Article

Pistil Biology of ‘WA 38’ Apple and Effect of Pollen Source on Pollen Tube Growth and Fruit Set

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Abstract: ‘WA 38’ (‘Enterprise’ × ‘Honeycrisp’) is an apple variety that is characterized by a peculiar self-thinning trait in which most of the fruitlets naturally shed within the first 8 weeks after bloom, leaving some clusters empty, but most with 1–2 apples. This study aimed to investigate potential causes for the relatively low fruit set observed in ‘WA 38’ by investigating its flower biology. This study comprised three objectives: (1) To characterize the effective pollination period (EPP) of ‘WA 38’ by studying stigmatic receptivity, pollen tube growth, and ovule longevity in ‘WA 38’ flowers, (2) to compare the pollen tube growth of 5 fully compatible pollinizer varieties in ‘WA 38’ pistils, and (3) to evaluate fruit and seed set resulting from controlled pollinations with 5 fully compatible pollinizer varieties. The results showed ‘WA 38’ EPP was approximately 3.2 days in 2019 and 1.4 days in 2020, and that differences in pollen sources did not attribute significant differences in fertility in ‘WA 38’ flowers. The results of this study suggest mechanisms other than pollination and fertilization, such as competition among fruitlets within a cluster or hormone signaling, may have a stronger impact on ‘WA 38’ fruitlet abscission.

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1. Introduction

‘WA 38’ is an apple (*Malus domestica* Borkh.) cultivar that Washington State University (USA) released to Washington growers in 2017 [1], and ‘WA 38’ fruit marketed as Cosmic Crisp® became available to the general public in 2019 [2]. ‘WA 38’ fruit has been lauded for its exceptional eating qualities, including a balanced flavor with a sugar/acid ratio similar to ‘Cripps Pink’ but lower than ‘Gala’ and ‘Fuji’, and firm, crisp, and juicy traits that are maintained well in cold storage [3]. Overall consumer liking of ‘WA 38’ apples from mature orchards exceeded that for ‘Honeycrisp’ apples in consumer sensory panels [4]. Commercial volume for Cosmic Crisp® apples is expected to more than double in 2021 and double again in 2022 [2], making ‘WA 38’ a prominent cultivar on the consumer market.

‘WA 38’ trees exhibit a type IV fruiting habit according to the Lespinasse classification system, which is characterized by acrotonic growth and a disposition to crop more distally from the trunk, leaving behind some unfruitful wood structures [5]. ‘WA 38’ does not require chemical thinning management due to its self-thinning trait that tends to leave only 1–2 fruitlets per cluster [1]. In one research block near Rock Island, WA, we observed that an average 38% of inflorescences did not retain fruit until harvest, and the majority of the fruitlets (83% of total fruitlets in 2020) naturally dropped within 8–9 weeks after full bloom over two years of observation (unpublished data). Previously, Celton et al. [6] investigated the reported self-thinning trait in INRA X3263 using a quantitative trait loci (QTL) approach and identified several genes, including those involved in sugar and hormone transport, secondary cell wall biosynthesis, and the development of the xylem vessel, which may be involved in early season fruitlet abscission. Iwanami et al. [7] and Ackerman and

Samach [8] reported that a low light environment can trigger a high fruitlet abscission rate, and cultivars with a small number of leaves per spur can also show high levels of early-stage fruitlet drop, probably due to the insufficient availability of nutritional resources to support fruitlet survival. The natural shedding of fruitlets is a complex mechanism that involves the interaction between nutritional and hormonal signaling in the fruit cortex, seeds, and in particular, the abscission zone [6,9,10], however, the specific mechanism controlling excessive early season fruitlet drop in ‘WA 38’ has not yet been determined.

Apples, like other deciduous tree fruit crops, are usually not self-fertile; they require viable and compatible pollen originating from a genetic source with different self-incompatibility alleles (S-alleles) to fertilize ovules, promote seed development, and achieve fruit set [11,12]. Insufficient cross-pollination by insect pollinators or low availability of compatible pollen are potential factors that could contribute to low productivity in apples. However, maternal factors could also play a role in low fruit set and retention. Williams [13] studied the effects of a nitrogen application on blossom quality and reported prolonged stigmatic receptivity and ovule viability associated with nitrogen treatment. In combination with pollen tube kinetics, Williams [13] introduced the effective pollination period (EPP) and identified the three main factors influencing it: Stigmatic receptivity, pollen tube kinetics, and ovule longevity. Since stigmatic receptivity was not a limiting factor in the study, Williams [13] reported: “The duration of the effective pollination period was determined by the longevity of the ovule minus the time necessary for pollen-tube growth”.

The duration of the EPP in tree fruit crops can range between 1 and 12 days, depending on the species, cultivar, and environment [14]. There can be significant year-to-year variations in the range of calculated EPP durations for a given species and cultivar [15]. Temperature, for example, is known to influence the processes that determine the EPP, and pollen tube growth rate increases with temperature [16,17] however, maturation and degeneration of the pistil also accelerate as temperature increases [14,18,19]. Conversely, lower temperatures prolong the viability of ovules [20] and stigmas [19], but also slow down pollen tube growth [16,20]. Thus, the interplay of the temperature affects each of the three major factors: Stigmatic receptivity, pollen tube growth, and ovule longevity, which ultimately defines the role of temperature in the EPP calculation. In addition to temperature, Sanzol and Herrero [14] outlined two more factors that can impact EPP durations: Flower quality, which includes factors like nutritional status [13], age of the tree and bearing wood [21], branch orientation [22], and chemical treatments, such as the application of plant growth regulators that affect hormone signaling [23], or those that alter nutritional status (boron, amines) [24–26]. Jahed and Hirst [27] investigated the effect of in vivo pollen tube growth from crabapple (‘Ralph Shay’ and *Malus floribunda*) and domestic apple (‘Delicious’ and ‘Golden Delicious’) pollen in ‘Honeycrisp’, ‘Fuji’, and ‘Gala’ maternal cultivars and found significant male $\times$ female interactions, suggesting that the genetic factors of either the paternal or maternal parent may also influence the duration of the effective pollination period. DeLong et al. [28] also found pollen tube growth rates differed depending on the maternal cultivar, with pollen tubes in ‘Golden Delicious’ styles exhibiting faster in vivo growth rates than those in ‘Fuji’, and found differential performances of paternal pollen sources based on in vivo pollen tube growth at a range of air temperatures. The interactions of factors impacting fertilization and fruit set complicate the ability to make broad conclusions about EPP for any given species and cultivar, which is highlighted by the year-to-year variation in EPP calculations reported in the literature [14,15]. Little is currently known about ‘WA 38’ pistil biology or how pollen source can affect the processes underpinning reproduction in this cultivar, including pollen tube kinetics, fruit set, and seed set (as a proxy for fertilization).

The objective of this study was to (1) calculate the effective pollination period (EPP) in ‘WA 38’ by determining the duration of stigmatic receptivity, ovule viability, and pollen tube growth to the ovule, and identify the limiting factor(s), (2) investigate the effect of pollen source on in vivo pollen tube growth in ‘WA 38’ styles, and (3) study the effect of pollen source on fruit set and seed set in ‘WA 38’. This study aims to shed light on potential

mechanisms that could explain instances of low fruit set observed in ‘WA 38’ and explore management approaches that could improve fruit set and retention in this self-thinning apple cultivar.

