Resource and Pollen Limitation in Hyacinthoides non-scripta: Impacts upon Fruit and Seed Development, plus Seed Maturation Pattern.

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Resource and Pollen Limitation in *Hyacinthoides non-scripta*: Impacts upon Fruit and Seed Development, plus Seed Maturation Pattern.

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Resource and Pollen Limitation in *Hyacinthoides non-scripta*: Impacts upon Fruit and Seed Development, plus Seed Maturation Pattern.

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Abstract

Resource limitation, pollen quality (involving self- or outcross-pollen) and pollen quantity limitations are known to affect seed development by increasing ovule abortion and reducing ovule fertilisation. It is therefore important to fully understand pollen and resource limitations as they have a significant effect upon plant fitness. *H. non-scripta* has linearly arranged ovules, and produces a general non-random seed maturation pattern, with increased seed development at the stylar end and increased seed abortion and unfertilised ovules at the basal end of the fruit. Although this pattern has been observed in many species, exploration of how resources and pollen quality and quantity influence the non-random seed maturation pattern has not before been performed. In this thesis I investigate the effects of additional resources and a range of pollen qualities and quantities upon fruit development, seed development and the seed maturation pattern.

A stochastic simulation model is used to assess how resources and pollen may impact seed development in *H. non-scripta*. Simulations reveal increasing resources and pollen quality and quantity should increase fruit set, and seed development. Resource manipulations in the field had little effect on fruit set or seed development, although field pollen manipulations produced similar results to those expected. Seed expansion increased with pollen quantity and quality. It is found that the non-random seed maturation pattern may be partly due to resources allocated to ovules fertilised first, but fewer resources may be allocated to the most stylar ovules in the fruit. Additionally, the non-random seed maturation pattern is stronger under mixed pollen qualities. Furthermore, open pollination appears to be composed of large quantities of a balanced mixture of outcross- and self-pollen. Together, the findings suggest resources and pollen are very important factors influencing the fruit development, seed development, and the non-random seed maturation pattern in *H. non-scripta*, and should be studied in conjunction. [302 words]

Statement of Copyright

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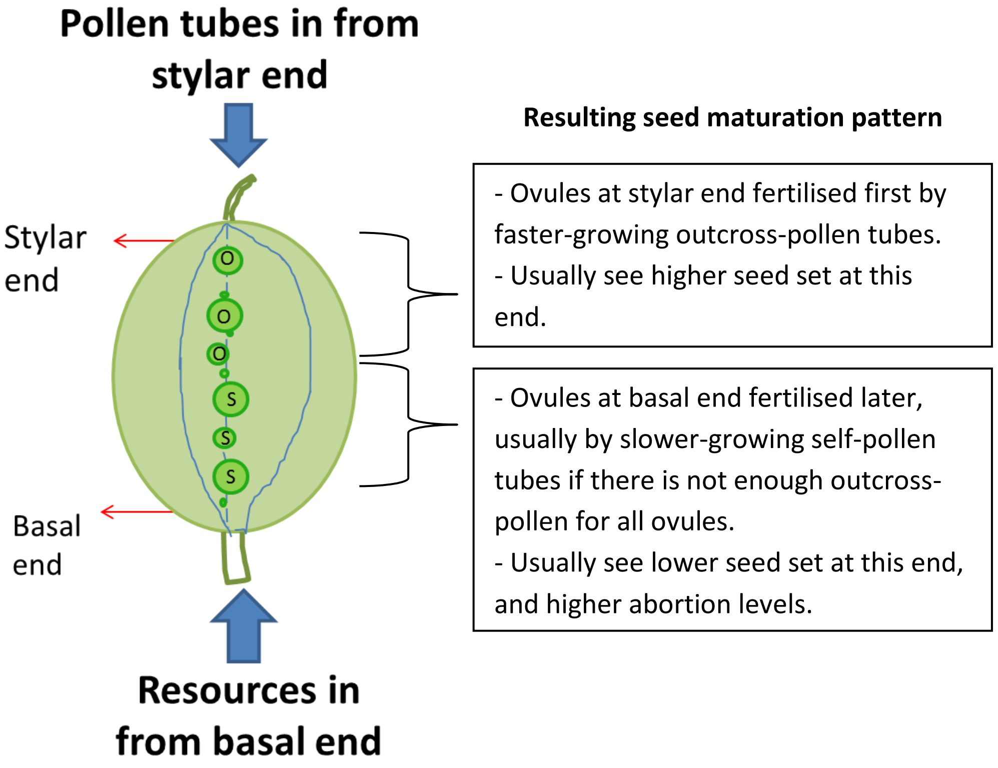
Chapter 1

Patterns of ovule maturation and abortion in *Hyacinthoides non-scripta*.

Introduction

In this thesis I investigate seed and fruit production in *Hyacinthoides non-scripta* (English Bluebell; Asparagaceae), a woodland bulbous perennial herb, whose fruit contain three locules with linearly arranged ovules. *H. non-scripta* is protected under the 1981 Wildlife and Countryside Act, as they are endangered by invasion of and hybridisation with *Hyacinthoides hispanica* (Spanish bluebell; Asparagaceae). As *H. non-scripta* plants are abundant in the local woods around the University of Durham, in the NE of England in the United Kingdom of Great Britain and Northern Ireland, and have linearly arranged ovules in their fruit, they are an ideal study species. Additionally, to the best of my knowledge there has only been one previous study on *H. non-scripta* pollen manipulation, by Corbet (1998), so much is yet to be learnt about *H. non-scripta*, which could ultimately aid in their current conservation effort. Furthermore, any new knowledge gained from them may also be applied to other fruiting plants and could aid in their conservation or agricultural propagation. This thesis aims to gain a more complete understanding of why ovule positional effects occur in *H. non-scripta*. It also aims to facilitate a better understanding of self- and outcross-pollen effects, pollen quantity effects and resource allocation and limitation effects in *H. non-scripta*.

Fruit in which ovules are arranged linearly along an axis (i.e. linear fruit), often have a non-random pattern of seed development (Marshall and Ellstrand, 1986; Nakamura, 1988; O'Donnell and Bawa, 1993; Corbet, 1998; Mena-Alí and Rocha, 2005a, b; Silveira and Fuzessy, 2014; Yuan *et al.*, 2014) (Figure 1.1). Some ovules are positioned closer to the stylar end of the ovary, where pollen tubes arrive in most linear fruit species, while others are alongside the basal end, where vasculature distributes resources. The ovules positioned closer to the stylar end of the ovary have improved chances of firstly being fertilised and secondly avoiding abortion, to accomplish full maturity (Nakamura, 1988; O'Donnell and Bawa, 1993; Mena-Alí and Rocha, 2005a, b; Yuan *et al.*, 2014). However, past research has not determined a clear rationale for this positional effect on fertilisation and abortion, although the involvement of pollen and resource limitation is assured. An answer to the question of why and how the non-random seed maturation pattern occurs in linear fruit may add to understanding of the processes involved in seed maturation in plants. In this chapter I examine the main mechanisms for ovule or seed abortion and development, and also the most likely theories explaining non-random patterns of seed maturation in linear fruit.



- Ovules at stylar end fertilised first by faster-growing outcross-pollen tubes.
- Usually see higher seed set at this end.

- Ovules at basal end fertilised later, usually by slower-growing self-pollen tubes if there is not enough outcross-pollen for all ovules.
- Usually see lower seed set at this end, and higher abortion levels.

Figure 1.1: The non-random seed maturation pattern in linear fruit. Pollen tubes and resources enter ovary from opposite ends in most plants and in *H. non-scripta*. It is hypothesised that outcross-pollen fertilises stylar ovules preferentially, leaving self-pollen to fertilise more basal ovules. Ovules marked with "S" have been self-fertilised. Ovules marked with "O" have been fertilised with outcross-pollen. The smallest ovules have remained unfertilised, the middle-sized ovules have been fertilised but were then aborted, and the largest ovules have been fertilised and have grown to become fully developed seeds.

It has long been observed that some of the ovules in an ovary of a flower are not matured into seeds (Bawa and Webb, 1984; Uma Shaanker *et al.*, 1988; Haig and Westoby, 1988; Casper and Niesenbaum, 1993; Burd, 1994; Larson and Barrett, 2000; Dogterom *et al.*, 2000; Moody-Weis and Heywood, 2001; Yang *et al.*, 2005; Aizen and Harder, 2007). It is not until relatively recently, however, that investigation into the causes and significance of this differential seed maturation has commenced. It is nearly forty years since Schemske (1977) examined differences in seed set in *Claytonia virginica* (Eastern spring beauty; Montiaceae) between self-fertilised and cross-fertilised hand-pollinated flowers. This study was the first to deduce possible factors which influence ovule development; of these, pollinator limitation and resource limitation are still front runners in modern theories (although "pollen limitation" is now recognised as an improved term for "pollinator limitation"). Since then, many studies have concluded inadequate pollination is a major explanation for seed abortion (Schemske, 1977; Schemske *et al.*, 1978; Schemske, 1980; Willson and Schemske, 1980; Bierzychudek, 1981; Bierzychudek, 1982; Petersen *et al.*, 1982; Gross and Werner, 1983; Bawa and Webb, 1984). Ovule abortion and seed abortion are terms used interchangeably in this thesis, and describe the occasion where an ovule has grown in size from that of unfertilised ovules, but has noticeably not grown as much as the large, fully developed seeds. Other studies have noted the continued abortion of immature fruits and undeveloped seeds under high pollination, indicating that the other possible factor, resource limitation, needs to be investigated further (Primack, 1979; Webb, 1979; Lloyd *et al.*, 1980; Stephenson, 1981; Aker, 1982; Lee and Bazzaz, 1982a, b; Haig and Westoby, 1988; Uma Shaanker *et al.*, 1988).

Resource limitation

Resource limitation occurs when an inadequate supply of resources is available to mature a number of fertilised ovules, zygotes, into seeds (Harder and Routley, 2006). It is thought plants have to divide their resources between necessary functions such as growth, maintenance and reproduction (Stephenson, 1981; Avila-Sakar *et al.*, 2001). Although resource allocations in plants are undeniably more complex, for example involving dynamic changes in available resources over time, and resources increasing year on year with growth, we may simply consider one time step in the life of the annual bulbous plant where there is a finite amount of resources produced from the leaves and stored in the bulb, and to behave optimally a plant should produce maximum seeds in this time step. In this scenario it could be inefficient to pre-allocate a certain amount of resources to unfertilised ovules when there is uncertainty in the number that will be fertilised, the number lost by herbivory, and in the amount of sunlight that will reach the plant for photosynthesis, to regain resources (Stephenson, 1981). The overproduction of ovules could therefore doubtless occur. This overproduction may be due to suboptimal resource allocation to ovule production, which is possible if resource conditions decline between ovule initiation and seed production (Harder and Routley, 2006). However, if a plant experiences embryo losses most breeding seasons, but extremely high seed set during rare excellent breeding seasons due to stochastic pollen or resource availability, overproduction of ovules could be a useful adaptation to take advantage of the unpredictably good conditions (Harder and Routley, 2006). Therefore the optimal resource allocation strategy for a plant in a stochastic environment may be to overproduce ovules.

Many studies have identified resource limitation during seed maturation by indicating that supplementation of nutrients and/or water enhances seed set (Van Andel and Vera, 1977; Willson and Price, 1980; McCall and Primack, 1985; Vaughton, 1991; Campbell and Halama, 1993; Worley and Harder, 1999). Further studies have shown that flower removal can increase seed set in the remaining flowers on the plant, as this increases resource availability for development of the remaining ovules (Lee and Bazzaz, 1986; Gorchov, 1988; Ehrlén, 1992; Yang *et al.*, 2005). Resources destined for developing fruit and seeds are often presumed to originate from leaves or bulbs (e.g. Corbet, 1998), but in a species like *H. non-scripta*, resources may also be created by the fruits themselves. This could occur in green immature fruits, as green tissue likely contains chlorophyll and is photosynthetic (Todd *et al.*, 1961; Smillie *et al.*, 1999; Lytovchenko *et al.*, 2011). For example, studies of *Solanum lycopersicum* (Tomato; Solanaceae) have shown that most resources used by developing fruit come from the leaves, however, it has been shown that photosynthesis of green immature fruit is important for the initiation of normal seed development (Lytovchenko *et al.*, 2011). Consequently, this thesis will take care to consider the possible sources of resources, in addition to investigation of how the resources are being allocated.

