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REGULAR PAPER

Title:

Flower strategy and stigma performance in the apple inflorescence

***Authors**:*

1. Juan M Losada$_1$. Aula Dei Experimental Station – CSIC.

2. *María Herrero. Pomology Department. Aula Dei Experimental Station – CSIC. Avda Montañana 1005. 50059. Zaragoza. Spain.

e-mail: mherrero@eead.csic.es

Telephone: 00 34 976716125

Fax: 00 34 976716145

* ***Corresponding author*:** Maria Herrero

$_1$**Present address**: Arnold Arboretum of Harvard University.1300 Centre Street, Boston MA. 02130. USA.

E-mail: juanlosada@fas.harvard.edu

Fax: 00 1 617.384.6531

HIGHLIGHTS

- Stigmatic receptivity is different in king and lateral flowers of the apple corymb
- King flowers show an intense and short stigmatic receptivity.
- Lateral flowers receive less pollen grains, but have a longer stigmatic receptivity
- This different performance may have different advantage in different scenarios.
- This provides a strategy to deal with environmental uncertainty assuring fruit set

Abstract

Flower gathering in inflorescences promote pollinator activity and assures seed and fruit set within the inflorescence. However, in this flower social behaviour, the possible contribution of each single flower gets diluted and has been overlooked. In this work we explore stigma receptivity in the different flower types of the apple corymb, an inflorescence with clear flower positions a central or king flower and four lateral flowers, where subsequent fruit set can be followed by the position along the flower axis. Flowers were receptive in turns, first in the king flower and thereafter in lateral flowers, prolonging in this way the whole inflorescence receptivity. But a closer look at pollen performance showed that king flowers had an intense but short stigmatic receptivity, whereas lateral flowers had a more discrete but much longer stigmatic receptivity. These divergences contribute to different strategies within a single inflorescence with different advantages under different scenarios. The king flower will have an advantage under good pollination conditions, whereas lateral flowers will have a better chance under poor pollination conditions. But in any circumstance these two stigma performances provide a strategy to deal with environmental uncertainty, ensuring a minimum of fruit production per inflorescence.

Keywords: apple, *Malus*, inflorescence, stigma receptivity, pollen germination.

1. Introduction

Grouping flowers in inflorescences enhances evolutionary angiosperm fitness, favouring a higher floral display for pollinator attraction (Jordan and Otto, 2012), and the evolution of inflorescence architectures (Prusinkiewicz et al., 2007; Prenner et al., 2009) may have played a clear part as modifier of pollinator behaviour and hence pollen movement among flowers. In natural conditions, pollen limitation has been shown to regulate seed and fruit set (Ashman et al., 2004), and encourages female success of individual flowers within inflorescences (Zhang et al., 2012).

But all flowers of the inflorescence do not set a fruit, and some flowers have more reproductive success than others (Wyatt, 1982; Webberling, 1992). Indeed some flowers just behave as males (Diggle, 1995; Torices and Méndez, 2011) and the contribution of each flower inside the cluster to either male or female function depends on internal factors as architectural constrains and resources allocation between flowers (Diggle, 1995; 1997; Torices and Méndez, 2010; Cao et al., 2011; Zeng et al., 2009). All this converts in a flower social behaviour within the inflorescence, where each flower contributes to the whole inflorescence success. But the individual contribution of each flower has been overlooked. In this context differences in receptivity between flowers may play an important part.

While no much attention has been focused on the influence of flower longevity, it could be an important drive in mating system evolution (Weber and Godwillie, 2012). Short receptive periods have been suggested under selection as a way to improve male genotype success (Castro et al., 2008). But also a delay in stigma receptivity will provide opportunities for gathering pollen landing and thus favouring pollen competition

(Hormaza and Herrero, 1992; 1994; Herrero and Hormaza, 1996). This has been related to the female control of pollination (Lankinen and Kiboi, 2007; Lankinen and Madjidian, 2011), suggesting that stigma longevity ultimately determines pollination opportunities, and consequently the possibility of fertilisation.

Stigmatic receptivity duration varies from few hours to days, depending on the species (Heslop-Harrison, 2000), and has a crucial relevance in economical important crops such as fruit trees (Sanzol and Herrero, 2001) because it conditions the effective pollination period (Williams, 1966). Due to the implications on the subsequent fruit set, the duration of stigmatic receptivity has been evaluated in several fruit tree species such as kiwifruit (González et al., 1995a,b), apricot (Egea and Burgos, 1992), pear (Sanzol et al., 2003), or almond (Yi et al., 2006), showing big fluctuation in this trait. In fact the duration of stigma receptivity may vary from year to year, between cultivars of the same species (Ortega et al., 2004), or even within a same genotype (Sanzol et al., 2003, Castro et al., 2008). Indeed, variability exists between flowers of the same cultivar at constant temperatures in peach (Hedhly et al., 2005), suggesting that some flowers are more receptive than others. This variability also occurs between the different pistils of a flower in pear trees, and the stigmas become receptive and loose receptivity sequentially, extending the receptive period in a single flower (Sanzol et al., 2003). It has further been reported that environmental factors, as temperature and humidity (Hedhly et al., 2003; 2005; 2009; Lora et al., 2011), also affect the duration of stigmatic receptivity. In sum, the chance for receptivity appears to vary within flowers of a plant and is also modified by the environment.