2. Materials and Methods

2.1. EPP (2019)

Forty ‘WA 38’ trees grafted on M9-Nic29 rootstock in their 7th leaf (in 2019) and trained to a V system [29,30] (7400 trees/hectare) were selected for the EPP study in Washington State University’s Sunrise Research Orchard (SRO) located near Rock Island, WA (USA). The durations of the stigmatic receptivity, pollen tube growth, and ovule longevity were evaluated over 10 consecutive days. On 24 April 2019, a total of 240 flower clusters (6 flower clusters per tree) were selected and tagged. Lateral flowers from all selected clusters were removed, and singularized apical (i.e., “king”) flowers at a pink balloon stage were emasculated and enclosed in protective sleeves (Kleenguard™ A20) to prevent uncontrolled cross-pollination. To each of the three factors investigated: Stigmatic receptivity, pollen tube growth, and ovule longevity, two flower clusters were assigned per tree, and four trees were assigned per time point for a total of 8 flowers per factor, per time point.

Pink balloon-stage flowers of ‘Granny Smith’ on ‘M.9-T337’ rootstocks were collected from a mixed-cultivar block that was planted in 2007 and trained to a spindle (spacing 0.9 m × 3 m) at SRO (Rock Island, WA, USA). Flowers were brought back to the lab at the Washington State University Tree Fruit Research and Extension Center (Wenatchee, WA, USA) and anthers were manually separated and air-dried until fully dehiscent (48–72 h) at approximately 25 °C.

2.1.1. Stigmatic Receptivity

Stigmatic receptivity was determined based on the ability of the stigma to support pollen adhesion and pollen germination. Beginning at the pink balloon stage (24 April 2019), 8 emasculated and isolated king flowers were manually pollinated with ‘Granny Smith’ pollen in 24-h intervals for up to 10 days. The time of pollination was kept consistent over the course of the 10 days (8:30 am ± 0:25). ‘Granny Smith’ pollen was collected and prepared as described above with the following exception: Pollen harvested on 24 April 2019, dried for 48 h, and stored in a refrigerator at 4 °C was used at the fifth time point (28 April 2019) due to a lapse in the availability of dried pollen with adequate germinability on that day. Pollinated flowers were harvested 24 h after pollination (beginning 25 April 2019) and immediately fixed in an ice-cold formalin aceto-alcohol solution (FAA, Ward’s Science, Rochester, NY, USA) composed of formaldehyde:ethanol:acetic acid:methyl alcohol (5.5%:46.3%:2.5%:3.5%) and stored at 4 °C. After 24 h, fixed samples were transferred to 70% ethanol and stored at 4 °C until further analysis.

Prior to microscopy, flowers were rinsed three times in distilled water, then softened in 8 M sodium hydroxide (NaOH) overnight, and transferred to a clearing solution of 5% potassium hydroxide (KOH) for 24 h. Cleared samples were then stained with a 0.1% (*w/v*) aniline blue solution (Acros Organics, Geel, Belgium) in 0.1 M dipotassium phosphate (pH 10) for 24 h. Styles stained in aniline blue were transferred to a microscope slide, and the stigmas were evaluated for pollen adhesion and pollen germination using confocal laser scanning microscopy (Leica SP-5, Leica Microsystems, Wetzlar, Germany). This analysis was performed at the Washington State University Franceschi Microscopy and Imaging Center (FMIC) in Pullman, WA. For each stigma, pollen adhesion and pollen germination were assessed on a binary scale (1 = yes and 0 = no). Logistic regression was used to determine the time point at which 50% of stigmas could support pollen germination. The number of stigmas with adhered and germinated pollen was also expressed as a percentage of total stigmas observed.

2.1.2. Pollen Tube Growth

Pollen tube growth kinetics were investigated by applying ‘Granny Smith’ pollen to ‘WA 38’ stigmas at the pink balloon stage (24 April 2019), and sampling in 24-h intervals to observe *in vivo* pollen tube growth over 10 consecutive days. Eight flowers were evaluated per time point for a total of 80 flowers in 2019. Sample collection and preparation (softening, clearing, and staining) was conducted as previously described in Section 2.1.1. Each flower was longitudinally cut in half with a scalpel and transferred to a microscope slide before being evaluated under an epi-fluorescence microscope (FM690 TC, AmScope, Irvine, CA, USA). Samples were scored on a binary scale (1 = yes and 0 = no) on the basis of whether pollen tubes were observed to reach ovules at that time point. A logistic regression was used to determine when 50% of the pistils evaluated had at least one pollen tube reaching at least one ovule.

2.1.3. Ovule Longevity

The duration of ovule longevity was determined by 24-h interval sampling of singularized, emasculated, and isolated unpollinated ‘WA 38’ flowers over 10 consecutive days (beginning on 24 April 2019 and ending on 4 May 2019), followed by viability staining. Protocols for sample fixation and storage were the same as in Section 2.1.1. Flower samples for ovule viability were rinsed in distilled water three times and softened for 6 h in 1 M NaOH. Ovules were extracted under a dissecting microscope and collected in 2-mL microcentrifuge tubes, cleared in 5% KOH for 5 days, then stained overnight (16–24 h) in 0.1% aniline blue in 0.1 M dipotassium phosphate (pH 10). Ovules were examined under an epi-fluorescence microscope (FM690 TC, AmScope, Irvine, CA, USA) outfitted with a UV filter cube (excitation bandwidth: 330–385 nm; dichroic longpass mirror: 400 nm; emission filter: 420 nm) and a 100-watt ultra-high pressure mercury lamp. Fluorescence emitted by stained ovules was an indicator of senescence, according to previous work investigating ovule fluorescence in rosaceous tree fruit species [31–33]. Ovules emitting fluorescence were considered senescent/non-viable [31–33], and each recovered ovule (ranging from 4 to 12 per flower) was scored on a binary scale (1 = yes fluorescence, 0 = no fluorescence). Logistic regression was used to determine the time point at which 50% of the ovules assessed were fluorescent (i.e., senescent or non-viable; Figure S2).

2.2. EPP (2020)

In 2020, all components of the EPP (stigmatic receptivity, pollen tube growth, and ovule longevity) were assessed on ‘Granny Smith’/‘M.9-T337’ trees that were planted in 2007 (0.9 m × 3 m = 3704 trees/ha) and top grafted with ‘WA 38’ scion in 2016. The methodology was similar to the one described above, with a few exceptions. First, the number of trees was reduced from four to three trees per time point. Second, the three components of the EPP were assessed over a period of 14 days compared to 10 days in 2019 to enable further observations. Lastly, the number of evaluated ovules per flower, to determine ovule longevity, was standardized by extracting and staining all ovules from a flower and rating only 6 ovules per flower as viable (non-fluorescent) or non-viable (fluorescent).