Pollen limitation

Pollen limitation was originally considered a cause for the reduction in seeds reaching maturation due to inadequate pollen transfer by vectors (Bierzychudek, 1981; Reed Hainsworth *et al.*, 1985; Ayre and Whelan, 1989; Ackerman and Montalvo, 1990; Johnston, 1991; Young and Young, 1992; Burd, 1994; Moody-Weis and Heywood, 2001). Put simply, not enough pollen is deposited on the stigma to fertilise all of the ovules of the flower (Aizen and Harder, 2007). Additionally, studies have shown that pollen tubes grown from pollen germinated on the same flower stigma competed for access to ovules (Snow, 1986; Bertin, 1990; Marshall, 1991; Dogterom *et al.*, 2000), and that there were differential pollen tube growth rates (Snow and Spira, 1991; Walsh and Charlesworth, 1992; Johnston, 1993; Snow and Spira, 1993; Burd, 1994). This pollen competition was discovered to result in better quality offspring, including larger and heavier fruit or seeds, and faster growth of seedlings (Davis *et al.*, 1987; Dogterom *et al.*, 2000). However, many studies during that period did not consider the pollen source (that is the relatedness of the pollen donor plant to the maternal plant) comprehensively enough, so resulting in inaccurate measures of pollen limitation.

Most papers investigating pollen limitation up to the late 1990s only considered “small” or “large” pollen quantities, without recognising differing pollen sources (Dogterom *et al.*, 2000). Modern pollen limitation definitions, however, encompass both pollen grain number deposited and pollen competition due to differing pollen quality. Accordingly, it is understood pollen limitation can occur when, either, some ovules within a flower remain unfertilised (pollen quantity limitation), or, when too few embryos avoid genetic death or herbivory to compete for resources (pollen quality limitation) (Harder and Routley, 2006). Pollen quality involves the pollen source, i.e. if pollen is self or outcross, and the genetic relatedness of the parental plants (if pollen is from the same plant, it is termed “self”, while pollen from a separate individual is termed “outcross”). Low pollen quality or selfing is often considered to increase levels of seed abortion, causing fewer seeds to develop (Aizen and Harder, 2007).

Under low-quality pollination conditions, self-fertilisation or increased genetic relatedness among parents may lead to reduced seed set due to self-incompatibility mechanisms, or inbreeding depression. Inbreeding depression reduces offspring fitness due to an increase in genetic load. It typically occurs after zygote formation, and refers to reductions in performance and fitness related trait values for individuals with increased homozygosity resulting from inbred mating (Montalvo, 1992). It can decrease seed production because embryos homozygous for deleterious alleles would die and abort during development (Aizen and Harder, 2007). This reduction in seed set could arise particularly when selfing occurs in a predominantly outcrossing species, due to less frequent purging of detrimental recessive mutations (Burd, 1994). Self-incompatible species are those which cannot produce viable progeny from self-pollen, whereas self-compatible species can. Since Darwin, it has been observed that in self-compatible species, self-pollen is competitively inferior to outcross-pollen (Darwin, 1976; Pfahler, 1965; Walsh and Charlesworth, 1992) and ovules fertilised by self-pollen produce fewer seeds (Sage *et al.*, 1999). Self-incompatibility mechanisms operate primarily prior to ovule fertilisation (Wiens *et al.*, 1987; Montalvo, 1992), and include mechanisms such as differential pollen germination, tube growth, and fertilisation (Lyons *et al.*, 1989; Montalvo, 1992). Differential pollen germination would occur for example, when outcross-pollen is more likely to germinate on the stigma than self-pollen. Similarly, outcross-pollen tubes would grow faster than self-pollen tubes, and these would be able to fertilise more ovules than self-pollen tubes.

It is clear that pollen limitation is a greatly significant cause of reduced seed set in fruit, and therefore has been studied in many papers. Pollen limitation has been experimentally measured many times, however, it is usually experimentally determined by the addition of abundant outcross-pollen to flowers subject to natural pollination conditions, which usually consists of a mixture of outcross- and self-pollen (Harder and Routley, 2006). As earlier stated, past papers tended not to consider sources of pollen (Dogterom *et al.*, 2000), and this resulted in differences found in seed set between non-supplemented and supplemented flowers being attributed to the additional pollen quantity, with no thought of how pollen quality was also increasing. They thereby falsely enlarged the estimates of quantity limitation (Aizen and Harder, 2007). In many modern papers considering pollen limitation, such as Corbet, 1998 and Ashman *et al.*, 2004, pollen quality is carefully considered as part of pollen limitation and any difference between seed set under self-pollen and outcross-pollen is measured. It is therefore clear that both pollen quality and quantity are very important factors to recognise when examining effects of pollen limitation. In this thesis I explicitly address the question of how pollen quantity and pollen quality affect plant fitness in terms of fruit and seed set.

Mechanisms acting on linearly positioned ovules

It is well known that seed maturation is affected by ovule location within linear fruits (Webb and Bawa, 1985; Lee and Bazzaz, 1986; Nakamura, 1988; O'Donnell and Bawa, 1993; Corbet, 1998; Mena-Alí and Rocha, 2005a, b; Calvino 2014; Silveira and Fuzessy, 2014; Yuan *et al.*, 2014). Most researchers have found a pattern where fertilisations and full seed development are more common towards the stylar end of the fruit, while aborted seeds are increasingly common towards the basal end of the fruit (Harris, 1915; Cooper *et al.*, 1937; Bawa and Webb, 1984; Lee and Bazzaz, 1986; Nakamura, 1988; Rocha and Stephenson, 1991b; O'Donnell and Bawa, 1993; Corbet, 1998; Mena-Alí and Rocha, 2005a; Susko, 2006; Susko and Clubb, 2008; Silviera and Fuzessy, 2014; Yuan *et al.*, 2014) (see Figure 1.1). This pattern is termed "the non-random seed maturation pattern" in this thesis. Less common patterns have also been recorded: a pattern of more abortions occurring in the stylar end of the fruit than in the middle or basal end of the fruit was observed in five species of tropical legumes (Wyatt, 1981). In *Medicago sativa* (Alfalfa; Fabaceae), another legume, a strange pattern was observed, where ovules at even numbered positions had a higher probability of seed set than ovules at odd numbered positions (Figure 1.2) (Horovitz *et al.*, 1976; O'Donnell and Bawa, 1993). Linck (1961) observed that in fruit of *Pisum sativum* (Pea; Fabaceae), peas had increased seed abortion rates at both ends of the pod. *Lupinus nanus* (California blue lupine; Fabaceae) exhibits a pattern where seed set increases towards the base of the fruit, except for a sharp decline at the ovule closest to the fruit base (Figure 1.2) (Horovitz *et al.*, 1976). See Table 1.1 for a comparison of species and seed maturation patterns. It is clear from that table that most plants tested for seed maturation patterns have been Fabaceae, probably due to the fact that Fabaceae has the most common linear fruit.

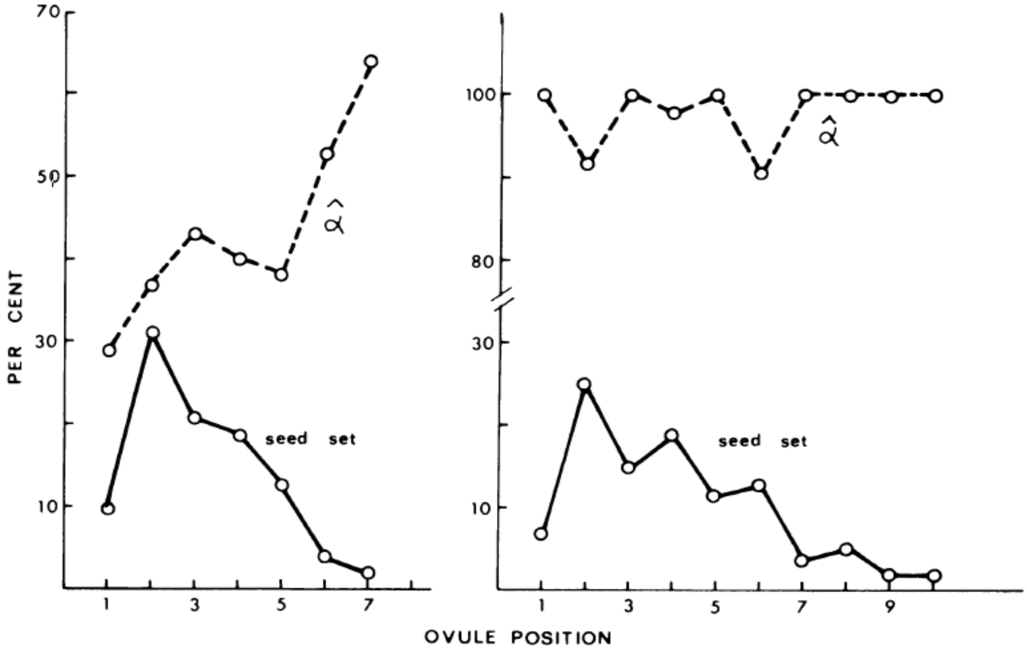


Figure 1.2: Distribution of seed set and outcrossing percentages, $\hat{\alpha}$, in different ovule positions: left, in *Lupinus nanus*, right, in *Medicago sativa*. Ovule positions are numbered in ascending order from base of ovary to style. Seed set graphs are based on only those seeds which developed into flowering progeny used for $\hat{\alpha}$ estimates. Figure is from Horovitz *et al.* (1976).

Table 1.1: Plant species and families showing different patterns of seed set from past papers. Note that most plants tested were Fabaceae and most did show the non-random seed maturation pattern.

Paper	Pattern of seed set	Plant family	Plant species
1) Cooper *et al.*, 1937.	Non-random seed maturation pattern – increased seed set at stylar fruit end, increased seed abortion at basal fruit end	1) Fabaceae	1) *Medicago sativa*
2) Bawa and Webb, 1984 (3 families, 7 species).		2) Fabaceae Bixaceae Bignoniaceae	2) *Bauhinia ungulata* *Caesalpinia eriostachys* *Dalbergia retusa* *Myrospermum frutescens* *Pterocarpus rohrrii* *Cochlospermum vitifolium*
3) Lee and Bazzaz, 1986		3) Fabaceae	3) *Cassia fasciculate*
4) Nakamura, 1988		4) Fabaceae	4) *Phaseolus vulgaris*
5) Rocha and Stephenson, 1991b		5) Fabaceae	5) *Phaseoulus coccineus*
6) O'Donnel and Bawa, 1993		6) Fabaceae	6) *Sophora japonica*
7) Corbet, 1998		7) Asparagaceae	7) *Hyacinthoides non-scripta*
8) Mena-Ali and Rocha, 2005a		8) Fabaceae	8) *Bauhinia ungulate*
9) Susko, 2006		9) Fabaceae	*9) Robinia pseudoacacia*
10) Susko and Clubb, 2008		10) Brassicaceae	10) *Hesperis matronalis*
11) Silviera and Fuzessy, 2014		11) Fabaceae	*11) Poincianella pyramidalis*
12) Yuan *et al.*, 2014		12) Fabaceae	*12) Robinia pseudoacacia*
Wyatt, 1981	Increased seed abortions at stylar end of fruit	Fabaceae	*Pentaclethra macroloba* *Swartzia simplex* *Cassia stenocarpa* *Cassia biflora* *Parkinsonia aculeata*
Horovitz et al., 1976	Ovules at even numbered positions had higher probability of seed set than ovules at odd numbered positions	Fabaceae	*Medicago sativa*
Horovitz et al., 1976	Seed set increases towards base of fruit, except for sharp decline at the ovule closest to the fruit base	Fabaceae	*Lupinus nanus*
Linck, 1961	Increased seed abortion at both ends of fruit	Fabaceae	*Pisum sativum*

When investigating seed abortion in linear fruit, such as legume pods, Wiens *et al.* (1987) presumed embryo abortions were due to genetic load, which would be a result of low pollen quality. However, another study, Bawa *et al.* (1989), argued against this occurring in linear fruit, as the seeds inside the fruit would exhibit a random distribution of aborted zygotes if this were the case. Yet, many studies have shown non-random patterns of abortion. Furthermore, Nakamura (1988) observed that in linear fruit, the developing embryos were ordered in proximity to the vascular system at the base of the fruit, presumably allowing them to receive first access to resources (Watson and Casper, 1984; Sage and Webster, 1987; Nakamura, 1988; Corbet, 1998). If all of the ovules are fertilised producing zygotes, this would lead to the closer, more basal zygotes gaining resources first, or in higher quantities, than more stylar zygotes further from the incoming vasculature (Mena-Alí and Rocha, 2005a). Consequently, further stylar embryos will have a higher probability of abortion (Lee and Bazzaz, 1982a, b). In theory, this would create an abortion pattern opposite to that observed in the studies mentioned above. Clearly, therefore, resource limitation cannot be the only mechanism at work creating positional seed abortion, as it would not create the pattern of seed development seen in the field.