The relevance of stigmatic receptivity on the subsequent fruit set sometimes is not easy to follow since evaluation of stigmatic receptivity implies a destructive method. Still the apple corymb is an excellent model system to evaluate this performance, since it has just five flowers -a number that can be easily followed- and the position of the flower and the subsequent fruit in the short inflorescence axis can be tracked. The apical flower -king flower- opens first, while lateral flowers open almost synchronically one to three days after the king flower (Pratt, 1988; Hancock et al., 2008). However, only a small proportion of flowers within the corymb set a fruit (Williams, 1966), suggesting distinct individual contributions of flowers during the reproductive phase in this species. With this perspective, studies on apple fruit abscission elucidated an apical dominance controlled by hormones (Dal Cin et al., 2005; 2009) as well as the genetic control of abscission (Bottom et al., 2010). But, before fruit set, the reproductive implication of the different flowers in the corymb to the reproductive outcome has been overlooked.

The aim of this work is to evaluate stigma performance in both king and lateral flowers within the apple corymb, and the subsequent implications in fruit set, to elucidate the possible contribution of each kind of flower to the general inflorescence strategy.

2. Materials and methods

2.1. Plant material

Apple trees (*Malus* x *domestica*, Borkh) cv Golden Delcious Spur were grown in an orchard located in the Aragón region on the North-East of Spain. The compatible cv Royal Gala was used as the pollen source. Before flower opening, at advanced balloon stage, 42 king and 42 lateral flowers were depetaled and emasculated leaving a 5mm length pedicel.

115 The flowers were placed in humid florist foam at room temperature of about 20ºC, and left
116 for 24 hours.
117 In the field, fifty king and fifty lateral flowers were selected at balloon stage to observe
118 their development. Each day, five king and five lateral flowers were weighed for six days
119 after anthesis. Field photographs were taken with an Olympus μ760 camera.
120
121 *2.2 Pollination procedures*
122 Since the cv Golden Delicious is self incompatible, pollen was obtained from flowers from
123 the compatible cv Royal Gala. Flower buds were picked at balloon stage, just prior to
124 flower opening. The anthers were removed and left on paper at room temperature of 22ºC
125 for 24-48 hours until dehisced. Then pollen was sieved using a 0.26 μm diameter mesh
126 and conserved at -20ºC until used.
127 Batches of six different Golden flowers - 30 stigmas - were hand pollinated with a paint
128 brush each day. One day after pollination, each batch of pistils was fixed in FAA -
129 formalin: acetic acid: 70% ethanol - (1:1:18) (Johansen, 1940) for at least 24 hours, and
130 then transferred to 70% ethanol.
131
132 *2.3. Microscopic preparations*
133 Stigmatic receptivity was evaluated through the ability of pollen grains to adhere, and
134 germinate on the stigma surface. With this aim, gynoecia were washed three times in
135 distilled water, for one hour each time, and then they were left in 5% sodium sulphite
136 overnight. The next day gynoecia were autoclaved for 10 min at 1kg cm^{-2} in 5% sodium
137 sulphite (Jefferies and Belcher, 1974), and finally individual styles were dissected and

138 squashed onto glass slides with 0,1% aniline blue in 0,1 N K_3PO_4 (Currier, 1957;
139 Linskens and Esser, 1957) to visualize callose and pollen tubes. Slides were observed
140 under an epifluorescent LEICA DM2500 microscope with a filter 340/425 nm.
141 Fluorescence photographs were taken with a CANON Power Shot S50 camera linked to
142 the CANON-Remote Capture software.
143 Stigmatic area of 30 styles from each flower type at anthesis was measured with the Leica
144 Application Suite software.
145
146 *2.4. Fruit set measurements*
147 To evaluate the final fruit set of king or lateral flowers in field conditions, 100 corymbs
148 were selected after June drop in branches oriented to all directions, and then the position
149 of the fruit in the corymb was recorded.
150
151 *2.5. Statistical analysis*
152 Statistical analyses were performed with the SPSS 17.0 software (SPSS Inc., Chicago,
153 USA). General ability of stigmas to adhere and germinate pollen grains was assessed by
154 comparison of mean percentages between flower types each day-after-pollination with one
155 way ANOVA at a P value ≤ 0.05. Same proof was used to evaluate mean number of
156 adhered and germinated pollen grains on stigmas among pollination days in each flower
157 type, and seeking for differences between number of adhered/germinated pollen grains
158 between flower types each pollination day. Finally, pollen germination percentage on both
159 flower types in regard of day of pollination was evaluated by same ANOVA mean
160 comparinson test after a data transformation into the $(\text{arcsen}\sqrt{\%\text{germination}})^{-1}$. When