2.3. Paternal Effect on Pollen Tube Growth in ‘WA 38’ Styles

2.3.1. Year 1 (2019)

Eighteen ‘WA 38’ trees grafted on M9-Nic29 rootstock in their 6th leaf (in 2019) and trained to a biaxis system [30,34] (3700 trees/hectare) in SRO were selected for the *in vivo* study of pollen tube growth of paternal cultivars. The experiment was designed to monitor pollen tube growth in ‘WA 38’ styles over 6 24-h time point intervals, and each time point consisted of 3 tree replicates. In each tree, 2 flower clusters per pollen source were selected for hand pollination with an artists’ paintbrush. At pink balloon stage (24 April 2019), flower clusters were tagged and thinned to a single king flower, emasculated, and hand-pollinated with one of the following pollen sources: ‘Evereste’, ‘Granny Smith’, ‘Indian Summer’, or ‘Snowdrift’. Except for ‘Granny Smith’ (*M. domestica*), the other paternal

cultivars are crabapples (*Malus* sp.), and all pollen sources were fully compatible with ‘WA 38’ [35,36]. Balloon stage flowers were harvested from a collection planted in SRO in 2016 and trained to a spindle (spacing 1.5 m × 3 m), except ‘Granny Smith’, which was sourced from the same planting previously described in Section 2.1. Pollen used for hand cross-pollination was prepared as described in Section 2.1. On 24 April 2019, a total of 144 ‘WA 38’ flowers were pollinated with the 4 pollen sources in a time span of approximately 75 min. All pollinated flowers were immediately covered with protective sleeves (Kleenguard™ A20) and secured with twist ties, and 6 flowers were harvested at 24-h intervals for 6 consecutive days. For each time point, the sampling was completed in a 20-min period and standardized at the same time of day for all pollen sources. Twenty-four flower samples were collected at each 24-h time point post-pollination, for a total of 144 flowers over 6 days of sampling. Flowers were fixed in the field following the same protocol described in Section 2.1.1. Due to random damages that occurred in the orchard during the *in vivo* trial (e.g., wind, tractor, broken branches, etc.), the number of flowers utilized for microscopy analysis was reduced to 5 flowers/time point/pollen source. For this experiment, a razor blade was used to separate the ovary of each flower from the pistil at the base of the styles.

Pistils were softened, cleared, and stained as described in Section 2.1.1. Stained styles were transferred to microscope slides and visualized by confocal laser scanning microscopy (Leica SP-5, Leica Microsystems, Wetzlar, Germany). The length of the longest pollen tube in each style was determined by recording the xy-coordinates of the pollen grain and the tip of the pollen tube. Then, the Euclidean distance between the two points was calculated. Microscopy was performed at Washington State University’s Franceschi Microscopy and Imaging Center in Pullman, WA (USA).

2.3.2. Year 2 (2020)

The evaluation of paternal cultivars on pollen tube growth in ‘WA 38’ styles was repeated in 2020 as described in Section 2.3.1 with the following exceptions. First, whereas 18 trees were used over 6 24-h time points (3 trees per time point) in 2019, 27 trees were used in 2020 to accommodate 9 24-h time points to enable further observation of pollen tube growth in the ovary. Second, ‘Mt Blanc’ was added as a paternal cultivar due to its pollen availability in 2020, whereas in 2019 it was excluded due to insufficient bloom in our field collection. Lastly, cross-pollinations were performed exclusively on lateral flowers (the “strongest” appearing flower within a cluster at balloon stage), due to the unavailability of king flowers at the required phenological stage at the time of pollination (15 April 2020).

2.4. Paternal Effect on ‘WA 38’ Fruit Set and Seed Set

In addition to *in vivo* pollen tube growth performance in ‘WA 38’ styles described in Section 2.3, pollen from the same paternal cultivars was also used in a pollination experiment to compare the fruit set of ‘WA 38’ after pollination and at harvest.

2.4.1. Year 1 (2019)

Fifteen mature trees of ‘WA 38’ grafted on M9-Nic29 trained to a V system [29,30] (7th leaf in 2019, planted at 7407 trees/hectare) were selected for this experiment. Flowers from pollinizer trees were collected and processed as described in Section 2.3.1 to obtain dry pollen for hand pollination. On 24 April 2019, hand pollination was performed with an artists’ paintbrush on balloon stage flowers, favoring the king flowers where possible. In cases where king flowers within a cluster were unavailable due to differences in phenology, the balloon-stage lateral flowers appearing the “strongest” within the cluster were used. All flowers were chosen on spurs and thinned to a single flower per cluster. In each experimental tree, 10 flowers were pollinated with one single pollen source for a total of 120 pollinated flowers (10 flowers/pollen source × 3 trees/pollen source × 4 pollen sources = 120 flowers for fruit set). The 4 pollen sources chosen to be tested were ‘Evereste’, ‘Granny Smith’, ‘Indian Summer’, and ‘Snowdrift’. ‘Mt Blanc’ was originally selected to be tested in this study, but its bloom was insufficient at pollination, so only 4 pollen sources

were compared in 2019. Each flower was enclosed in protective sleeves (Kleenguard™ A20) for 7 days post-pollination and then the sleeves were removed from all flowers. Fruit set was assessed on flower basis on 14 May 2019, 14 June 2019, and at harvest, on 24 September 2019 (150 days after full bloom (DAFB), with 27 April 2019 as the full bloom date) and expressed as a percentage for each time point. Fruit set percentages were determined for each tree replication by calculating the number of fruit present at the time of assessment divided by the difference between the number of flowers pollinated and the number of fruit lost by mechanical damage in the orchard, then averaging the fruit set percentage (%) for the 3 trees (reps).

Hand-pollinated 'WA 38' apples were stored after harvest at 1 °C in regular air. Apples were processed for size, mass, and seed analysis approximately 6 weeks after harvest. Each fruit was weighed (g) on a digital balance (Navigator® XT Portable Balance, Ohaus®, Parsippany, NJ, USA), fruit diameters were sized by a customized loop fruit sizer (Crangston Machinery CO., Oak Grove, OR, USA), and cut equatorially to assess the number of carpels and seeds. All types of seeds were removed from carpels and classified as either mature-healthy seeds [37] or underdeveloped seeds (including non-developed and non-fertilized) and counted.

2.4.2. Year 2 (2020)

Pollination in the second year (2020) was performed on 16 April 2020. The experiment was conducted as previously described for 2019, except in 2020, all crosses were performed on the “strongest” lateral flower at a balloon stage within a cluster, and 'Mt Blanc' was added as a pollen source. The fruit set was assessed on 15 May 2020, 24 June 2020, and at the time of harvest, 17 September 2020 (152 DAFB with 18 April 2020 as the full bloom date). The harvest and seed analysis protocol were repeated as in Section 2.4.1 with the following additions. During Year 2, the distribution of the seeds inside carpels was assessed based on Sheffield [38] criteria, and all the mature-healthy seeds/fruit were weighed to obtain the average mature-healthy seed weight (mg).

2.5. Statistics

For the 2019 stigmatic receptivity data set, the percentage of stigmas with adhered pollen grains and percentage (%) of stigmas with germinated pollen grains were analyzed using the single flower as a replication (8 flowers per time point). Percentages ranging from 0 to 100% were transformed with arcsin transformation according to Gomez and Gomez [39] prior to statistical analysis, then analyzed with PROC GLM in SAS (SAS Institute Inc., Cary, NC, USA) using type III SS and a Student-Newman-Keuls (SNK) post-hoc test to discriminate means.