Nakamura (1988) also observed differences in abortion patterns between selfed and outcrossed flowers. In the study, increased seed abortion and stunted seed set in basal fruit positions only appeared in selfed and more inbred crosses; when plants were outcrossed, embryo survivorship patterns were replaced with randomly positioned fully developed seeds, and random patterns of abortion. It has been suggested that pollen tube growth rates may vary according to pollen genotypes (Sari Gora *et al.*, 1975; Mulcahy, 1979; Bookman, 1984; Nakamura, 1988), which may lead to preferential fertilisation of stylar ovules by faster developing outcross-pollen tubes (Nakamura, 1988). This has been termed the pollen competition hypothesis (O'Donnell and Bawa, 1993); outcross-pollen fertilises ovules first due to faster-growing pollen tubes compared to self-pollen (a type of self-incompatibility mechanism); ovules fertilised by outcross-pollen are also superior to those fertilised by self-pollen, as they are less likely to experience inbreeding depression (O'Donnell and Bawa, 1993; Mena-Alí and Rocha, 2005a, b; Silveira and Fuzessy, 2014; Yuan *et al.*, 2014).

The pollen competition hypothesis can be extended to encompass the order of ovule fertilisation in the ovary in linear fruits. The gamete selection hypothesis proposes that stylar ovules are preferentially fertilised first by faster-growing outcross-pollen tubes; pollen tubes sequentially fertilise more basal ovules as they grow down towards the base of the ovary (O'Donnell and Bawa, 1993). As embryos in stylar positions have a longer time to develop, they are larger and heavier than basal seeds. Some evidence for the hypothesis was found, as ovules in fruit of *Phaseolus coccineus* (Runner bean; Leguminosae) fertilised by the fastest-growing pollen tubes were more likely to fully develop into seeds, and matured into faster growing and larger seedlings than those ovules fertilised by more sluggish pollen tubes (Rocha and Stephenson, 1991a, b). These two theories suggest that pollen tube competition, derived from pollen quality differences, could lead to the pattern of maturation and abortion seen in studies; higher seed development in the stylar end of fruits and higher seed abortion in the basal end of fruits.

More recently, the microgametophytic competition hypothesis has been proposed to explain positional seed maturation (Mena-Alí and Rocha, 2005b, Silveira and Fuzessy, 2014). This theory enhances the pollen competition and gamete selection hypotheses by including resource limitation as well as pollen quality limitation. Under resource limitation and multiple pollen donors, this hypothesis suggests that stylar embryos fertilised by faster, stronger pollen tubes, will have a higher probability of reaching maturity, as they are able to gather more maternal resource, or at a higher rate, than more basal ovules (Mena-Alí and Rocha, 2005a, b; Silveira and Fuzessy, 2014). Consequently, it envisages a higher likelihood of zygote abortion by the mother plant towards the base of the fruit due to a lower competitive ability and garnering of resources (Bawa and Webb, 1984; Rocha and Stephenson, 1991a; Mohan Raju *et al.*, 1996; Mena-Alí and Rocha, 2005a, b). The microgametophytic competition hypothesis allows for the pattern of abortion seen in previous studies, where there is higher seed development at the stylar end of the fruit and higher seed abortion at the basal end of the fruit.

State-of-the-art, implementation and thesis plan

Although the microgametophytic competition hypothesis is supported by data in many studies, the underlying mechanisms of pollen and resource limitation are only partially understood. This thesis will investigate the interacting mechanisms other researchers have suggested that can create non-random patterns of abortion and maturation, in order to test the theories proposed above. This will be accomplished by using local populations of *H. non-scripta* as a model species. It will focus on the effects of pollen quality and quantity by differing mixtures of self- and outcross-pollen, and comparing fruit set and the seed development patterns produced to patterns seen in fruits under open pollination conditions. It will also focus on effects of resource limitation by comparing fruit set and seed maturation patterns between flower positions up the raceme, and by effectively increasing resources by removing certain ovaries of the plant before they are able to develop into fruit.

Chapter 2 presents a model of seed development that incorporates various hypotheses regarding pollen tube growth and resource allocation. The model predicts patterns of ovule fertilisation and seed development that are expected under certain ratios of self- and outcross-pollen, and under plentiful and limited resources. These predictions will be tested using experimental studies of *H. non-scripta*. Chapter 3 investigates the effects of freeing up resources by ovary removal on the fruit development and seed maturation pattern. Chapter 4 investigates the effects of varying pollen quality and quantity on fruit and seed maturation pattern. Chapter 5 provides conclusions from the thesis as a whole, comparing results from all chapters and suggests future directions for continuation of this study.

Chapter 2

Modelling effects of pollen and resource limitation on spatial patterns of fertilisation and seed development within the ovary.

Introduction

Final seed set in a fruit results from a sequential process which begins with unfertilised ovules (Calviño, 2014). Pollen and resource limitations lead to ovules remaining unfertilised and seed abortion, and in fruit with ovules linearly arranged along an axis (termed linear fruit), non-random patterns in seed development (Corbet, 1998; Mena-Alí and Rocha, 2005a; Calvino, 2014; Silveira and Fuzessy, 2014) (See Figure 1.1). Mechanisms that may explain non-random seed development patterns within ovaries have been proposed (see Chapter 1), but rarely have they been modelled to examine their effects on seed production. This study aims to gain understanding of seed development patterns by considering resource and pollen limitation, through creating a stochastic simulation model of *H. non-scripta* seed development.

As discussed in Chapter 1, pollination may limit seed development via two factors: pollen quantity and pollen quality, and resources may limit the number of fertilised ovules able to mature into seeds, leading to embryo competition and abortion. Past research has determined that a reduction in either pollen import or resources would lead to decreased seed numbers, viability, and possibly decreased seed size (Casper and Neisenbaum, 1993; Corbet, 1998; Silveira and Fuzessy, 2014). Pollen- and resource-limitation may also influence the spatial pattern of seed maturation within linear fruits through several proposed mechanisms. Two of these will be considered here, both of which involve resource allocation. The first mechanism involves microgametophytic competition (see Chapter 1), and is here termed the “first-fertilised first-served” resource allocation mechanism. This model of resource allocation to developing ovules suggests that ovules towards the stylar end of the ovary are better able to garner resources as they are typically fertilised first and have more time to access maternal provisioning. The second resource allocation mechanism suggests that ovules that are in closest proximity to the basal end of the ovary, from which resources are distributed, would be better able to garner resources. This is termed the “bottom-up” resource allocation mechanism.

Corbet (1998) investigated seed development in the linear fruits of *H. non-scripta*, and found under natural pollination, higher seed production at the fruit’s stylar end and greater abortion at the basal end. She also found “a degree of effective self-incompatibility”, as a difference in the mean proportion of ovules that are matured between plants that were selfed (33.33 ± 21.08 in laboratory experiment) and plants that were outcrossed (79.56 ± 6.84 in laboratory experiment) (Corbet, 1998). Research such as that on pollen tube attractants has since been completed, and new knowledge has been incorporated into our model.

Once pollen tubes have entered the ovary they are attracted to ovules by a signal produced by two synergid cells, which form part of the female gametophyte (Okuda *et al.*, 2009; Okuda and Higashiyama, 2010; Kessler and Grossniklaus, 2011; Lu *et al.*, 2011). The synergid cells are able to guide the pollen tubes so that the two sperm cells can be delivered to the egg cell and the central cell during double fertilisation (Kessler and Grossniklaus, 2011; Lu *et al.*, 2011; Takeuchi and Higashiyama, 2011). There is also evidence that after a pollen tube has entered an ovule and the gametes have fused, other pollen tubes are no longer attracted to the fertilised ovule; this has been termed a block to polytubey (Beale *et al.*, 2012). Here we model the sequence of fertilisations by assuming that pollen enters the stylar end of the ovary, ovules attract pollen tubes, ovules at all positions attract nearby pollen tubes to the same degree, and tubes are more likely to be attracted to the closer unfertilised ovules.

The model presented in this chapter simulates seed development in linear fruit (e.g., *H. non-scripta*). The linear arrangement of ovules and the simulation of both pollen- and resource-limitation have not previously been incorporated into a stochastic simulation model. The model will investigate possible processes involved in creating the seed maturation patterns which have been observed in the literature. Various parameters are manipulated, and by exploring results from the model, the present study will allow an increased understanding of the processes underlying non-random seed abortion and maturation patterns in linear fruit. The model provides predictions of seed maturation patterns under varying pollination and plant-resource conditions, which will be tested by manipulating *H. non-scripta* plants in field experiments (see Chapters 3 and 4).

Methods

The stochastic simulation model described here was implemented using R version 3.1.2 (R Core Team, 2014). The model simulates self- and outcross-pollen deposited onto the stigma of flowers, pollen tube growth down the style, and the resulting ovule fertilisation, abortion and seed development in the fruit. Model parameter values were based on Corbet (1998) and data collected from local populations of *H. non-scripta* in the spring and summer of 2014, Durham, UK. The model describes the fates of ovules positioned inside three locules of *H. non-scripta* fruit. 12 ovules were linearly arranged in each locule. Ovule position 1 was located at the stylar end of the fruit (see Figure 1.1). Ovules had seven possible fates, which were coded as follows:

1) Unfertilised (U)
2) Fertilised by self-pollen then aborted due to resource limitation (RLS)
3) Fertilised by outcross-pollen then aborted due to resource limitation (RLX)
4) Fertilised by self-pollen then aborted due to inbreeding depression (IDS)
5) Fertilised by outcross-pollen then aborted due to inbreeding depression (IDX)
6) Fertilised by self-pollen and fully developed into a seed (DS)
7) Fertilised by outcross-pollen and fully developed into a seed (DX)

For each pollinated flower, the model simulated the fates of the 12 ovules per locule under various pollen and resource limitation conditions. Base-line model parameters are shown in Table 2.1. The sequence of events simulated are shown in Figure 2.1.

Table 2.1: Model parameters and their definitions, with the baseline values used in the model. If the values were changed from the baseline, this was noted in the graphs produced.

Parameter	Definition	Baseline parameter value
N_F	Number of flowers sampled	5000
N_O	Number of ovules per locule	12
N_s	Number of self-pollen grains that successfully germinate on the stigma	8
N_x	Number of outcross-pollen grains that successfully germinate on the stigma	8
g_s	Probability a self-fertilised ovule starts to develop into a seed after fertilisation	0.33
g_x	Probability an outcross-fertilised ovule starts to develop into a seed after fertilisation	0.8
q	Probability a pollen tube accepts each free ovule's signal	0.35
σ	Standard deviation of order of fertilisation of ovules by pollen tubes.	0.01 = sequential fertilisation, or 100 = random fertilisation
O_{max}	Number of ovules that could be developed (due to level of resource limitation).	12

(1) Self- and outcross-pollen grains land on the stigma of a flower.

(2) Self- and outcross-pollen grains germinate on the stigma, producing pollen tubes (self-pollen grains may not germinate so commonly as outcross-pollen grains due to self-incompatibility mechanisms of the plant).

(3) Self- and outcross-pollen tubes grow down the style towards the ovary (self-pollen tubes may grow slower than outcross-pollen tubes again due to self-incompatibility mechanisms of the plant, σ=0.01 or 100).

(4) Self- and outcross-pollen tubes sequentially fertilise ovules going down to the base of the fruit (due to step 3 above, self- and outcross-pollen tubes may arrive at the same time or outcross-pollen tubes may arrive first).

(5) Ovules signal to pollen tubes to be fertilised (strength of signal related to q, probability a pollen tube does not fertilise each ovule=1-q). A fertilised ovule is termed a zygote.

(6) For self-fertilised ovules, the proportion of zygotes which will go on to develop into seeds is g_s (the proportion of self-fertilised ovules aborted due to inbreeding depression is 1-g_s).

(7) For outcross-fertilised ovules, the proportion of zygotes which will develop into seeds is g_x (the proportion of outcross-fertilised ovules aborted due to inbreeding depression is 1-g_x).

(8) Both self- and outcross-fertilised ovules may abort due to resource limitation if there are not enough resources for all fertilised ovules to develop.

(9) Those zygotes which have not been aborted may continue to become fully developed seeds.

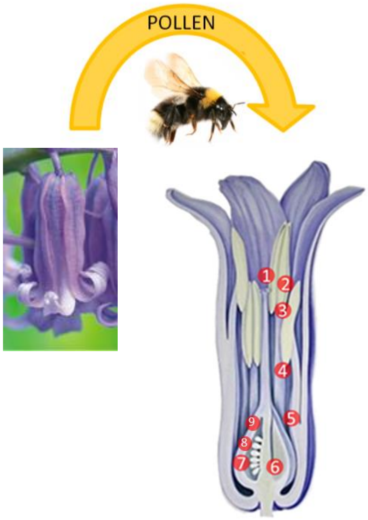


Figure 2.1: The sequence of events simulated (see table 2.1 for definitions of parameters).

