire nectar volume can be replaced several times throughout anthesis. This may be an adaptation to nectar robbers or ineffective pollinators, increasing the possibility of repeat visits to a single flower. We have commonly observed both honey bees and bumble bees nectar robbing the flowers, and there is evidence that many legitimate pollinator visits do not deposit sufficient pollen grains for full fertilization of the ovaries (Frier et al. 2016).

The results of this study suggest that Haskap flowers are likely generalized in their pollinator attraction strategy and may be pollinated by a wide variety of insect species and functional groups. Haskap flowering occurs very early in the year when few pollinators are active, and the characteristics described here may reflect adaptations to capitalize on every opportunity to receive a successful pollination visit. The flowers, which open throughout the day (and perhaps the night as well), remain open for up to four days, but successful pollination triggers early senescence. Nectar is produced immediately upon anthesis, potentially beginning in the large bud stage. After initial nectar production the volume is held relatively constant until senescence and any nectar removed during this period is replaced. The anthers begin to dehisce immediately after the flower opens and continue over the first day. The stigma appears receptive in the bud stage, and could potentially be pollinated before the flower opens.

As a plant with polyphilic flowers, Haskap is less likely to be pollinator limited than more specialized species, and it may be effectively pollinated by a wide variety of managed and wild insects. This means that Haskap could be successfully cultivated in many habitats and geographic locations, and not be limited to distributions or commercial use of specific pollinators. As the flowers are observed to open in the evening, it is likely that nocturnal insects such as moths are important pollinators of this crop, not just diurnal bees and flies. Haskap growers should take advantage of this generalist system by developing and maintaining healthy pollinator habitat in and around Haskap orchards, as species rich pollinator populations may be essential to realizing optimum fruit yields from this crop (Frier et al. 2016).

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