For the pollen tube growth measurements, each pistil was considered a replication, so 5 pistils (each pistil usually comprised 5 styles if the flower was complete) for each pollen source and time point were utilized. Pollen tube length was analyzed by pollen source, time point (1 day after pollination (DAP) to 3 or 4 DAP depending on the year), and pollen source × time point with PROC GLM in SAS (SAS Institute Inc., Cary, NC, USA) using type III SS and a Student-Newman-Keuls (SNK) post-hoc test to discriminate means. Since the interaction pollen source × time point was highly significant ($p < 0.001$), data were presented as the average pollen tube length for each day and each pollen source. Moreover, within each time point, the pollen tube length was compared across the 5 pollen sources (4 pollen sources in 2019), and for each pollen source, the length of the pollen tubes was compared from 1 DAP to 4 DAP (where available). QQplot was used to check the residuals, and with the exception of a few outliers, the results indicated acceptable normality.

EPP was calculated by subtracting the time required for pollen tubes to reach the ovules from the ovule longevity [13]. In addition, the duration of stigmatic receptivity was calculated based on adhesion and germination of 'Granny Smith' pollen grains on 'WA38' stigmas. The duration of each component was calculated using R software [40]. Logistic regressions were fitted using the base function 'glm' with a binomial error distribution.

The durations of stigmatic receptivity, pollen tube growth kinetics, and ovule longevity were defined as the period after which 50% of the stigmas lost the ability to support pollen germination, the period required for pollen tubes to reach the ovules in 50% of the samples, and the period after which 50% of the ovules showed callose fluorescence, respectively.

Fruit set percentage values per tree were transformed into Arcsin according to [39] before statistical analysis. Statistical analysis was first conducted by comparing the fruit set percentages across pollen sources within each assessment time (May, June, or September for both years) and then comparing the fruit set (%) between the three assessments within each pollen source. Fruit set means were then separated (where significance was present) with the SNK post-hoc test ($\alpha = 0.05$) within each pollen source by fruit set assessment time (May, June, and September), with different letters indicating significant differences between fruit set (%) in the different months of assessment.

3. Results

3.1. EPP

3.1.1. Stigmatic Receptivity

Regarding the duration of receptivity of 'WA 38' stigmas during the 2019 bloom, all stigmas observed between the first sampling time point (Day 1) and the third sampling time point (Day 3) had retained pollen adhesion and supported pollen germination. There was a significant decrease in the percentage of stigmas hosting germinated pollen at Day 4, but pollen adhesion did not significantly differ from Days 1–3 (Figure S1). Both pollen adhesion and germination on stigmas between Day 5 and Day 9 were not statistically different from Days 1–3 (Figure S1). Thus, the decrease in stigmatic germinability on Day 4 was attributed to the quality of the pollen used at that time point and likely unrelated to stigmatic characteristics. A dramatic loss of stigmatic receptivity between Day 9 and Day 10 was observed (Figure 1A). Whereas 88% of stigmas carried both adhered and germinated pollen on Day 9, on Day 10, only 8% of stigmas were found with adhered pollen, and no stigmas were observed with germinated pollen (Figure S1).

In 2020, all stigmas from anthesis to 5 days post-anthesis carried adhered and germinated pollen, and on Day 6, only one stigma did not carry adhered or germinated pollen (data not shown). Loss of stigmatic receptivity continued through Day 7, and on Day 8 we observed the first pistil on which none of the 5 stigmas carried germinated pollen (data not shown). By Day 11, none of the stigmas observed from any of the pistils sampled could support pollen adhesion and germination (Figure 1B).

Overall, stigmatic receptivity of 'WA 38' flowers, based on the ability to support pollen adhesion, was 9.6 days in 2019 and 2020. However, 'WA 38' stigmas lost the ability to support pollen germination after 9.2 and 8.1 days in 2019 and 2020, respectively (Figure 1).

3.1.2. Pollen Tube Growth

In 2019, at 6 days after pollination (DAP), none of the 'WA 38' pistil samples were found to have 'Granny Smith' pollen tubes in the vicinity of the ovules however, by 7 DAP all samples were found to have pollen tubes that had reached ovules (Figure S2). Logistic regression estimated 6.5 days between the time of pollination and when pollen tubes reached the ovules in 2019 (Figure 1C).

'WA 38' pistils in 2020 were more variable among time points with respect to the status of pollen tube growth to ovules. Pollen tubes reached ovules by Day 6 in one sample, but only half the samples showed pollen tubes reaching ovules by Day 7 (data not shown). Whereas pollen tubes were observed reaching ovules in all samples by Day 7 in 2019, pollen tubes were observed reaching ovules in all samples by Day 8 in 2020 (data not shown). Thus, despite the earlier instance of pollen tubes reaching ovules in 2020, the estimated pollen tube growth duration (6.8 days) was slightly longer than in 2019 (6.5 days; Figure 1).

Figure 1. Scatter plots of (A,B) stigmatic receptivity, (C,D) pollen tube growth, and (E,F) ovule senescence with fitted regression curves. (A,B) are based on the stigmatic capacity to support adhesion and germinability. (C,D) are based on the progression of pollen tube growth into the ovary and reaching the ovules. (E,F) are based on the duration of ovule viability. (A,C,E) represent 2019 data, while (B,D,F) represent 2020 data.

3.1.3. Ovule Longevity

In 2019, fluorescent ovules were first observed on Day 9 however, by Day 10 non-fluorescent ovules were still detected in the sample set. Logistic regression analysis showed an estimated ovule longevity of 9.7 days (Figure 1E). Since this duration was close to the 2019 experimental sampling window of 10 days, in 2020, the sampling was extended to 14 days to define better the timeline development of senescence in ‘WA 38’ ovules. In 2020, fluorescent ovules were first observed on Day 8 (Figure 1F), and about 53% of the ovules

observed at that time point were considered non-viable (data not shown). By Day 9, over 80% of the ovules observed were scored as non-viable, and from Day 11 to Day 14 all ovules stained and visualized were considered non-viable (Figure 1F). The logistic regression of the 2020 data showed an estimated period of ovule longevity of 8.2 days (Figure 1F), which was notably shorter than the estimated ovule longevity in 2019. Variation in fluorescence among ovules within a pistil sample was limited in both years but more prevalent in the 2020 data set.

The EPP for ‘WA 38’ was estimated at 3.2 days in 2019 and 1.4 days in 2020 based on stigmatic receptivity, pollen tube growth, and ovule longevity in the described experimental conditions.

3.2. Paternal Effect on Pollen Tube Growth in ‘WA 38’

In 2019, pollen from ‘Evereste’, ‘Indian Summer’, ‘Granny Smith’, and ‘Snowdrift’ was utilized in a hand-pollination experiment to compare pollen tube growth performances of different pollen sources in ‘WA 38’ styles. Within each year, the statistical analysis took into account the interaction pollen $\times$ day after pollination (DAP) and because the interaction was significant at $p < 0.001$ and $p < 0.05$ in 2019 and 2020, respectively, data were presented by pollen source and DAP (Table 1). At 1 DAP, the average lengths of the longest measurable pollen tube of ‘Granny Smith’ and ‘Indian Summer’ were significantly longer than either ‘Evereste’ or ‘Snowdrift’. At 2 DAP, ‘Granny Smith’ reported the longest average pollen tube length (3.74 mm) followed by ‘Evereste’ (2.79 mm), ‘Snowdrift’ (2.43 mm), and the shortest was ‘Indian Summer’ (0.46 mm; Table 1). At 3 DAP, ‘Snowdrift’ pollen tubes (6.43 mm) and ‘Granny Smith’ (5.99 mm) were the longest, while ‘Indian Summer’ pollen tubes were the shortest among the pollen sources. At 4 DAP, ‘Snowdrift’ pollen tubes had already passed the base of the style (pollen tube length > 11.2 mm, the average length of ‘WA 38’ styles). The statistical comparison at 4 DAP considered the remaining 3 pollen sources and determined ‘Evereste’ and ‘Granny Smith’ pollen tubes to be significantly longer (8.39 and 8.08 mm, respectively) than ‘Indian Summer’ pollen tubes (4.78 mm) in ‘WA 38’ styles. All four pollen sources also showed significant differences in pollen tube lengths (mm) between 1 DAP and 4 DAP ($p < 0.001$). Among the four pollen sources considered, ‘Indian Summer’ emerged as the only pollinizer whose pollen tube length did not seem to increase between 1 DAP and 2 DAP (Table 1).