We can therefore manipulate the degree of self-incompatibility, the degree of inbreeding depression, the degree of resource limitation, and the pattern of resource allocation, i.e. first-fertilised first-served or bottom-up resource allocation. For example, in a scenario where a mixture of self- and outcross-pollen grains landed on the stigma of an *H. non-scripta* flower, it is thought that some self-incompatibility mechanisms would occur. This is due to results from Corbet (1998), however, the exact mechanisms and how they are taking place has not yet been discovered in *H. non-scripta* to the best of my knowledge so it will suffice to know for this model that some occur.

We can alter the degree of self-incompatibility by altering the difference between parameters N_s (number of self-pollen grains that successfully germinate on the stigma) and N_x (number of outcross-pollen grains that successfully germinate on the stigma). It is then possible to simulate the degree of inbreeding depression by manipulating the parameters g_s (probability a self-fertilised ovule starts to develop into a seed after fertilisation) and g_x (probability an outcross-fertilised ovule starts to develop into a seed after fertilisation). Higher inbreeding depression would be simulated if g_s was much lower than g_x. It is possible to simulate the differential likelihood of fertilisation of ovules at positions 1-12 from the stylar end to the basal end of the fruit by manipulating q (probability a pollen tube accepts each free ovule's signal) and σ (standard deviation of order of fertilisation of ovules by pollen tubes). If σ is 100 then there is random fertilisation at all ovule positions, increasing q then increases the likelihood of fertilisation at all ovule positions equally. However, if σ is 0.01 then there is sequential fertilisation, and the ovules are sequentially fertilised from stylar ovule positions to basal positions. In this case, decreasing q increases the spread of the ovule positions fertilised by causing each ovule position to have decreased chance of attracting a pollen tube.

The model simulates seed abortion due to resource limitation by using the parameter O_{max} (the number of ovules that could be developed by amount of resources available). The number of ovules aborted due to inbreeding depression = O_{max} - the number of ovules fertilised. The model was able to simulate first-fertilised first-served resource allocation in its baseline state as ovule abortion due to resource limitation was highest at the ovules that were latest fertilised. For bottom-up resource allocation the probability of ovule abortion due to resource allocation was highest at the most stylar positions. However, the probability of abortion due to resource limitation was equal for self-fertilised and outcross-fertilised ovules, and so reflects the ratio of N_x to N_s in the model results (rather than the ratio of outcross-fertilised ovules developed to self-fertilised ovules developed). For each model simulation of different scenarios, the model ran 5,000 times and probabilities of ovule fates at each ovule position from 1-12 was calculated, and graphs could then be produced. See Appendix A for a copy of the code used to create the model and test graphs in R Studio.

In order to provide predictions of pollen maturation patterns for the later field experiment chapters, the way the model results were collated into graphs was changed. Here there were five seed maturation stages measured and calculated as proportions:

1) Ovules unfertilised (Proportion of ovules that were not fertilised out of total ovules).

2) Ovules expanded (Proportion of ovules that have just been fertilised out of total ovules, seen as an initial expansion, the ovule would be slightly larger than unfertilised ovules but not as large as developed ovules).
3) Ovules developed (Proportion of ovules that fully developed into seeds out of those that initially expanded).
4) Ovules aborted (Proportion of ovules that went on to be aborted out of those initially expanded. They have been aborted either due to inbreeding depression or limited resources).
5) Seed set (Proportion of ovules that fully matured into a seed out of total ovules. This is different to ovules developed).

These measurements relate to each other such that:

Ovules developed = Ovules expanded – Ovules aborted, and

Ovules unfertilised + Ovules expanded = 1

Additionally, the pollen quality and pollen quantity was changed for the results predictions so that they were varied by changing the ratios of self- and outcross-pollen. The ratios used were under a lower quantity 6, self-pollen: outcross-pollen ratios 0:6, 2:4, 4:2 and 6:0, and under a higher quantity 12, self-pollen : outcross-pollen ratios 0:12, 4:8, 8:4 and 12:0. The four ratios represent pollen qualities 1, 2/3, 1/3 and 0.

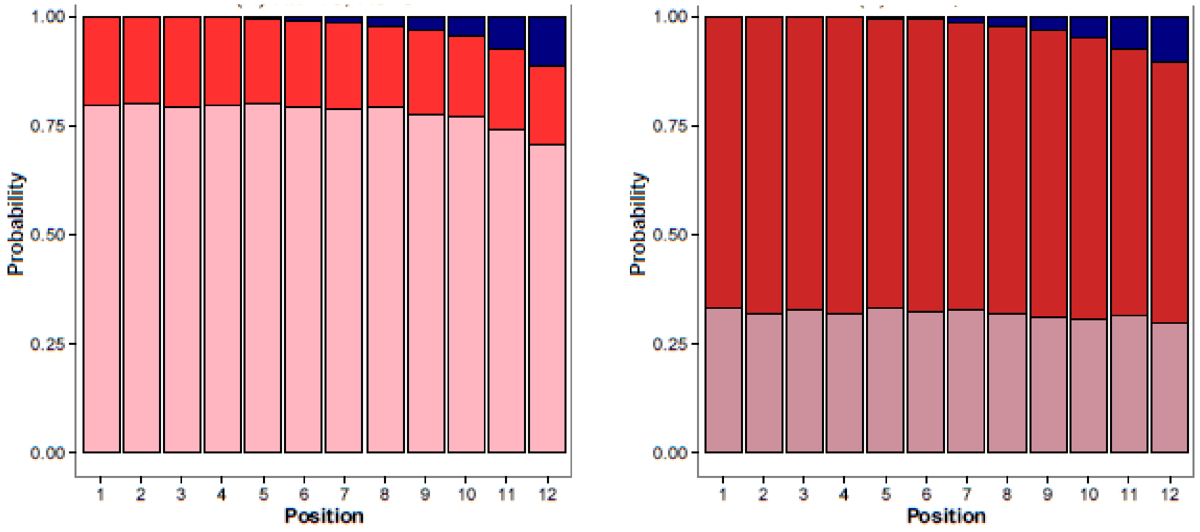
Results

Figures 2.2, 2.3 and 2.4 show the effects of altering model parameter values on ovule maturation patterns, and what is expected under experimental field manipulations. Figure 2.2 shows the outcome of changing factors relating to unfertilised ovules, seed development, and seed abortion due to inbreeding depression. In this case resources are not assumed to be limited. Figure 2.3 illustrates the effects of resource limitation. Figure 2.4 predicts seed development patterns that would be expected from manipulating pollen quality and quantity, which is to be done in field experiments.

Figure 2.2 illustrates large differences in seed set between selfed and outcrossed flowers. Panel A has exclusively outcross-pollen and B purely self-pollen, creating a flattened line of seed development in both. Outcross-fertilised ovules have a higher probability of survival after fertilisation than self-fertilised ovules as g_x (probability an outcross-fertilised ovule starts to develop into a seed after fertilisation) $> g_s$ (probability a self-fertilised ovule starts to develop into a seed after fertilisation), owing to a higher probability of inbreeding depression causing abortion in self-fertilised ovules. This creates a greater proportion of seeds developed in A than B. These seed proportions developed are the same values as g_x and g_s (i.e. 0.8 and 0.33) in positions where there is no probability of ovules being left unfertilised. However, development then drops towards the basal end due to pollen limitation. Panel C simulates an equal mixture of self- and outcross-pollen, which enters the ovary in a random sequence. This also results in flat seed development, as ovules down the ovary are fertilised randomly by self- or outcross-pollen; the proportion of ovules developed (when no ovules are left unfertilised) is averaged between g_x and g_s according to the ratio in which self- and outcross-pollination occurs. Here there are equal proportions of self and outcross, so the proportion of seeds developed is halfway between g_x and g_s, 0.57.

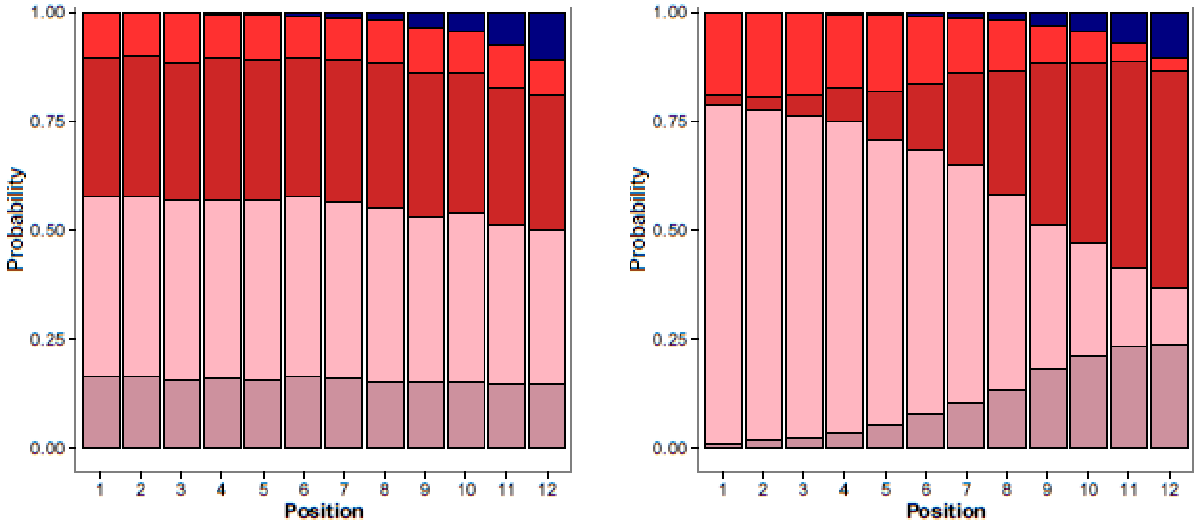
Panel D describes the same conditions as C but with a non-random sequence of outcross- and self-pollen entering the ovary. Outcross-pollen enters the ovary first, simulating differential pollen tube growth due to self-incompatibility mechanisms. Changing this one parameter greatly alters the pattern of development, increasing the downward gradient of the curve of seeds developed from the stylar fruit end to the base, which is the general pattern of seed development seen in a previous field study (Corbet, 1998). This gradient is due to a greater segregation of self- and outcross-fertilised ovules; the darker colours in the panel reveal an increasing proportion of ovules self-fertilised towards the base of the fruit.

Panels A to D have equal proportions of ovules left unfertilised (dark blue), but these are changed in panels E and F. The only parameter that has changed between these panels is q (probability a pollen tube accepts each free ovule's signal), which is effectively the strength of the attractive signal produced by the ovule; lower q results in a higher proportion of ovules being unfertilised. The proportion left unfertilised increases towards the basal fruit end due to ovules towards the stylar end capturing the limited number of pollen tubes. More interestingly, q (probability a pollen tube accepts each free ovule's signal) also influences the segregation between self- and outcross-pollen. The higher the q value, the more segregation occurs, so a low q value flattens the curve of proportion of seeds developed.



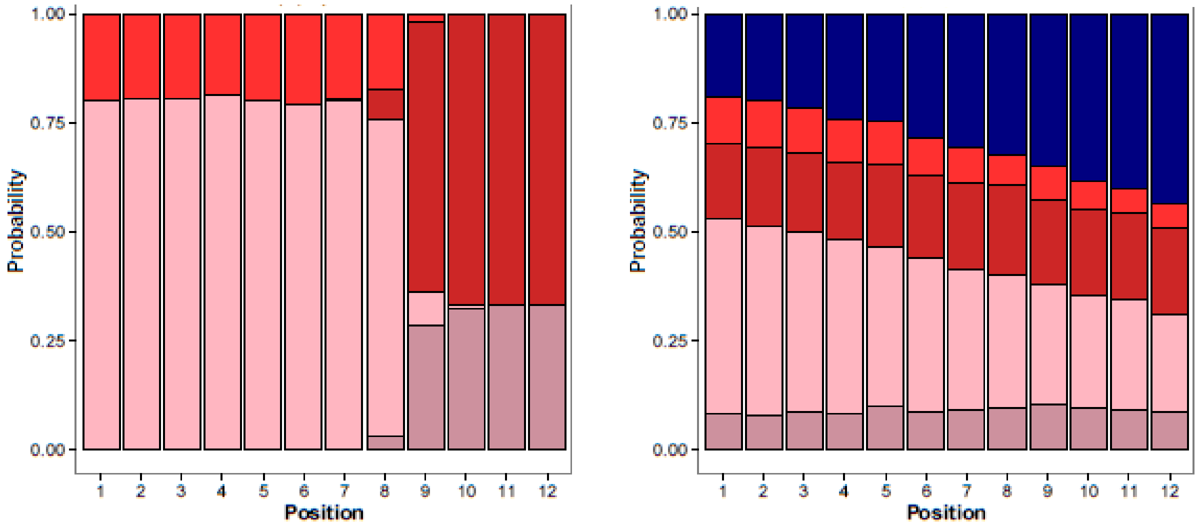
(A) N_x=16, N_s=0

(B) N_x=0, N_s=16



(C) σ=100

(D) Baseline parameters



(E) q=0.9

(F) q=0.1

Figure 2.2: Simulated seed development patterns averaged over 5000 fruit under changing conditions, but without resource limitation. All parameters are baseline values unless stated. Panels A and B are under complete outcross-pollen and self-pollen respectively. Panel C is under random allocation of self and outcross pollen tubes. Panel D is under baseline parameter values. Panels E and F are under high and low probabilities of each unfertilised ovule attracting a pollen tube. Blue=U, red=IDX, dark red=IDS, pink=DX, dark pink=DS.