161 possible, significant independent groups were separated by Duncan multiple range test at
162 the 95% confidence level.
163 Flower weights were correlated with pollination day with a T pair comparison proof, and
164 thereafter, mean weights between flower types were compared by one way ANOVA each
165 pollination day. Finally, ANOVA test served to compare fruit set percentages between
166 fruit types at a P value ≤ 0.05.
167
168 **3. Results**
169 *3.1. Stigmatic receptivity*
170 Monitoring flower development in field conditions showed that king flowers lasted for
171 four days, when petal wilting occurred concomitantly to stigma browning (Fig. 1). Lateral
172 flowers had a slower developmental pace and lasted for five days. King flowers opened
173 ahead of lateral flowers (Fig. 2A,B), but hand pollinating both kinds of flowers at anthesis,
174 showed a surprising different pollen performance. Pollen grains abundantly germinated on
175 stigmas of king flowers (Fig. 2C), contrasting to lower levels of pollen germination on
176 stigmas of lateral flowers (Fig. 2D). However, when pollination was performed on flowers
177 that had been opened for three days after anthesis, king flowers had a very poor pollen
178 germination (Fig. 2E), while lateral flowers showed a high pollen germination (Fig. 2F).
179 Quantifying the proportion of flowers with at least one pollen grain adhered or germinated
180 confirmed microscopy observations. All king flowers could adhere pollen on their stigmas
181 for two days after anthesis (Fig. 3A), while this capability remained for six days after
182 anthesis in lateral flowers, with a statistically significant drop the fourth day after anthesis.
183 Pollen grain germination followed the same trend and also diverged among flower types

(Fig. 3B): whereas the percentage of receptive king stigmas showed a quick reduction three days after anthesis, in lateral flowers this capability lasted longer and there were significant differences between both flowers types on the third and fourth days after anthesis.

3.2. Pollen performance

Quantifying the number of pollen grains per stigma showed a more precise image. Clear differences were observed between both kinds of flowers in pollen grain adhesion. At anthesis some 150 pollen grains adhered on king stigmas, compared to 40-60 pollen grains in lateral flowers (Fig. 4A). High pollen adhesion in king stigmas was maintained just for two days, severely dropping three days after anthesis. On the contrary, pollen adhesion on lateral flowers increased from anthesis to a maximum number of 80 pollen grains thee days after anthesis, and thereafter decreased although a certain pollen adhesion was maintained for five days after anthesis. Pollen grain germination followed the same pattern (Fig. 4B). A high number of pollen grains germinated on the stigma of king flowers for two days after anthesis, and then germination significantly decreased. However, in lateral flowers pollen germination reached a maximum of some 75 germinated pollen grains three days after anthesis, decreasing thereafter although receptivity was mantained for five days. These differences in the number of germinated pollen grains appear to be derived of prior differences in the number of adhered pollen grains, since percentage of pollen germination (Fig. 4C) was very similar and optimum for both flower types and pollination days for two days after anthesis, while it was significantly higher in lateral flowers in older flowers.

206 Therefore, pollen performance on apple stigmas was different depending on the flower
207 position within the cluster.
208
209 *3.3. Flower morphology and fruit set within the corymb*
210 The different adhesion ability between king and lateral flowers led to search whether a
211 different stigmatic surface could account for these differences. But the stigmatic area was
212 not significantly different between both flower types (n=50; μ=0.565; σ=0.188). However,
213 the whole gynoecium weight was higher in king flowers at anthesis and for the subsequent
214 three days (r=0.322; $P \leq 0.05$) (Fig. 5A). Thereafter gynoecium weight decreased in both
215 flower types probably due to degeneration.

216 Differences were also recorded for fruit set depending on the position in the corymb (Fig.
217 5B) and king flowers set fruits four times more than lateral flowers.
218
219 **4. Discussion**
220 Results in this work show differences between king and lateral flowers in the apple
221 inflorescence. Both kinds of flowers differed in fruit set, receptivity times, and stigma
222 performance, resulting in different flower strategies to assure fruit production within the
223 cluster.
224
225 *4.1. Fruit set chance within the apple inflorescence*
226 Under conditions of pollen abundance, fruits set preferentially in the king flower. The
227 excellent stigmatic receptivity of these flowers could account for these results. Stigmatic