The same experiment repeated in 2020 reported some different results. The difference in pollen tube lengths between pollen sources was significant only at 2 DAP in 2020, where ‘Snowdrift’ pollen tubes were the longest among the five pollen sources compared (4.47 mm), similar to what was reported at 3 DAP in 2019 (Table 1). At 2 DAP in 2020, ‘Indian Summer’ was statistically similar to ‘Snowdrift’, with a slightly lower mean (4.09 mm in ‘Indian Summer’ compared to 4.47 mm in ‘Snowdrift’; Table 1), then in descending order of pollen tube length at 2 DAP in 2020 followed ‘Granny Smith’ and ‘Mt Blanc’ (statistically similar), and the lowest was ‘Evereste’ (2.66 mm). At 3 DAP, however, no significant differences in pollen tube length were found between the five pollen sources, with average lengths ranging from 7.28 mm to 8.57 mm (Table 1). By 4 DAP, all the pollen tubes had reached the base of the ‘WA 38’ style, one day earlier than in 2019. All five pollen sources also showed significant differences in pollen tube lengths (mm) between 1 DAP and 4 DAP ($p < 0.001$), with clear statistical discrimination between each day of sampling (Table 1).

3.3. Paternal Effect on ‘WA 38’ Fruit Set and Seed Set

3.3.1. Paternal Effect on ‘WA 38’ Fruit Set

In 2019, no significant differences were found between the four pollen sources used for hand pollination of ‘WA 38’ at the three times of assessment: In May (T1 = 3 weeks after pollination), in June (T2 = 7 weeks after pollination), and in September (T3 = 20 weeks after pollination = harvest; Figure 2A). The initial range of fruit set at T1 in 2019 was 73% (‘Indian Summer’) to 93% (‘Granny Smith’ and ‘Snowdrift’), but we observed significant fruit drop in the following 4 weeks. The final fruit set at harvest ranged from 20% (‘Snowdrift’) to

37% ('Evereste') with no statistical differences between pollen source treatments. Statistical analysis within each of the four pollen sources and comparing the times of assessment revealed that the difference in fruit set between May (T1) and June (T2) was significant for all pollen sources, which was expected based on the fruit abscission common in most *M. domestica* varieties in that season ("June drop"; Figure 2A). High variability across replications was reported for 'Indian Summer' in May (T1) and 'Granny Smith' in June (T2) and September (T3). In the second year (2020), starting at T1 (4 weeks after pollination; 15 May 2020), no significant differences in fruit set across the five pollen sources emerged. Fruit set ranged between 70% ('Indian Summer') and 87% ('Granny Smith'; Figure 2B). On average, the fruit retention decreased in the following months of assessment, but no significant differences were found across the five pollen sources within each month of assessment until harvest (T3). The reduction in fruit set between May (T1) and June (T2) was only significant in 'Granny Smith' crosses ($p = 0.0239$; Figure 2B). The retention of 'WA 38' fruitlets originating from 'Granny Smith' crosses dropped from 87% in May (T1) to 43% in June (T2). High variability across replications was reported for 'Evereste' in all three time point assessments. Nearly stable fruit retention was observed between June (T2) and harvest (T3), indicating that the majority of the apples that were retained on the trees after "June drop" persisted until harvest (T3, Figure 2B).

Table 1. 'WA 38' pollen tube length measurements in 2019 and 2020: Comparison between four pollen sources in 2019 and five pollen sources in 2020 for 4 and 3 days after pollination (DAP), respectively (1 DAP = 24 h). Significance of the model reported in the legend: *** $p < 0.001$ (proc GLM in SAS 9.4). Statistical letters originated from Student-Newman-Keuls post-hoc test with alpha = 0.05. Lowercase letters discriminate pollen tube length means across pollen sources within each day (vertically), while capital letters discriminate means for each pollen source across the 4 days (significance across days, horizontally). 'WA 38' styles' average length was 11.2 mm in 2019 and 10.2 mm in 2020. The number of styles utilized for the pollen tube length measurements ranged from 17 to 25 for each day and pollen source, in 2019 and between 25 to 30 in 2020; avg = average). NS = not significant.

Pollen Sources—2019	N Pistils (Avg 5 Styles)	Pollen Tube Length (mm)								Significance Across Days ←
		1 DAP		2 DAP		3 DAP		4 DAP		
Evereste	5	0.45	bD	2.79	bC	4.80	bB	8.39	aA	***
Granny Smith	5	0.81	aD	3.74	aC	5.99	aB	8.08	aA	***
Indian Summer	5	0.70	aC	0.46	dC	1.15	cB	4.78	bA	***
Mt Blanc	-	-	-	-	-	-	-	-	-	-
Snowdrift	5	0.24	bC	2.43	cB	6.43	aA			***
Significance across pollens ↓		***		***		***		***		

Pollen Sources—2020	N Pistils (Avg 5–6 Styles)	Pollen Tube Length (mm)								Significance Across Days ←
		1 DAP		2 DAP		3 DAP		4 DAP		
Evereste	5–6	0.96	C	2.66	cB	8.33	A			***
Granny Smith	5–6	0.91	C	3.67	bB	7.58	A			***
Indian Summer	5–6	0.91	C	4.09	abB	8.57	A			***
Mt Blanc	5–6	0.83	C	3.81	bB	7.66	A			***
Snowdrift	5–6	0.92	C	4.47	aB	7.96	A			***
Significance across pollens ↓		NS		***		NS				

3.3.2. Paternal Effect on 'WA 38' Seeds Set

'WA 38' apples that were retained on trees from experimental crosses (Section 3.3.1) were harvested in September and refrigerated in regular air (RA) storage at 1 °C until analysis. Fruit diameter and mass were measured and recorded, and seeds were harvested, categorized, and weighed. The average fruit diameter ranged from 77.3 mm ('WA 38' × 'Granny Smith') to 81.2 mm ('WA 38' × 'Snowdrift') in 2019, and from 79.4 mm ('WA 38' × 'Granny Smith') to 85.4 mm ('WA 38' × 'Snowdrift') in 2020. Still none of the differences in fruit diameter by paternal cultivar were statistically significant (Table 2). Average fruit mass in 2019, ranged from 206 g ('WA 38' × 'Granny Smith') to 244 g