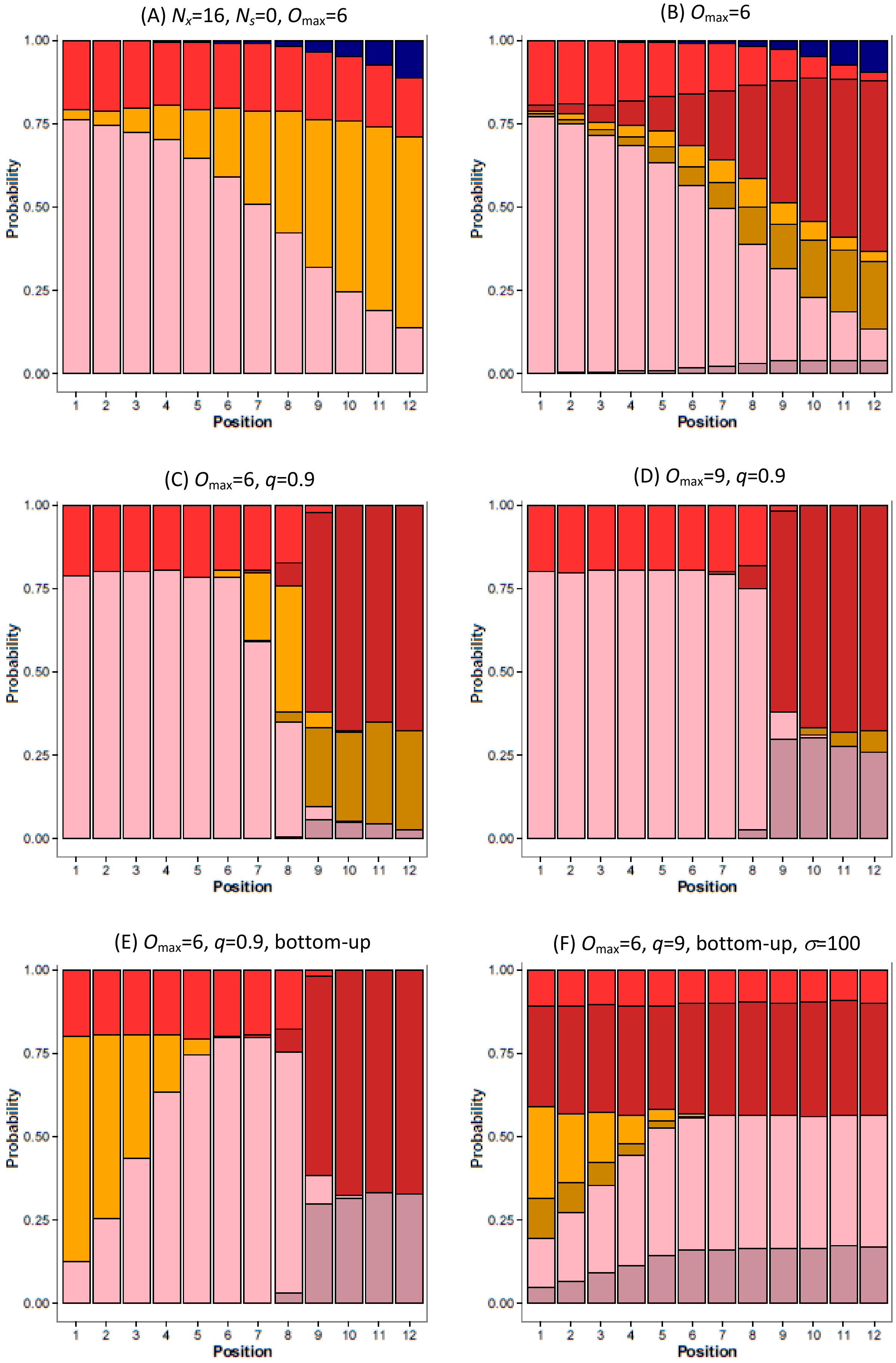


Figure 2.3: Simulated seed development patterns including resource limitation averaged over 5000 fruit under changing conditions. All parameters are baseline values unless stated. Panel A is under complete outcross-pollen with resource limitation. Panel B is under baseline parameter values with resource limitation. Panel C is under high probability an ovule attracts a pollen tube with resource limitation. Panel D is under high probability an ovule attracts a pollen tube under decreased resource limitation. Panel E is under high probability an ovule attracts a pollen tube, under bottom-up resource allocation and under resource limitation. Panel F is under high probability an ovule attracts a pollen tube, bottom-up resource allocation, and under resource limitation, with random allocation of self- and outcross-pollen tubes. Blue=U, red=IDX, dark red=IDS, orange=RLX, dark orange=RLS, pink=DX, dark pink=DS.

Figure 2.3 shows how resource limitation reduces the proportion of ovules that would otherwise have gone on to develop. Panels A and B in Figure 2.3 have the same parameters as panels A and D in Figure 2.2 apart from having the maximum of developing ovules, $O_{\max}$ (number of ovules that could be developed due to level of resource limitation) being reduced from 12 to 6. Here resources are allocated in the order of fertilisation. Resource limitation results in a decreased proportion of seeds developed especially towards the basal end. Resource limitation results in spatially similar seed development patterns as observed above and observed in the literature. Changing q (probability a pollen tube accepts each free ovule's signal) under this form of resource limitation adjusts the ovule positions likely to show abortion. Panel C shows that the ovules aborted by resource limitation are predominantly at the basal end, showing again that q is important in segregating effects within the ovary. Panel D shows that decreasing the amount of resource limitation in the fruit reduces the probability of ovules being aborted due to resource limitation.

Panel E in Figure 2.3 shows ovule fates when resources are preferentially allocated to the basal end of the ovary. Now, seed development drops at both ends of the fruit for different reasons. The ovules aborted due to resource limitation are now those at the stylar end and those aborted due to inbreeding depression and pollen-limitation increase towards the basal end. Bottom-up resource allocation also occurs in panel F, but the added variable of a random sequence of self- and outcross-pollen entering the ovary creates a different pattern. The proportion of seeds developed is flat at all positions, except for the fact that the bottom-up resource allocation results in a drop in seeds developed at the stylar end of the fruit. The random sequence of pollen entering the ovary desegregates the pollen fertilisations up the fruit which would have occurred due to the high q value (probability a pollen tube accepts each free ovule's signal).

The model is also used to predict possible patterns of seed development that would be observed in field data (i.e. when it is unknown if self- or outcrossed-fertilisation has occurred). Specifically, we plot the proportion of seeds expanded (due to fertilisation), seeds set, seeds aborted (out of those expanded), and the proportion of seeds developed (out of those initially expanded). Panels A to D in Figure 2.4 show the outcross- : self-pollen ratios 12:0, 8:4, 4:8 and 0:12 respectively, under high pollen quantities. Panels E to H show the same ratios, but under low pollen quantities which are 6:0, 4:2, 2:4 and 0:6. For panels A to D, there are very high proportions of ovules that have expanded, and as the proportion of self-pollen increases, the seed set decreases. The higher proportion of seeds aborted can be seen to creep from the basal end of the fruit to the stylar end as the proportion of self-pollen increases. In panels E to H half of all twelve ovules are expanded as there are only six pollen grains entering the ovary in all circumstances. Due to the high q value (probability a pollen tube accepts each free ovule's signal), the unfertilised ovules all lie at the base of the ovary. Panels E to H are very similar to A to D, except that as the proportion of self-pollen increases, the proportion of seed set decreases only from the more stylar position 6 towards position 1 rather than from position 12. This means that for the panels with mixtures of self- and outcross-pollen, F and G, the proportion of ovules aborted is highest in the middle of the fruit and drops off at both ends.

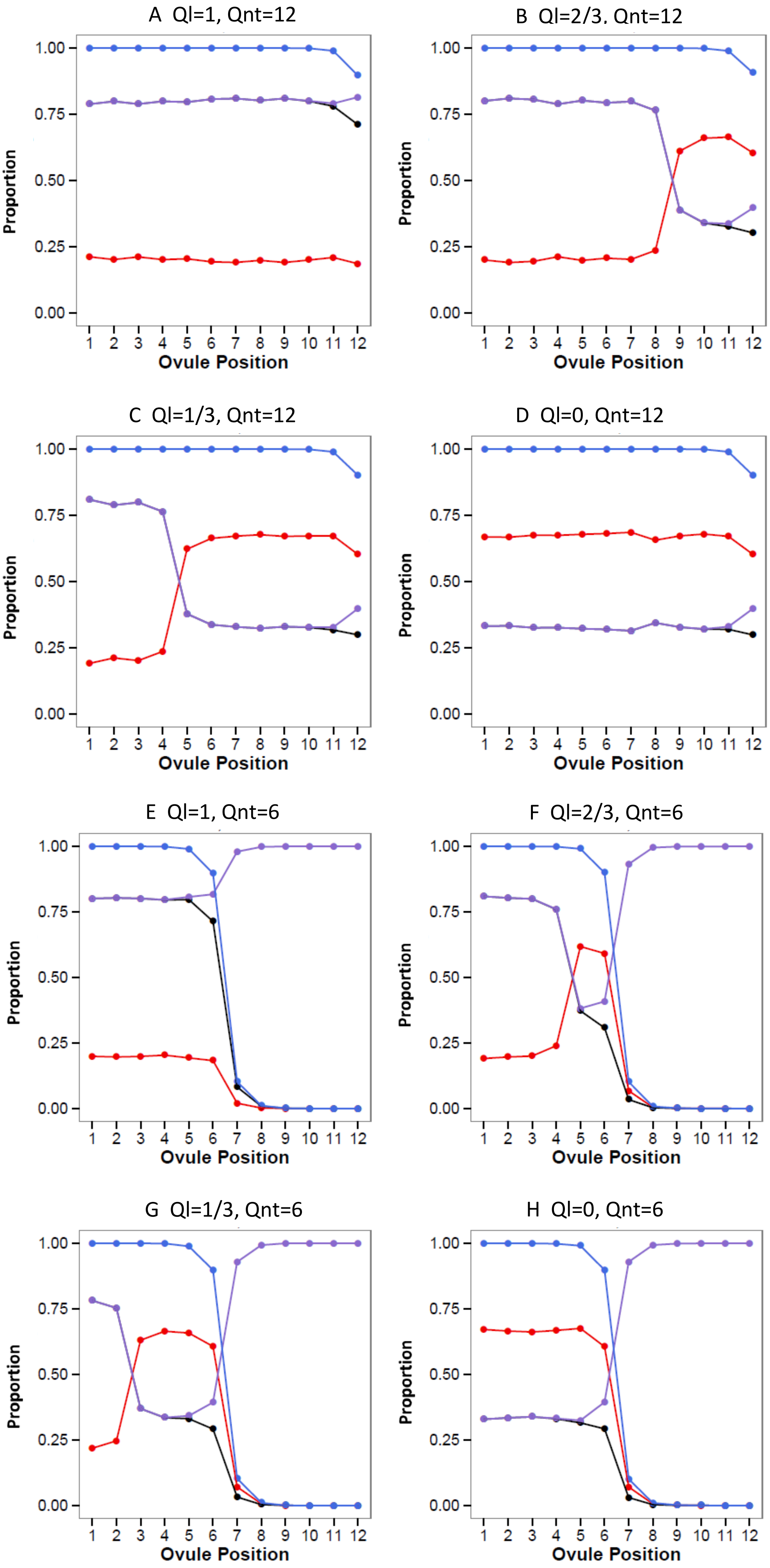


Figure 2.4: Simulated seed development patterns averaged over 5000 fruit under differing outcross- : self-pollen ratios and pollen quantities, which may be expected from the field data. Ql is pollen quality, referring to the ratios of self- to outcross-pollen, and Qnt is pollen quantity. In panels A-D pollen quantity is 12 pollen grains, and in panels E-H pollen quantity is 6 grains. Red=ovules aborted, black=seed set, purple=ovules developed, blue=ovules expanded. For A-H, *q*=0.9.