228 receptivity was not related to a larger stigmatic surface, but the gynoecium of king flowers
229 had a higher weight during three days after flower opening. King flowers in apple have
230 been traditionally considered as a sink for resources (Lauri et al., 1996), which could be
231 related to hormonal control of apical dominance, as it occurs with the ethylene gradient
232 during fruit abscission in this species (Dal Cin et al., 2009). Apical directed reserves
233 towards the king flower could result in a better flower quality, and differences in flower
234 weight related to fruiting success have been recorded in very different species as apricot
235 (Rodrigo and Herrero, 2002; Julián et al., 2010) or avocado, (Alcaraz et al., 2010). These
236 differences appear to be associated to differences in the time of flower opening in relation
237 to pistil development (Rodrigo and Herrero, 2002), and gender biased flower position
238 (Seifi et al., 2008), giving support to the idea of 'ontogenetic contingency' understood as
239 the joint effects of position, previous developmental history, and environment (Diggle,
240 1994; 1995).
241 While it is clear that inadequate pollination of all stigmas may result in differential seed
242 set and fruit asymmetry (Matsumoto et al., 2012), our results also support a differential
243 fruit set within the apple cluster, where stigma receptivity of the different flower types has
244 a clear bearing.
245
246 *4.2. Coordination of flower receptivity in the apple corymb*
247 The stigmatic receptivity of both kinds of flowers entered in the scene in turn, providing a
248 longer receptive period for the entire inflorescence (Fig. 6). Stigmas from king flowers
249 were receptive for two days after anthesis, whereas lateral flowers started to be receptive
250 just after king flowers lost their ability, and remained receptive for a longer period.

King flowers would attract pollinators first, favouring cross pollination. Later, concomitant opening of lateral flowers extend the floral display promoting pollinator visits, although it may limit reproductive success (Sun et al., 2009). Species with particularly high dense clusters often contain a number of sterile flowers (Jin et al., 2010), supporting the idea of different roles for flowers composing inflorescences (Wyatt, 1982; Harder et al., 2004), where fitness position accounts from an ecological perspective (Vallius, 2000), attracting pollinators at anthesis. This gender positional predisposition in inflorescences has been suggested to be the result of flower competence in crop species (Seifi et al., 2008). In apple, the dual stigmatic performance reported here could play a part determining gender potentialities.

Stigmas in apple were receptive at flower opening (Losada and Herrero, 2012), but maximum stigmatic receptivity varied in a flower positional dependent fashion. King flowers had maximum receptivity at anthesis, while lateral flowers showed a maximum receptivity three days after anthesis. This may be related to differences in development of the gynoecium upon flower opening. In other species, differences have been encountered in the maximum stigma receptivity peak. Maximum receptivity at anthesis has been observed in apricot (Egea et al., 1991), or kiwifruit (González et al., 1995b), whereas in apple close related species such as pear, maximum stigmatic receptivity was attained four days after anthesis (Herrero, 1983; Sanzol et al., 2003). In apple, even being multicarpelar, stigmas were receptive at the same time. This may be due to the perfect syncarpy observed in some apple cultivars (Sheffield et al., 2005) with same probability of all stigmas to fertilise an ovule.

While the idea of a sequential flower opening has implications prolonging the receptivity of the inflorescence, results in this work show a finely tuned intra inflorescence stigmatic receptivity that results in an extended stigmatic receptivity for the inflorescence. But the differences in stigma performance could also provide an opportunity for different flower strategies.

4.3. Two stigma strategies

Stigma performance was different between king and lateral flowers. King flowers had an excellent stigmatic receptivity, gathering all at once over twice the number of germinating pollen grains than lateral flowers. But this receptivity was really short, just two days. In contrast lateral flowers had a more conservative approach, with less capacity to gather pollen grains at once, but with an extended receptive period. These two different stigma performances may result in a different advantage in different scenarios. It might be expected that under good weather and pollination conditions, king flowers would have an advantage, as this was the case in this work. However, when pollination conditions may be threatened, either by inappropriate weather or by scarce insect activity, lateral flowers – with a longer stigmatic receptivity- may have a clear advantage.

In apple, flowering is accelerated at warming winters (Tooke and Battey, 2010), threatening the synchrony with pollinators observed for some varieties (Das et al., 2011), and an extended receptive period would mitigate such circumstances. The threat of climate change, which may lead to an asynchrony between plants and the environment, is especially relevant in temperate climates where life cycles are season dependent (Sherry et al., 2007, Hedhly et al., 2009). While a post pollination male-female synchrony is required

for a successful mating and fruit production (Herrero, 2003), stigmatic receptivity at pollination time is also crucial for a successful fruit production. The two different strategies for the two kinds of flowers in the apple inflorescences may have a different advantage in different scenarios. But in any circumstances both of them provide a strategy to deal with environmental uncertainty and to assure fruit set within the inflorescence.

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FIGURE LEGENDS:

Fig. 1. Phenological stages of Malus x domestica flowers within the corymb. King flowers (KING) opened one day after balloon stage and developed to middle anthers dehisced, all anthers dehisced, brown stigma, and reached petal fall four days after anthesis. Lateral flowers (LAT), went through the same stages at a slightly slower pace, reaching petal fall five days after anthesis.