(‘WA 38’ × ‘Snowdrift’), and in 2020, ranged from 230 g (‘WA 38’ × ‘Granny Smith’) to 287 g (‘WA 38’ × ‘Snowdrift’), but no statistical differences in fruit mass by paternal cultivar were found in either year (Table 2). Harvested seeds were categorized by appearance as either mature-healthy or aborted/underdeveloped on the basis of size, color, and morphology [37]. The average number of healthy-mature seeds per fruit ranged from 8.88 (‘WA 38’ × ‘Indian Summer’) to 10.33 (‘WA 38’ × ‘Granny Smith’) in 2019, and from 8.73 (‘WA 38’ × ‘Mt Blanc’) to 9.56 (‘WA 38’ × ‘Snowdrift’) in 2020. Underdeveloped seeds were less frequent among all groups in both years, with average underdeveloped seeds per fruit ranging from 0.78 (‘WA 38’ × ‘Granny Smith’) to 1.75 (‘WA 38’ × ‘Indian Summer’) in 2019, and from 0.46 (‘WA 38’ × ‘Granny Smith’) to 1.27 (‘WA 38’ × ‘Mt Blanc’) in 2020. The average number of total seeds per fruit (including both healthy/mature and aborted or underdeveloped types) ranged from 10.45 (‘WA 38’ × ‘Evereste’) to 11.11 (‘WA 38’ × ‘Granny Smith’) in 2019 and from 9.92 (‘WA 38’ × ‘Granny Smith’) to 10.06 (‘WA 38’ × ‘Evereste’ and ‘WA 38’ × ‘Snowdrift’) in 2020. No statistical differences in the average number of healthy/mature seeds per fruit, the average number of underdeveloped seeds/fruit, or the average total number of seeds per fruit were identified among any pollen source treatments in either year.

Figure 2. Cont.

Figure 2. ‘WA 38’ fruit set (%) by pollen source in May (T1), June (T2), and September (T3) in (A) 2019 and (B) 2020. Ten flowers on each of three trees per pollen source were pollinated by hand, and the fruit set is presented here as the average of three trees ($n = 3$). Error bars represent the standard error of the mean (SE). Statistical significance for the comparison of fruit set (%) across pollen sources within each time of assessment is reported in the legend, and significance for the comparison of fruit set (%) across time point assessments within each pollen source is indicated above the braces ($* = p < 0.05$, $*** = p < 0.001$ NS = not significant). When the model showed significance, means were then separated with the SNK post-hoc test within each pollen source by fruit set assessment (T1, T2, and T3), where different letters indicate significant differences between fruit set (%) at the different time points of assessment. In 2019: T1 = 14 May 2019, T2 = 14 June 2019, and T3 = 24 September 2019; in 2020: T1 = 15 May 2020, T2 = 24 June 2020, and T3 = 17 September 2020.

Table 2. ‘WA 38’ apple size (mm), weight (g), and seeds analysis (healthy-mature, underdeveloped, and total seeds) in 2019 and 2020: Comparison between four pollen sources in 2019 and five pollen sources in 2020. Significance (across pollens) of the model is reported in the legend: NS = not significant (proc GLM in SAS 9.4).

Pollen Sources—2019	<i>n</i> Apples	Avg Apple Diameter (mm)	Avg Apple Weight (g)	Avg Number of Healthy-Mature Seeds/Apple	Avg Number of under-Developed Seeds/Apple	Avg Number of Total Seeds/Apple
Evereste	11	77.8	211	9.64	0.82	10.45
Granny Smith	9	77.3	206	10.33	0.78	11.11
Indian Summer	8	80.8	241	8.88	1.75	10.63
Mt Blanc	—	—	—	—	—	—
Snowdrift	6	81.2	244	9.67	1.17	10.83

Table 2. Cont.

Pollen Sources—2019	<i>n</i> Apples	Avg Apple Diameter (mm)	Avg Apple Weight (g)	Avg Number of Healthy-Mature Seeds/Apple	Avg Number of under-Developed Seeds/Apple	Avg Number of Total Seeds/Apple
Significance across pollens ↓		NS	NS	NS	NS	NS
Pollen Sources—2020	<i>n</i> Apples	Avg Apple Diameter (mm)	Avg Apple Weight (g)	Avg Number of Healthy-Mature Seeds/Apple	Avg Number of under-Developed Seeds/Apple	Avg Number of Total Seeds/Apple
Evereste	16	80.5	252	9.13	0.94	10.06
Granny Smith	13	79.4	230	9.46	0.46	9.92
Indian Summer	14	83.1	277	9.27	0.67	9.93
Mt Blanc	15	83.1	275	8.73	1.27	10.00
Snowdrift	18	85.4	287	9.56	0.50	10.06
Significance across pollens ↓		NS	NS	NS	NS	NS

4. Discussion

4.1. Effective Pollination Period of ‘WA 38’

The ability to support pollen adhesion and germination on the stigmatic surface is critical to successful reproduction and is the first post-pollination factor that must be addressed in the EPP model. Our investigation of ‘WA 38’ stigmatic performance revealed a receptivity duration of about 9.5 days from anthesis to loss of pollen adhesion in both the 2019 and 2020 experiments (data not shown). The capacity for adhered pollen to germinate on the ‘WA 38’ stigmatic surface was also studied, and we found the duration for which stigmas could support pollen germination was shorter. Using a logistic regression approach with a 50% threshold (Figure 1), we found stigmatic germination persisted until approximately 9.2 days after anthesis in 2019. Since pollen adhesion and germination were sustained on ‘WA 38’ stigmas for over 9 days in 2019, we extended the sampling protocol in 2020 up to 14 days to account for potential year-to-year variation in stigmatic performance that might extend stigmatic receptivity. However, the duration of time in which stigmas could support pollen germination was shorter in 2020 (8.1 days, Figure 1). The difference in stigmatic pollen germinability between the 2019 and 2020 experiments was approximately 1 day, suggesting additional factors, such as pollen quality or nutritional differences possibly impacting stigmatic pollen germinability. Previous authors [41] have investigated the complex role of stigmatic exudates in supporting pollen adhesion and germination, suggesting multiple factors are involved with stigmatic receptivity. Losada and Herrero [42] compared king and lateral flowers within an apple inflorescence and found distinct differences in stigmatic receptivity. Whereas king flowers were highly receptive at anthesis and the couple days following, pollen germination on the stigmatic surface was higher in lateral flowers by the third day of pollination [42], which indicates there may be a biological hedging of reproductive strategies among flowers within an inflorescence. In our investigation of the ‘WA 38’ effective pollination period, we narrowed our focus to king flowers because of their competitive advantage over lateral flowers [43] that often result in larger fruit [44], which may be more desirable to consumers [11]. However, in some countries, “moderate”-sized apples are preferred [45], so it may be important to investigate the biology of lateral flowers in future studies. In our 2019 and 2020 experiments, stigmatic receptivity was not the limiting factor in the ‘WA 38’ EPP calculation. This finding is consistent with other studies on apple that identified the interactions of pollen tube growth rates and ovule degeneration as limiting factors in the EPP [13,32,33].