Discussion

The stochastic simulation model demonstrates several important factors that determine seed development patterns under pollen and resource limitation, but above all it demonstrates the importance of fertilisation quality. Outcross-pollen is of higher quality than self-pollen, as outcross-fertilised ovules are shown to produce a much higher proportion of seed set than self-fertilised ovules (Figure 2.2A, B). The higher the proportion of outcross-fertilised to self-fertilised ovules in the simulation, the larger the proportion of seeds that will go on to develop as seeds set. Assuming absolute seed production primarily determines plant fitness, it is in the plant's best interest to have the highest fertilisation quality possible for its ovules (Figure 2.2A).

Fertilisation from outcross-pollen tubes increases seed production. Some simulated mechanisms produce a better reproductive outcome for the plant than others. Considering the parameter *q* (probability a pollen tube accepts each free ovule's signal), the best reproductive output comes from a high value as this decreases the proportion of ovules left unfertilised and also increases the segregation of fertilisation qualities. Lowering unfertilised ovule numbers is beneficial to the plant as it increases chances of seed set. Therefore, under mixed pollen quality, a high *q* value produces the best outcome, as seen in Figure 2.2E. A high *q* value (probability a pollen tube accepts each free ovule's signal) increases the segregation of ovules fertilised as the model presumes that outcross-pollen tubes grow faster and reach ovules before self-pollen tubes. The resulting pattern of high segregation, where stylar ovules are purely outcross-fertilised and basal ovules purely self-fertilised, becomes advantageous under resource limitation, as the first-fertilised first-served resource allocation mechanism means basal embryos are more likely to be aborted. Consequently, under mixed pollen qualities, first-fertilised first-served resource allocation combined with a high *q* value are the optimum conditions for the plant, as these ensure self-fertilised embryos have the highest chance of being aborted while those left to develop are most likely outcross-fertilised. This is illustrated in Figure 2.3C, when compared to Figure 2.3E.

The results from the model also help with how to interpret seed development patterns, particularly by studying the curve delineating the proportion of seeds developed. The curve slopes downwards towards the base of the fruit in nearly all of the situations created. The strength of the downward slope can help reveal the distribution of pollen quality fertilisations down the fruit, as a strong slope indicates a high proportion of outcross-fertilised seeds at the stylar end of the fruit compared to the basal end. Outcross-fertilised seeds have a lower probability of abortion due to inbreeding depression than self-fertilised seeds, creating a slope under segregating conditions. Resource limitation would also create this decreasing slope when first-fertilised first-served resource allocation occurs, as it leads to a lowered probability of seeds developing at the basal end of the fruit. If the curve is low at both ends of the fruit with the highest seed set towards the middle, then it implies that resource limitation has occurred, suggesting that bottom-up resource allocation has taken place, as none of the other mechanisms modelled were able to produce this pattern (see Figure 2.3E).

Although two resource allocation mechanisms were investigated using the model, and first-fertilised first-served resource allocation can provide the optimal conditions for the plant, I think it would be plausible for the fruit to experience a different resource allocation mechanism. There must be a limit to the number of ovules produced, as there are finite ovules in each fruit. Presumably, therefore, there is a limit to the resources for producing them, and the resources may be slightly reduced in the most stylar ovules, even if there is still a general increase in development towards the stylar end due to better quality fertilisations. In this case, I suggest that ovules in linear fruit under mixed pollen quality loads experience a combination of principally first-fertilised first-served resource allocation, with a small amount of bottom-up resource allocation only at the extreme stylar positions. This could be termed “top-limited resource allocation”.

Results from previous studies support top-limited resource allocation. In Figure 7 in Corbet (1998), two of the three pruning treatments show that there was a general decrease in seed maturation % from the stylar end to the basal end, but the seed maturation % drops noticeably at the most stylar ovule (see Figure 2.5 A). Also in Bawa and Webb (1984), in graphs 3.1 (of *Bauhinia ungulata*) and 3.7 (of *Myrospermum frutescens*), we see that the probability of seed development drops towards the basal end of the fruit, but there is again a small but noticeable drop at the most stylar ovule (see Figure 2.5 B and C).

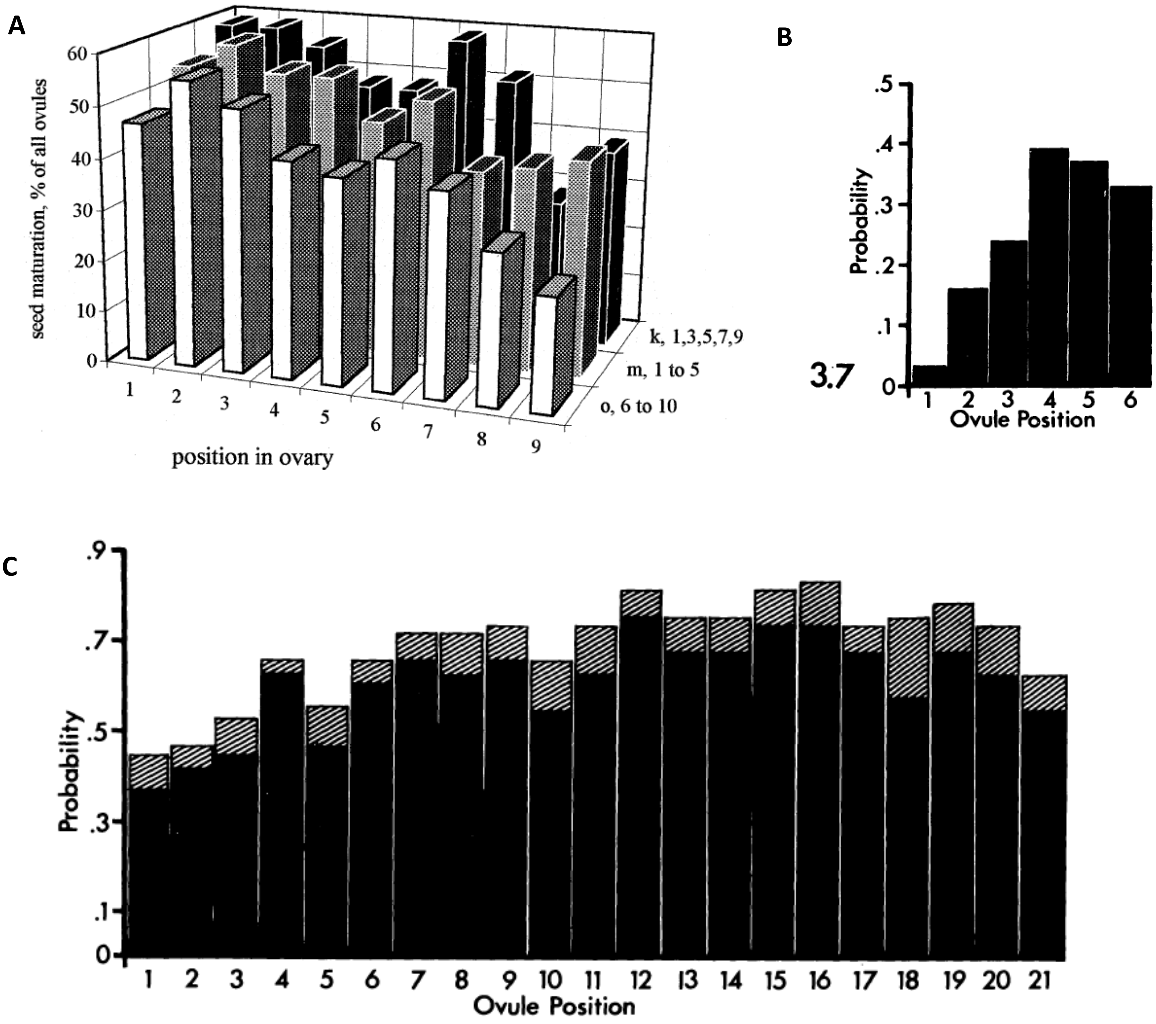


Figure 2.5: A – Effect of position in the ovary on percent of ovules producing mature seeds in relation to position in the ovary, from position 1 at the stylar end, in racemes pruned to five buds in alternate positions (ranks 1,3,5,7 and 9, k), basal positions (ranks1-5, m), or terminal positions (ranks 6-10, o), under cross-pollination (Figure 7 bottom graph from Corbet, 1998). B – Probability that a seed is developing for immature pods at different ovule positions, numbered from base (=1) to distal end, for *Myrospermum frutescens*, 67 immature pods (Figure 3.7 from Bawa and Webb, 1984). C – Probability that a seed has developed for mature pods at different ovule positions, numbered from the base (=1) to distal end, for *Bauhinia ungulata*, 38 mature pods, shaded area represents seeds partly developed but aborted (Figure 3.1 from Bawa and Webb, 1984).

The predictions presented in Figure 2.4 illustrate the optimum responses expected under hand-pollination experiments. Seed production was highest when *q* (probability a pollen tube accepts each free ovule's signal) was set high, which, as discussed, should give high seed development under mixed pollen loads. Under these conditions it should be possible to estimate g_s (probability a self-fertilised ovule starts to develop into a seed after fertilisation) and g_x (probability an outcross-fertilised ovule starts to develop into a seed after fertilisation) for *H. non-scripta* in the field, and it may even be possible to estimate whether *q* is as high as is predicted. Clearly, the most favourable scenario is one where the plant is not restrained by resource limitation, however, if resources are limited, the results from the field should allow recognition of which resource allocation mechanism is likely taking place, either: first-fertilised first-served, bottom-up, or possibly top-limited resource allocation, the combination of both allocation mechanisms.

The optimal plant strategy predicted by the model is inconsistent with some development patterns observed in the literature. A pattern of more abortions occurring in the stylar end of the fruit than in the middle or basal end of the fruit was observed in five species of tropical legumes (Wyatt, 1981). Our model suggests that this pattern may be caused by bottom-up resource allocation. Additionally, Linck (1961) observed that fruit of *Pisum sativum* (Pea; Fabaceae) had increased seed abortion rates at both ends of the pod. My model can explain this pattern being due to mixed, low amounts of self- and outcross-pollen fertilising ovules, combined with high resource limitation under bottom-up resource allocation, as seen in Figure 2.3E.

Nonetheless, the simulation model presented here generally agrees with seed development patterns observed in the literature, and has created deeper understanding of possible mechanisms of pollen and resource effects on seed maturation patterns. It has allowed prediction of results from field manipulations and will allow comparison between the real seed development of a plant to its ideal seed development in the model which can reveal how efficiently a plant produces seeds. However, the model could be improved in several ways.

The model presented here only considers seed development patterns at the fruit level, so future models should bring in more of the complexities of realistic fruit and seed development by considering patterns at the plant level. This would be achieved by incorporating ovule development in fruits at different positions up the raceme, and also investigating whole fruit development, and the effect of fruit position on this. Furthermore, future models should simulate effects of multiple pollinator visits to the stigma carrying varying pollen quantities and mixes of self- and outcross-pollen, which is more common in wild situations. It could also be modified to simulate the seed patterns of other plant species, in order to determine how plant species differ in seed maturation patterns, and in effects of pollen and resource limitation. If developed enough, and when verified by field data, this stochastic simulation model could prove extremely valuable to society by predicting the seed yield for crop plants with linear fruit, such as beans and peas, under changing pollinator abundances and changing environments.

Chapter 3 presents field experiments that test the model's predictions. Firstly, the model predicts that the higher amount of resources available, the more seeds will develop. This will be tested in Chapter 3 by freeing up plant resources via ovary removal. The results from Chapter 3 may allow the recognition of which resource allocation mechanism takes place out of those discussed in this chapter. It should also determine if resource limitation occurs in the *H. non-scripta* plants studied, and how altering resources available influences the seed development pattern.

Chapter 3

Manipulating resources available to developing seeds by removing flower ovaries in *H. non-scripta*.

Introduction

It is rare that all fruit or all seeds from a plant fully mature. Even under high outcross-pollination, some fruits and seeds may be aborted. Limited resources such as water or nutrients, and their distribution within the plant, may contribute to fruit and seed abortion. Resource limitation may result in the basipetal fruit development patterns often observed on inflorescences (Diggle, 1995; Ashman and Hitchens, 2000; Diggle, 2003; Cao *et al.*, 2011). In Chapter 2, I show how resource limitation may contribute to non-random seed maturation patterns in linear fruit. Investigating the prevalence of resource limitation and its link with abortion in natural communities is important because of its effect on plant fitness. Such studies could potentially be of use to many fields of plant research, such as agriculture and conservation (Ayre and Whelan, 1989; Bawa *et al.*, 1989; O'Donnel and Bawa, 1993; Ghazoul, 2005; Silveira and Fuzessi, 2014). This chapter looks for evidence of resource limitation occurring in a natural population of *H. non-scripta* and how it influences patterns of fruit and seed maturation.