Fig. 2. Pollen performance on the stigma of King and Lateral apple flowers. (A) King flower at anthesis. (B) Lateral flowers at anthesis. (C) High pollen germination on stigmas of king flowers when pollinated at anthesis (P0). (D) Reduced pollen germination on stigmas of lateral flowers, when pollinated at anthesis (P0). (E) Stigmas of king flowers loose the ability to germinate pollen grains when pollinated three days after anthesis (P3). (F) In contrast, pollination at this time in lateral flowers resulted in a higher level of pollen germination. C-F. Squash preparations of apple styles stained with aniline blue. Scale bars = 50µm.

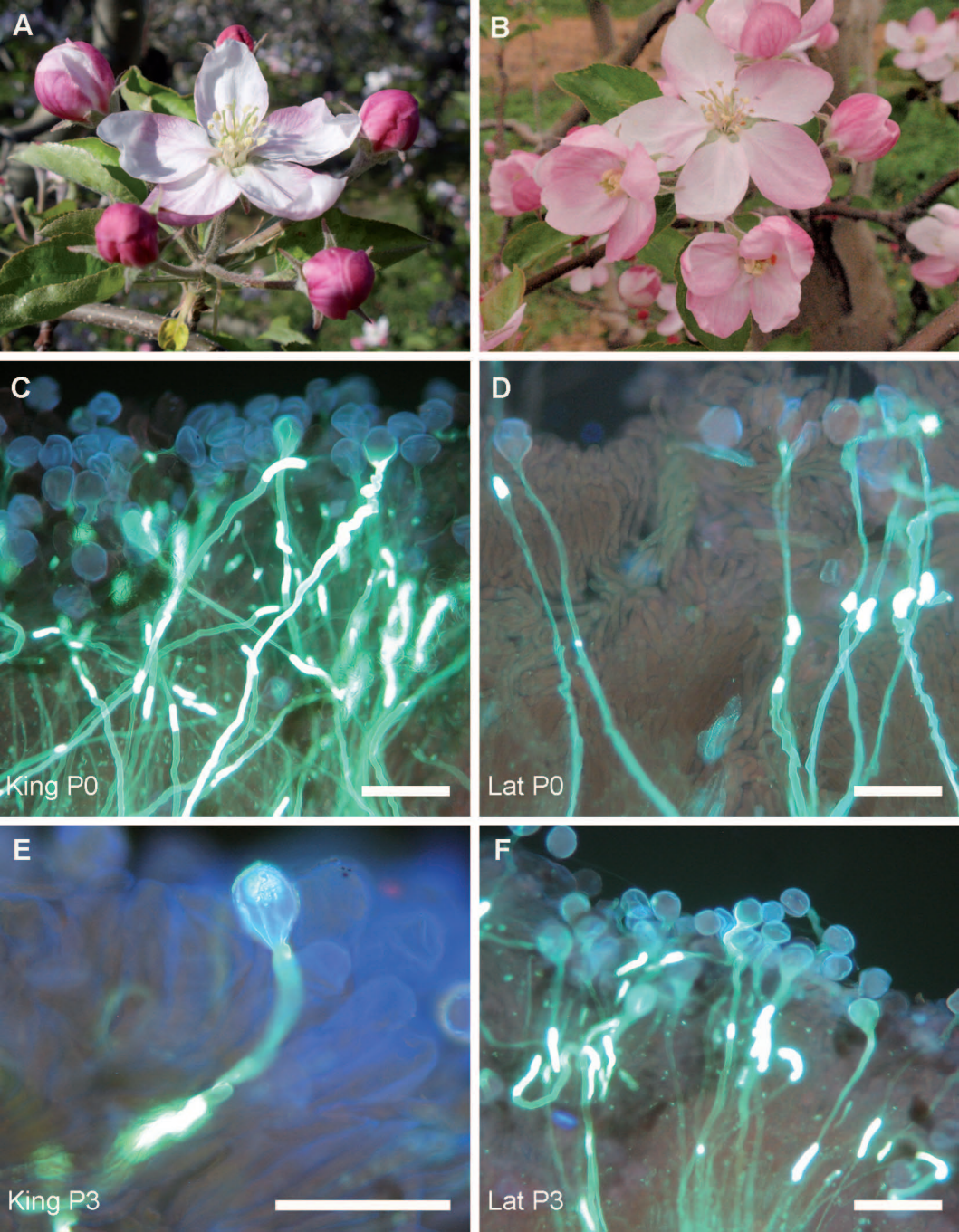
Fig. 3. Percentage of receptive stigmas from king and lateral apple flowers, with adhered (A) and germinated (B) pollen grains. (A) While stigmas from lateral flowers supported pollen grain adhesion for six days after anthesis, in king flowers all stigmas were able to adhere pollen grains just for two days after anthesis. (B) A high percentage of stigmas from lateral flowers supported pollen grain germination until the fifth day after anthesis, whereas in stigmas from king flowers this proportion decreased from the second day after anthesis. Values with * indicate a significant difference between flower types for the same pollination day at a $P \leq 0.05$.