In vivo pollen tube growth was studied in ‘WA 38’ pistils to estimate the time required for pollen tubes to reach ovules. Previous research reported paternal differences in stylar pollen tube growth rates [28,46] that could affect the EPP model for any given maternal cultivar. Since it was not feasible to replicate the EPP experiment with a broad range of paternal cultivars, we standardized both 2019 and 2020 experiments with ‘Granny Smith’

pollen. ‘Granny Smith’ is a *M. domestica* cultivar with a reported self-incompatibility genotype of S_3S_{23} [47], which renders it fully compatible with ‘WA 38’ (S_5S_{24}) [36] and a suitable pollen source for this experiment. In 2019, ‘Granny Smith’ pollen tubes reached ‘WA 38’ ovules in all samples 7 days after pollination (DAP), and logistic regression estimated pollen tubes reached ovules in 50% of the samples 6.5 DAP (Figure 1). In 2020, the time required for pollen tubes to reach ovules was slightly longer (6.8 days; Figure 1). Our results were similar to previous EPP studies in apple [13,15,33] that determined a range of approximately 5–7 days required for pollen tubes to reach the ovules.

Ovule longevity was estimated by the relative fluorescence signal detected in aniline blue-stained ovules extracted from ‘WA 38’ flowers sampled over a period of 10 days post-anthesis in 2019 and 14 days post-anthesis in 2020. Highly fluorescent ovules were indicative of callose accumulation, which is associated with the onset of senescence [48]. This approach for estimating the duration of ovule viability was previously used in sweet and sour cherry [31,48,49], pear [25], and apple [32,33]. Zhang et al. [31] categorized sweet cherry ovule maturation and degeneration by the percentage of fluorescence coverage however, in the present study, we did not observe notable localization of fluorescence in our observations of ‘WA 38’ apple ovules. Instead, fluorescence developed relatively homogeneously throughout the ovule, and differences between fluorescent and non-fluorescent ovules were marked by differences in fluorescence signal intensity more than the percentage coverage of the ovule surface area. Thus, data presented in this paper were collected in a binary manner on the basis of judging fluorescence or non-fluorescence. This approach is useful because it enables a logistic regression analysis that can be used to estimate time points at a chosen percentage threshold. However, the primary limitation of this approach is that subtle changes in fluorescence cannot be accounted for in data collection. In 2019, ovule longevity lasted 9.7 days based on the logistic regression results, while in 2020, it lasted 8.2 days (Figure 1).

The present results on ovule longevity of ‘WA 38’ unpollinated king flowers were similar to those previously reported for different cultivars like ‘Golden Delicious’, ‘Olsentwo Gala’, and ‘Rubinstar’ in 2019, with ovule longevity equal to or greater than 9.3 days [33]. On the contrary, Prieto et al. [32] found variability in the decline of ovule viability within the first 96 h after pollination over two seasons in ‘RedChief’ and ‘Golden Delicious’ cultivars. However, Prieto et al. [32] utilized pollinated flowers to assess ovule longevity and performed their experiment under different environmental conditions than the current study or the study published by Roeder et al. [33], which limits our ability to make direct comparisons between them. It has been previously reported in the literature that environmental conditions have a key impact on the rate of ovule degeneration [14], and more specifically, cooler temperatures at bloom can prolong ovule longevity [31]. In the present study, we plotted the cumulative growing degree hours (GDH; Figure S3) based on days after treatment, with a baseline of 41 °F from the SRO AgWeatherNet weather station. This graph shows that between 3 days after treatment and 4 days after treatment from the beginning of the experiments, an inversion of GDH accumulation between 2019 and 2020 was registered. After day 4, more GDH were accumulated in 2020 with respect to 2019, which could have played a role in the shorter ‘WA 38’ ovule longevity observed in 2020.

Based on the duration of the 3 factors (stigmatic receptivity, pollen tube growth, and ovule longevity) contributing to the reproductive process, EPP for ‘WA 38’ was calculated to be 3.2 days in 2019 and 1.4 days in 2020. For ‘WA 38’, the limiting factor in the EPP is not the stigmatic receptivity (at least 8 days) but instead the interaction between ovule longevity and pollen tube growth. Another aspect worth mentioning as a contributor to the EPP and inconsistent fruit set is the quality of the flowers. Flower quality can vary within a tree, and this variability can be influenced by factors resulting from orchard management practices, such as branch orientation and bearing wood selection, canopy architecture, light microclimate, and nutrition [13,14,22]. Williams [13,50] reported that summer N applications could improve the apple fruit set (cv. Worcester Pearmain) in the following season by supplementing nutritional demands to developing flower buds.

Williams [13] reported the ovule longevity of “*n* treated” flowers was approximately 12–13 days [13]. The author also reported an EPP of 2 days for “normal” flowers versus 6 days for “strong flowers” (originated from “plump buds” with early budbreak and fast leaf growth). Rodrigo et al. [51] also investigated the role of pre-stored starch in apricot flowers as a supporting resource for the blossoms at anthesis and then decreasing with the completion of flower development and ovary senescence; highlighting variability between starch content in flowers at anthesis as an additional aspect potentially contributing to EPP variability. However, the role of starch in flowers as it relates to EPP has not yet been demonstrated in the *Malus* species, although it is known that the pollen tube is partially supported by nutrients and cell wall materials in the transmitting tissue of the pistil as it extends to the ovules [52,53]. Moreover, in the *Prunus* species like apricot, flowers at anthesis are not supported by photoassimilates generated by the emerging leaves in the mixed flower buds as in apple [51]. Apple flowers, indeed, are less dependent upon reserves once petals show color; at this phenological stage, leaves become the main source of carbohydrates supporting flowers and fruit growth [54,55].

4.2. Paternal Effect on Pollen Tube Growth in ‘WA 38’ Styles

In many modern planting systems, flowering crabapples are used as pollinizers (trees with the dedicated purpose of supplying compatible pollen to the crop cultivar), and regional availability, overlapping bloom period, and cross-compatibility with the cropping cultivar are the driving factors for pollinizer selection. With respect to apple production in the Pacific Northwest region of the USA, some of the most common crabapple varieties propagated and sold as pollinizers include ‘Manchurian’, ‘Snowdrift’, ‘Indian Summer’, ‘Mt Blanc’, and ‘Evereste’. ‘Manchurian’ (S_5S_{39b} ; [35]) also shares the S_5 allele with ‘WA 38’ (S_5S_{24} ; [36]), and this semi-compatibility indicates half of the pollen produced by ‘Manchurian’ flowers is unable to fertilize ‘WA 38’ ovules, making it potentially less desirable than other fully compatible pollinizers [56,57].