For plants that produce inflorescences it is common for flowers to open in an acropetal sequence, from basal to distal flowers (Diggle, 1995, 2003; Ashman and Hitchens, 2000; Ortiz *et al.*, 2003; Zhao *et al.*, 2008; Cao *et al.*, 2011). Many acropetally-opening hermaphroditic species show a basipetal fruit development pattern and have a reduced female function in later flowers towards the apex of the inflorescence (Mazer and Dawson, 2001; Ishii and Sakai, 2002; Brookes *et al.,* 2010). This pattern of development is usually accompanied by reduced flower size and fruit set, and reduced ovule number and seed set towards the top of racemes (Herrera, 1991; Ashman, 1992; Ashman and Baker, 1992; Wolfe, 1992; Diggle, 1993, 1995, 1997; Brunet and Charlesworth, 1995; Guitián and Navarro, 1996; Ashman and Hitchens, 2000; Medrano *et al.*, 2000; Vallius, 2000; Kudo *et al.*, 2001; Ishii and Sakai, 2002; Buide, 2004; Guitián *et al.*, 2004; Kilber and Eckert 2004; Zhao *et al.*, 2008; Brookes *et al.*, 2010). These patterns are thought to result from a decline in the allocation of resources among distal flowers (Stephenson, 1981; Thompson, 1989;

Wolfe, 1992; Ashman and Hitchens, 2000; Vallius, 2000; Zhao *et al.*, 2008; Brookes *et al.*, 2010).

One likely explanation for the decrease in availability of resources towards the raceme apex is given by the resource pre-emption hypothesis, which contends two points (Zhao *et al.*, 2008; Brookes *et al.*, 2010; Cao *et al.*, 2011): firstly, earlier flowers towards the base of the inflorescence are pollinated first, so fruit and seeds develop and draw upon limited resources earlier. Secondly, basal flowers are closer to the source of resources, either leaves or root storage structures, and are therefore more able to garner resources than more distal flowers. Experiments changing the amount of resources available have demonstrated effects on the pattern of fruit development, which supports the resource pre-emption hypothesis. For example, Susko and Lovett-Doust (1999) found an increase in resource allocation to distal fruit when basal fruit were removed in *Alliaria petiolate* (Garlic Mustard; Brassicaceae).

Resource distribution also affects the non-random pattern of ovule maturation in fruit, as discussed in Chapters 1 and 2. Often a higher proportion of seeds set at stylar positions compared to a higher proportion of ovules aborted or unfertilised at basal positions. Previous papers have realised resources may be distributed to maturing ovules via the first-fertilised-first-served resource allocation or the bottom-up resource allocation mechanisms (see Chapter 2). However, the results of the model reported in Chapter 2 illustrated that the common non-random seed maturation pattern is most likely to result from the first-fertilised-first-served resource allocation mechanism. In this chapter I test this prediction by observing how manipulation of resources alters the seed development patterns within *H. non-scripta* fruit.

Furthermore, it is expected that resource manipulation will not affect the proportion of ovules fertilised (seen as ovules initially expanded). This thesis hypothesises that there are two main steps involved in an unfertilised ovule maturing into a seed, which are both affected by different environmental factors; pollen quantity and quality may affect the first step of ovule fertilisation, and resources and pollen quality may affect the second step of growth of a zygote into a fully developed seed. These deductions seem evident as pollen quantity typically alters the number of ovules fertilised, and pollen quality alters the number of ovules that expand through self-incompatibility mechanisms (acting prezygotically) (Aizen and Harder, 2007; Eckert *et al.*, 2010). Furthermore, resource abundance and allocation strategies may change abortion rates, and pollen quality may also alter ovule abortion through early-acting (pre-dispersal) inbreeding depression (Charlesworth and Charlesworth, 1987; Montalvo, 1992; Corbet, 1998; Aizen and Harder, 2007), hence both affect seed development. Therefore, it is hypothesised that resource manipulation will not affect the proportion of ovules fertilised (seen as ovules initially expanded) but will affect the proportion of ovules that go on to develop into seeds.

Many studies have observed that increasing resources (either through addition of nutrients or water, or when compared to reduction of foliage, fruit, or root storage structures) generally increases seed and fruit set in different plant species (Lee and Bazzaz, 1986; Gorchov, 1988; Ehrlén, 1992; Gedge and Maun, 1992; Koptur *et al*., 1996; Susko and Lovett-Doust, 1999; Yang *et al.*, 2005; Ne'eman *et al.*, 2006; Brookes *et al.*, 2008; Cao *et al.*, 2011). This has also been tested in *H. non-scripta*, where Corbet (1998) observed that raising available resources by fruit removal increased both fruit set and seed set. However, some plants can store resources in organs between seasons, for example bulbous perennial plants such as *H. non-scripta*, which may negate an effect of resource manipulation (Corbet, 1998). Furthermore, fruit that remains green until seeds are fully mature may produce sufficient photosynthate to support their seeds with minimal input from the maternal plant vasculature (Byrne and Mazer, 1990). As *H. non-scripta* plants demonstrate both traits, it is ambiguous whether resource manipulation through flower removal would have any effect on the fruit and seed production, at least in the same season (Corbet, 1998; Brookes *et al*., 2008).

Resources are crucial in fruit and seed production, and this study aims to testify their level of importance in a wild population of *H. non-scripta*. The resources available to each flower may decrease up the raceme, hence the effect of flower position was studied. Resources available to each ovule may decrease towards the stylar end of the fruit, hence the effect of ovule position was also studied. Resources were further manipulated within plants by removing ovaries of opened flowers before the fruit developed, hence increasing available resources to remaining ovaries. Plant density and flower number per inflorescence were noted as these could influence plant pollination and resource use. Plants were studied in three different sites within the same woodland to quantify any local variation in resource limitation.

Methods

Study species and site

A population of *H. non-scripta* was studied in Great High Wood, County Durham, United Kingdom (coordinates: 54°45'42.8"N 1°34'13.1"W). The woodland comprises semi-ancient oak and beech, where *H. non-scripta* were in high densities and had relatively large inflorescences made up of between 1 and 31 flowers which were observed. *H. non-scripta* are spring-flowering bulbous perennials which can reproduce by seed and bulb division (Blackman and Rutter, 1954; Wilson, 1959; Kohn *et al.*, 2009), and they are considered self-compatible to some extent (Corbet, 1998). Their insect pollinators are mostly bumblebees *Bombus* species and hoverflies, Syrphid family (Kohn *et al.*, 2009), but some butterflies of *Aglais* species and bee flies of *Bombyliidae* species were also spotted visiting the *H. non-scripta* flowers. Bulbs normally produce one inflorescence, so one inflorescence was considered analogous to one plant.

Plant manipulations

Inflorescences in three sites were tagged in pairs, every treated plant having a neighbour that was left untreated as the control plant. The pairs were randomly chosen, but had to be roughly the same size of inflorescence and be in the same stage of development, growing as close together as possible, usually less than 20cm apart. Both partners were left to be open-pollinated. In treated plants, half of the open flowers had stigmas and ovaries removed using forceps, while leaving the petals in place, which left a wound at the base of the flower. The petals were left in place in order to have a minimal effect on pollinator attraction.

Alternate flowers up the raceme were treated: for half of the plants, these were the odd flowers, and for the other half, these were the even flowers. All of the plants in each site were treated on the same day. This meant that some of the racemes did not have all of their flowers open, and treatment did not occur on unopened flowers.

There were three sites containing 30 pairs of plants in each. The sites were situated down a valley in the woodland, and were all at least 10m apart. The top-most site, 1, was treated first, on 22nd April 2015, the middle site, 2, on 4th May, and the bottom-most site, 3, on 14th

May. The racemes of fruit were collected when the fruit appeared to have reached full size but the seeds were still green and attached to the ovary axil. The racemes of both treated and untreated plants were collected from the top site on 28th May 2015, the middle site on 5th June, and the bottom site on 15th June.

Data collection and statistical analysis

Fruit position up the raceme and fruit fate were recorded. The fruit were recorded as either developed fruit, which had significantly increased in size compared to an undeveloped ovary (these had at least one developed ovule inside), or undeveloped fruit, which appeared not to have developed much or at all as determined from the ovary size (these had no developed ovules inside). Fruit development was therefore a categorical variable as they either developed or did not.

Flower positions were numbered from the base to the apex of the raceme, and included failed fruit. All three locules from the developed fruits were opened and had ovule number, position and fate recorded. The ovule positions were numbered from the stylar end to the basal end of the fruit. Ovules were numbered as the same positions in each locule as it was seen that ovules occurred in the same location in all three locules rather than alternatively along the fruit. The ovules fates were either:

1) undeveloped (these ovules appeared not to have grown at all)
2) partially expanded (these ovules appeared to have undergone an initial expansion but not grown as much as fully developed ovules, they were at least half as small as the fully developed ovules)
3) fully developed (these ovules had grown to the largest size).

Here, the ovules that had expanded partially were assumed to have had the potential to become fully developed if they were not limited by some limiting factor, as they had increased in size from the undeveloped ovaries. Additional factors were also measured: the density of bluebells with at least one flower open within $1m^2$ centred around each plant (plant density), and the number of flowers on the inflorescence (inflorescence size).

General linear mixed models were fit by maximum likelihood using the binomial family of the GLMER framework (logistic regression was performed). Generalised linear mixed models were used to evaluate how the plant identity (random factor), ovule or flower

position (covariate), treatment (discrete, fixed factor), site (discrete, fixed factor), plant density (covariate), and inflorescence size (covariate) impacted:
(1) fruit set (probability of a flower turning into a fruit)
(2) ovule expansion (probability of an undeveloped ovule growing into a partially developed ovule)
(3) ovule development (probability of a partially developed ovule growing into a fully developed ovule).

The formulas of the models used in the analysis were as follows:

1) Fruit set = flower position + treatment + flower position*treatment + site + plant density + inflorescence size + plant identity

2) Ovule expansion = ovule position + flower position + ovule position*flower position + treatment + flower position*treatment + site + plant density + inflorescence size + plant identity

3) Ovule development = ovule position + flower position + ovule position*flower position + treatment + flower position*treatment + site + plant density + inflorescence size + plant identity

The interactions between fruit or ovule position and the ovule expansion, and ovule development were also considered. Plant identity was treated as a random factor to deal with repeated sampling of flowers per plant. An independent unit in this experiment was specified as a plant. All analyses were completed using R version 3.1.2 (R Core Team 2014). The mixed models were fitted using “glmer” in the “lme4” package. The slope values of the effects were evaluated and coefficients with a *p*-value < 0.05 were considered to be statistically significant.

In order to display results in graphs, measurements were used for:
1) fruit set (proportion of flowers that matured into fruits)
2) ovules expanded (proportion of undeveloped ovules that matured into partially developed ovules)
3) ovules developed (proportion of partially developed ovules that matured into fully developed ovules)

4) ovules aborted (proportion of partially developed ovules that did not mature into fully developed ovules)
5) seed set (proportion of all ovules that matured into fully developed ovules).

Results

Fruit set

The probability of flowers setting fruit for untreated plants was very similar to that of treated plants, and the mean values were 0.53 ±0.008 and 0.56 ±0.009 (±SE), respecitvely. Probability of fruit development varied with flower position and site, but did not appear to change if the plant was treated or untreated (see Table 3.1 and Figure 3.1). Results from the generalised linear mixed model predict that flowers higher up the raceme, racemes at site 3, and flowers on smaller racemes, had a lower probability of fruit development; however, treatment and plant density had no effect on fruit set (see Table 3.1). As is clear in Figure 3.1, site 3 had a significantly lower probability of fruit set than sites 1 and 2.

Table 3.1: Results of a generalised linear mixed model of the effects of variables on probability of fruit development. d.f. indicated degrees of freedom associated with the likelihood ratio test. β is the estimated effect size.