Fig. 4. Number of adhered (A) and germinated (B) pollen grains in the stigma, and percentage of pollen germination (C) in king and lateral flowers. Letters over bars show significant differences between days after pollination for the same flower type on either adhered or germinated pollen grains on the stigma. Asterisks mean significant differences in germination percentages between both flower types at a given pollination day at a $P \leq 0.05$. Mean separation by Duncan multiple range test at a $P \leq 0.05$.

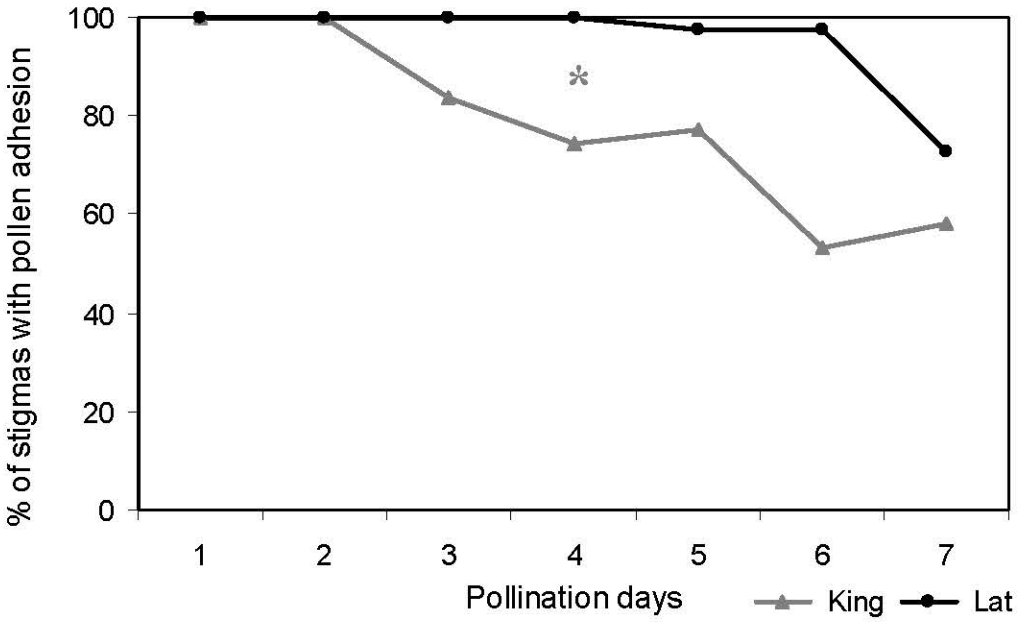
Fig. 5. Flower weight and fruit set percentage between king and lateral flowers. (A) Flower weights were significantly different from anthesis to three days later (asterisks). (B) Percentage of fruit set further show a much higher percentage for king flowers than lateral flowers ($P \leq 0.05$). Bars correspond to SE. Asterisks show significant differences between weights of both flower types each day at a $P \leq 0.05$.

Fig. 6. Schematic representation and pictures of corymb development in apple. While stigmatic receptivity in king flower decreased two days after anthesis, stigmatic receptivity in lateral flowers started after the king flower receptive period and lasted four more days. In sum, inflorescence has a total stigmatic receptivity of six days.