For the EPP experiment, ‘Granny Smith’ pollen was used for determining stigmatic receptivity and pollen tube growth rates in ‘WA 38’ styles, but because pollen germination and tube growth rates were reported to vary in vivo depending on the paternal cultivar [28,46], we further investigated the effect of pollen source on growth rates in ‘WA 38’ styles. In 2019, ‘Snowdrift’ pollen tubes were significantly longer than ‘Evereste’, ‘Granny Smith’, or ‘Indian Summer’ at 3 DAP, and by 4 DAP ‘Snowdrift’ pollen tubes had passed the base of the style (11.2 mm average style length in 2019), while the other pollen sources passed the base of the style at 5 DAP. Our results in 2019 were in contrast to the pollen tube growth data reported by DeLong et al. [28], who found that ‘Snowdrift’ pollen tube growth was generally shorter in ‘Cripps Pink’, ‘Fuji’, and ‘Golden Delicious’ styles than other pollen sources, including ‘Evereste’ and ‘Indian Summer’. However, our results in 2020 suggested pollen tubes from all paternal cultivars evaluated were not statistically different at 3 DAP and by 4 DAP, pollen tubes from all paternal sources had passed the base of the style (10.2 mm average style length in 2020). The present results indicated variability in pollen tube performances between paternal cultivars, primarily in 2019 (Table 1), which agrees with the observation of the differential performance of pollen sources reported by previous authors [28,46], but it also highlights year-to-year variability whereas in 2020, less time on average was required to reach the base of the style with respect to 2019, regardless of the paternal cultivar. Although the average style length was shorter in 2020 (10.2 mm compared to 11.2 mm in 2019), length measurements of pollen tubes 4 DAP in ‘Evereste’ and ‘Granny Smith’ in 2019 were similar to pollen tube lengths measured at 3 DAP in 2020 (Table 1). ‘Indian Summer’ pollen tubes grew at a significantly slower rate than the other pollen sources evaluated in 2019, measuring only 4.78 mm 4 DAP, whereas in 2020, ‘Indian Summer’ pollen tubes measured 8.57 mm and were not statistically different from any of the pollen sources assessed. Some of this observed variation between years may be attributed to environmental and climatic conditions as reported in the literature [14,58], or differences in flower type used for this experiment in 2019 (king) and 2020 (“strongest”

lateral). Other factors that may contribute to the variability of these results include the nutritional status of both the maternal and paternal trees, scion-rootstock combination, tree age, and variability of the carbohydrate balance of sources and sinks among trees, but these factors were not investigated in this study. Moreover, it is worth noting that the rate of pollen tube growth is not continuous inside the pistil and can vary in the different portions from the stigma to the ovules, impacting the time of fertilization [52]. Furthermore, maternal factors influencing pollen tube growth inside the pistil are not yet fully understood [53]. Several mechanisms at the cellular and molecular levels are crucial to achieving adequate fertilization, so although maternal and paternal contributions can be kept constant under experimental conditions for two consecutive seasons, numerous other factors related to their interaction can influence the final results.

4.3. Paternal Effect on ‘WA 38’ Fruit Set and Seed Set

Our study sought to better understand the potential differential effect of paternal selection on ‘WA 38’ fertility by investigating fruit and seed set in response to manual hand pollination with a range of compatible pollen sources. Despite identifying differences between paternal cultivars in pollen tube growth progression in ‘WA 38’ styles (Table 1), fruit set at each time point did not statistically differ between pollen sources in 2019 (Figure 2A) or in 2020 (Figure 2B), which suggests that pollinizer selection is unlikely to be the cause of the self-thinning trait of ‘WA 38’. Despite the lack of significant differences in fruit set between pollen sources in either year, initial fruit set (in May, T1 in Figure 2) in 2019 was generally higher than in 2020 (average of 85% vs. 78% respectively) however, by harvest, overall fruit set was higher in 2020 compared to 2019 (average of 52% vs. 29% respectively, Figure 2). These apparent differences in fruit set and retention between years may be explained by differences in reproductive performance between king (2019) and lateral (2020) flowers, or other biological or environmental variables that were not accounted for between years. Under our experimental conditions, we selected paternal cultivars and manually pollinated ‘WA 38’ flowers accordingly, which allowed us to orchestrate fully-compatible crosses and eliminate partial or full cross-incompatibility as a factor contributing to poor fruit set. However, a critical aspect of the experiment was that we were unable to control the number of pollen grains deposited on each stigma. As few as 10 viable and compatible pollen grains are required to produce an apple with a full seed set [59], but our approach in the field was to prioritize the certainty of pollination over the mimicking of pollinator pollen transfer, which likely resulted in higher pollen deposition on the stigmatic surface in our experiment than what would typically occur under natural pollination conditions. Previous research has demonstrated the effects of excessive pollen deposit on the stigma. Schneider et al. [57] found that manual pollination in excess can overcome a low fruit set associated with semi-compatibility in apple, and higher pollen densities on stigmas were associated with higher fruit set, seed set, and fewer misshapen fruit in comparison to lower pollen density treatment in *Pyrus pyrifolia* [60]. Competition between pollen tubes has also been shown to lower the likelihood of seed abortion [61]. It is, therefore, possible that the saturation of pollen on the stigmatic surface could compensate for any potential shortcomings in pollen performance inherent to the paternal cultivar in our current study. The pollen sources utilized in this trial did not show significant differences in resulting fruit set, fruit size, shape, or seed set in ‘WA 38’. In general, ‘WA 38’ shows an abundant number of fully developed healthy/mature seeds per apple, with a maximum of 14 seeds observed. Microscopy of transverse sections of ‘WA 38’ pistils revealed a “perfectly syncarpic” gynoeceium, displaying a compitum that allows for a uniform distribution of pollen tube growth to the ovary, even under poor pollination conditions when all stigmas may not receive pollen [62]. ‘WA 38’ apples did not show any trends of misshapeness in relation to a particular pollen source and, in general, fruit reported a high number of healthy-mature seeds. Similar results on fruit size and the effect of pollen source on the developed fruit were reported by Jahed and Hirst [27] for ‘Honeycrisp’, ‘Fuji’, and ‘Gala’ with three different pollen sources (crabapples ‘Ralph Shay’ or *Malus floribunda*, ‘Red

Delicious', and 'Golden Delicious'). The same study reported a significant difference in the number of fully-developed seeds inside each maternal variety based on the pollen source, an effect the authors attributed to possible semi-compatibility however, the S-genotypes of the cultivars involved were not reported in that study.

5. Conclusions

This study investigated factors controlling the effective pollination period (EPP) in 'WA 38', a self-thinning apple cultivar, and found notable year-to-year variation in duration. We characterized the EPP in 'WA 38' king flowers as 3.2 days in 2019 and 1.4 days in 2020. These results are consistent with previous EPP studies in tree fruit crops. We further investigated the paternal role on pollen tube growth rates in 'WA 38' styles and on fruit set and seed set in the field and found pollen sources performed similarly within a year, but results between years were somewhat inconsistent. Although a shorter EPP may contribute to a lower fruit set, the calculated EPPs in this study were based only on the performance of king flowers, which have a temporal advantage over lateral flowers. Given the self-thinning tendencies of 'WA 38' and the potential for preferentially setting lateral flowers under some disadvantageous conditions at the time of king anthesis, the performance of 'WA 38' lateral flowers may be worthy of future investigation. The cymose inflorescence in apple allows for a longer period of bloom for the apple tree as a whole than what may be inferred by the reported EPP. It is likely that differences in calculated EPP and pollen tube growth rates between 2019 and 2020 are more strongly influenced by variations in environmental factors than biological factors. Future research would benefit by exploring the impact of nutrition on EPP, a deeper comparison of king and lateral flower biology, and a survey of EPP in other apple cultivars in the Pacific Northwest as a comparison to the self-thinning 'WA 38' cultivar investigated in the present study.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/agronomy12010123/s1>, Figure S1: Stigmatic receptivity of 'WA 38' flowers ($n = 8$) in 2019. Figure S2: Microscopy approaches to determine the effective pollination period (EPP) in 'WA 38' flowers. Figure S3: Line chart showing the trend of cumulative growing degree hours (GDH) as a function of days after treatment in both seasons (2019 and 2020) presented in this study.

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