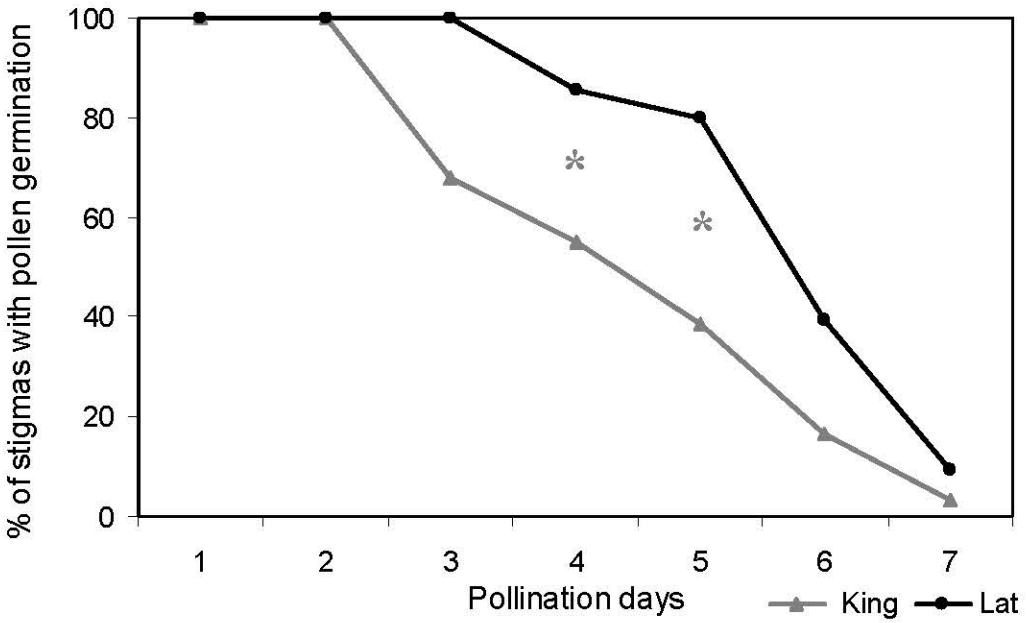

KING
LAT

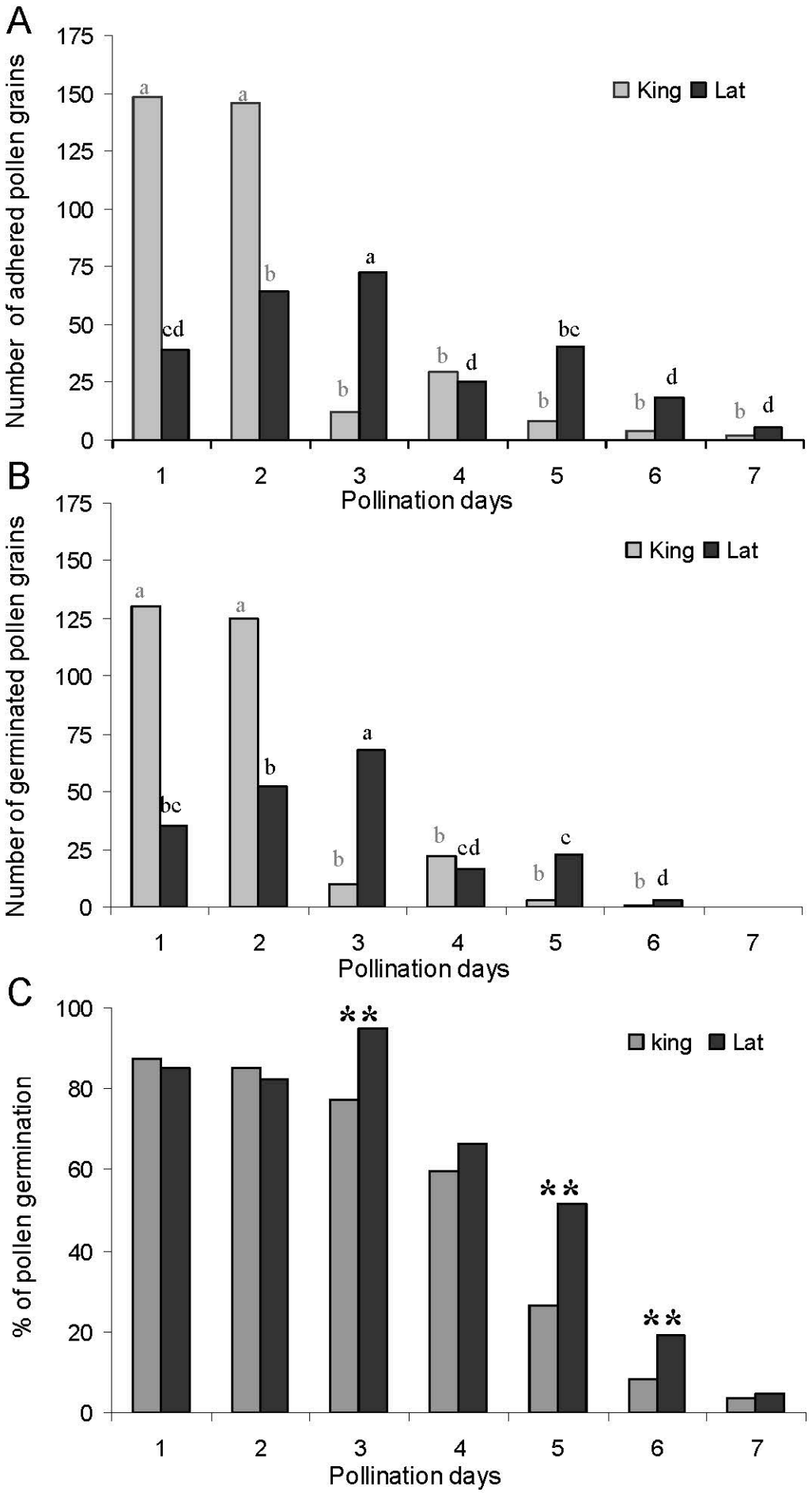

A
B
C
King P0
D
Lat P0
E
King P3
F
Lat P3

A

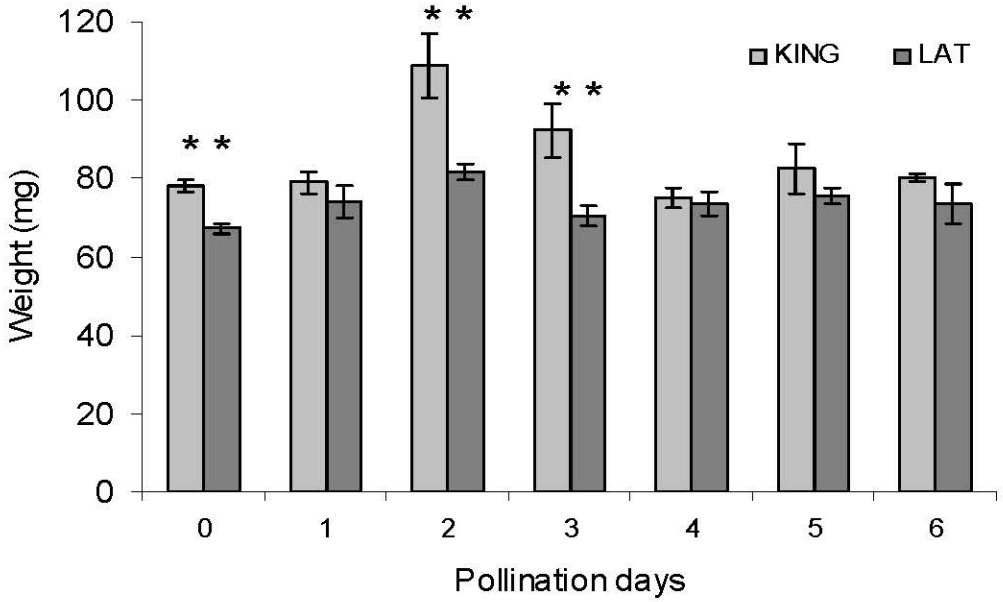

100
80
60
40
20
0
% of stigmas with pollen adhesion
*
1
2
3
4
5
6
7
Pollination days
King
Lat

B

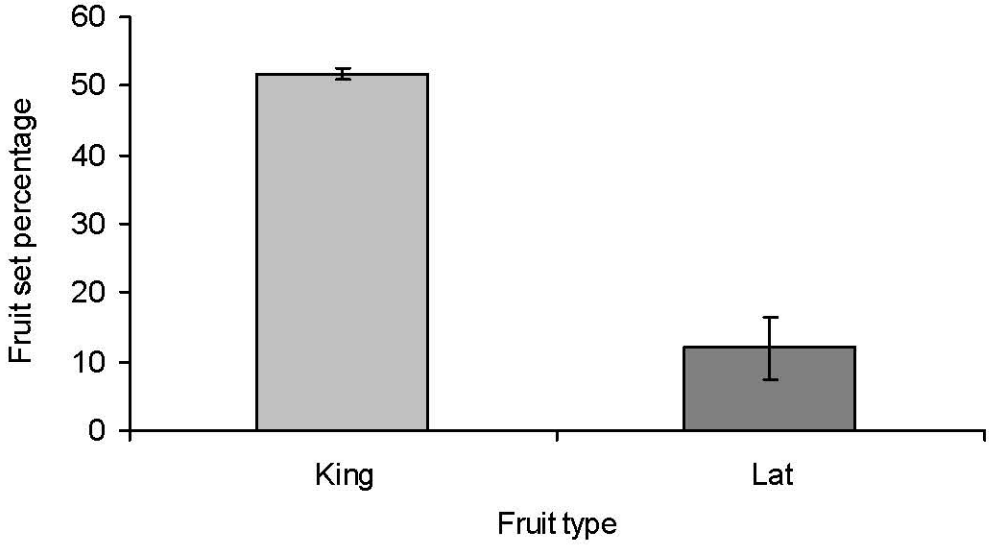

100
80
60
40
20
0
% of stigmas with pollen germination
*
*
1
2
3
4
5
6
7
Pollination days
King
Lat


A
Number of adhered pollen grains
175
150
125
100
75
50
25
0
King
Lat
a
a
cd
b
b
a
b
d
b
bc
b
d
b
d
1
2
3
4
5
6
7
Pollination days
B
Number of germinated pollen grains
175
150
125
100
75
50
25
0
King
Lat
a
a
bc
b
b
a
b
cd
b
c
b
d
1
2
3
4
5
6
7
Pollination days
C
% of pollen germination
100
80
60
40
20
0
king
Lat
**
**
**
1
2
3
4
5
6
7
Pollination days

A


120
100
80
60
40
20
0
Weight (mg)
* *
* *
KING
LAT
0
1
2
3
4
5
6
Pollination days

B


60
50
40
30
20
10
0
Fruit set percentage
King
Lat
Fruit type

K King stigma receptivity duration

L1
L2
L3
L4

Lateral stigma receptivity duration

Stigmatic receptivity duration for the whole corymb

Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6