Variable	*G*	d.f.	*P*	β
Flower position *Treatment	0.20	1	0.656	-
Flower position	290.87	1	<0.001	-0.325
Site	7.63	2	0.022	Site 2: -0.389 Site 3: -0.946
Treatment	0.80	1	0.372	-
Plant density	0.58	1	0.447	-
Inflorescence size	24.82	1	<0.001	0.187

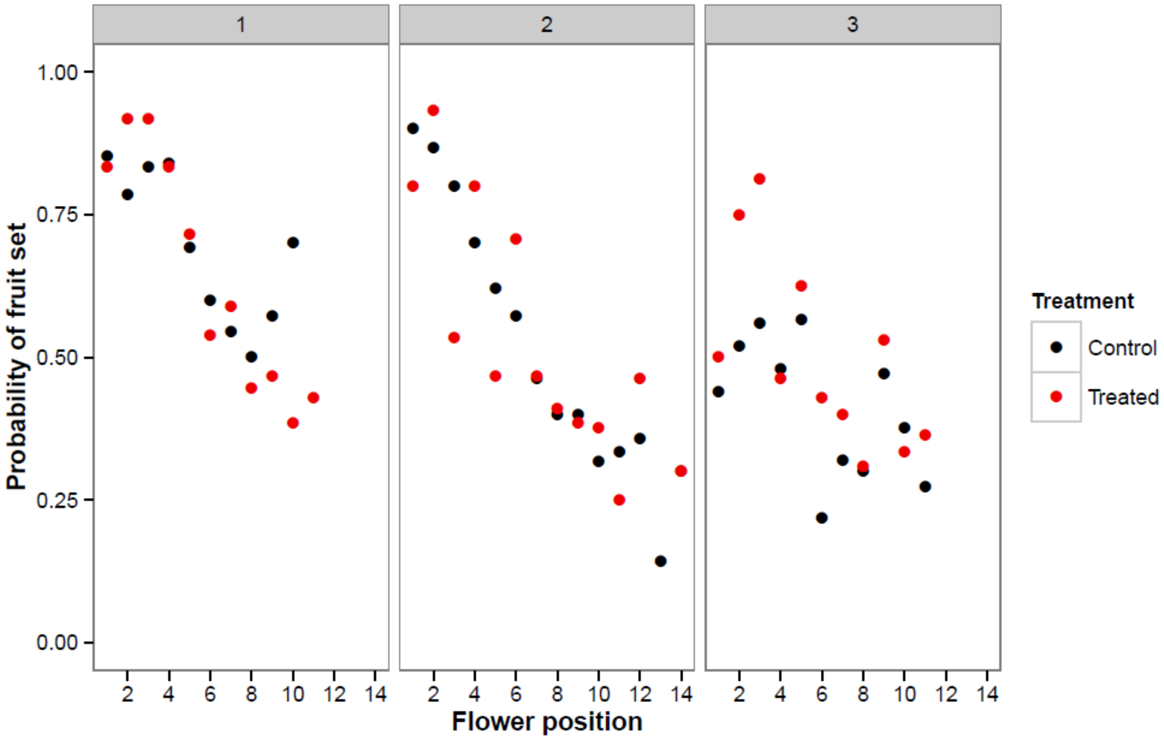


Figure 3.1: The probability of fruit development at absolute flower positions up the raceme (position 1 is at the base of the raceme, position 14 at the top) compared between control plants (black circles) and treated plants (red circles) at three sites. Treated plants had every other flower ovary of open flowers removed up the raceme. Panels represent plants at the numbered sites. For the control plants, n=772 fruit, and for the treated plants, n=500 fruit.

Ovule expansion and ovule development

The mean number of ovules per locule was 9.84 ±0.038. The generalised linear mixed models indicated that five variables: ovule position, treatment, plant density, site, and the interaction between flower position and treatment, affected the probability of ovules expanding, while only two, the ovule and flower position, affected the probability of ovules developing (once they had initially expanded) (see Table 3.2). It is interesting that flower position affected probability of ovules developing, but not the probability of ovules expanding, while the interaction between flower position and treatment only occurred in relation to ovules expanding, not to ovules developing. There was no interaction between ovule position and fruit position with relation to ovules expanded or developed, and the total number of flowers in the inflorescence had no significant effect on the probability of ovules expanding or developing. The parameter estimates imply that ovules had a higher probability of expanding at the stylar end of the ovary, in denser patches, at site 1 and 2 compared to site 3, if the plant was untreated, and in flowers higher up the raceme but only if the plant was treated. Ovules had a higher probability of becoming fully developed at the stylar end of the ovary, but also in fruit lower down the raceme. The mean probability of any ovule fully maturing into a seed in an untreated plant was 0.32 ±0.00041, and the mean probability of any ovule fully maturing into a seed in a treated plant was a similar value, 0.30 ±0.00049 (±SE).

Figure 3.2 illustrates the effect of flower position and treatment on ovule expansion and ovule development. The results from Table 3.2 and from Figure 3.2 together indicate that ovule expansion increased significantly with increasing flower position in treated plants, but ovule expansion was not affected by flower position in control plants. However, both treated and untreated plants experienced the same decreasing effect of flower position on ovule development but treatment had no effect on seed set (i.e. the proportion of ovules that fully develop out of all ovules).

Figure 3.3 shows how ovule position and treatment related to the proportion of ovules expanded and the proportion that developed. The results from the treated and untreated plants appear nearly identical, however, the proportion of ovules expanded may be slightly decreased in the treated plants. Figure 3.3 also shows that while ovule expansion and seed set clearly decrease with ovule position, ovule abortion and development seem unaffected by ovule position.

Table 3.2: Results of two generalised linear mixed models showing the effects of variables on ovules expanded and on ovules developed.

	Ovules Expanded				**Ovules Developed**			
Variables	***G***	**d.f.**	***P***	**β**	***G***	**d.f.**	***P***	**β**
Ovule position *Flower position	1.09	1	0.297	-	0.086	1	0.77	-
Ovule position	97.93	1	<0.001	-0.056	6.64	1	0.010	-0.031
Flower position	<0.01	1	1	-	5.69	1	0.017	-0.024
Flower position *Treatment	24.12	1	<0.001	Control* flower position: 0.015 Treated* flower position: 0.074	0.059	1	0.809	-
Treatment	5.91	1	0.015	-0.389	1.22	1	0.270	-
Site	22.76	2	<0.001	Site 2: -0.287 Site 3: -1.012	0.86	2	0.652	-
Inflorescence size	0.073	1	0.788	-	2.45	1	0.118	-
Plant density	5.086	1	0.024	0.009	<0.01	1	0.965	-

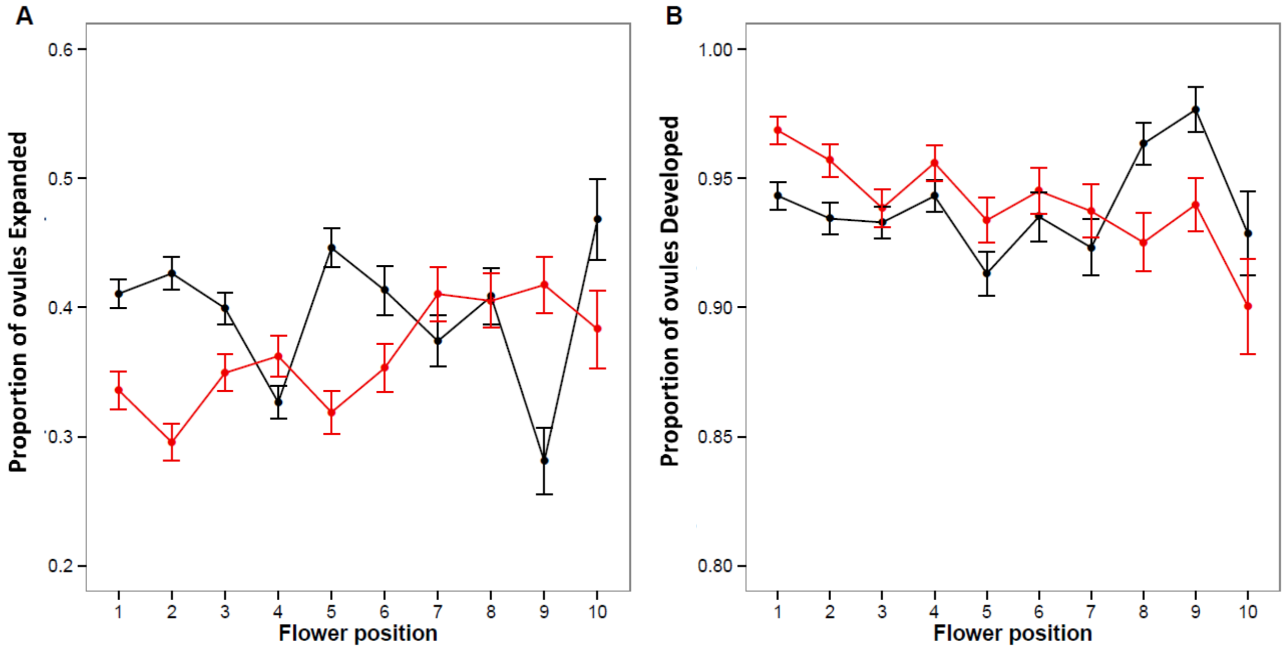


Figure 3.2: Effect of flower position on (A) mean proportion of ovules expanded, and (B) on proportion of ovules developed, between treated and untreated (control) plants. Black circles represent control plants and red circles represent treated plants. Error bars represent ±SE. For the control plants n=9841 ovules, for the treated plants, n=7387 ovules.

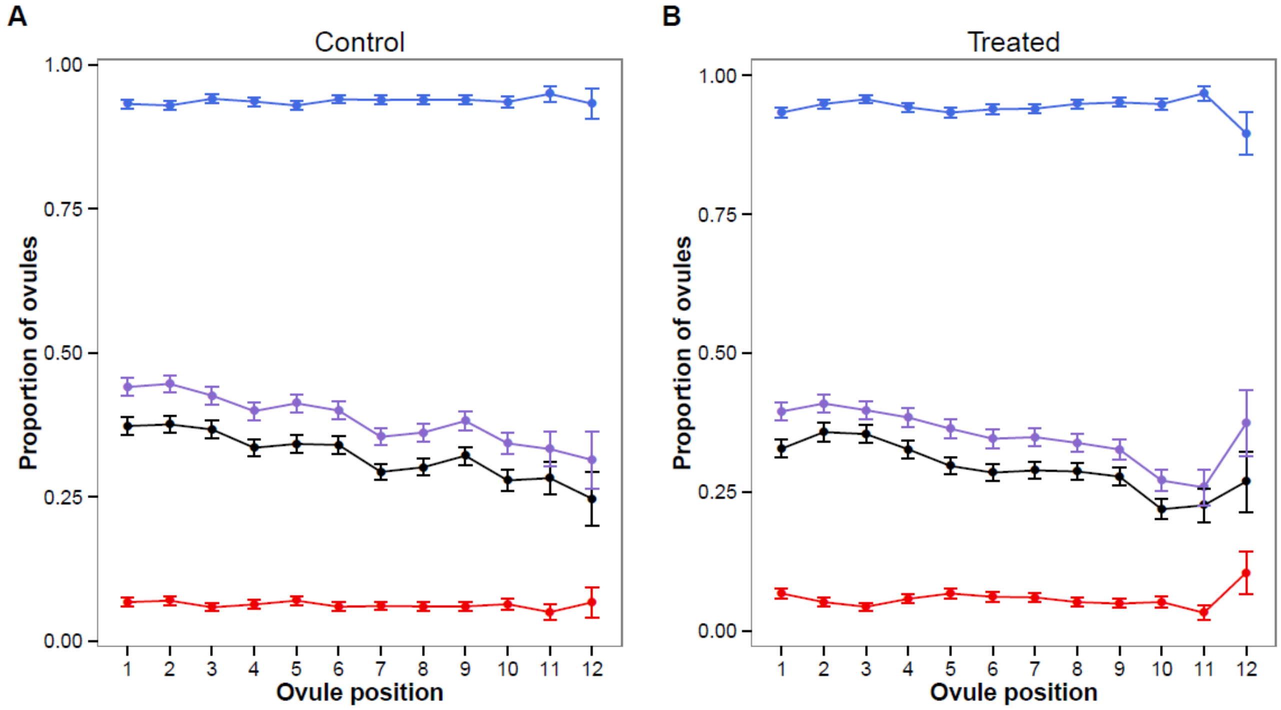


Figure 3.3: The effect of ovule position on the mean proportion of ovules under certain fates for (A) control plants and (B) treated plants. Red points represent the proportion of ovules aborted. Black points represent the proportion of seeds set. Purple points represent the proportion of ovules expanded. Blue points represent the proportion of ovules developed. Error bars represent ±SE. For the control plants, n = 10165 ovules. For the treated plants, n = 8140 ovules.

Discussion

The general pattern of fruit development and ovule development here is similar to that observed in the *H. non-scripta* population studied by Corbet (1998). Such patterns of higher fruit development at the base of the raceme and higher seed set at the stylar end of the fruit have also been seen in many other plant species (e.g. Lee and Bazzaz, 1986; Hossaert and Valéro, 1988; Lee, 1988; Nakamura, 1988; O'Donnell and Bawa, 1993; Diggle, 1995). The ovary removal treatment did not significantly affect ovule development; however, there was an interaction between the treatment and flower position on ovule expansion, which suggests that the removal of ovaries may have influenced pollination of the remaining flowers.

Ovary removal treatment

The limited effect of the removal of up to half of the ovaries on fruit or seed set could indicate several possibilities. Either there was no resource limitation in the plants tested, or the additional resources freed up by the treatment went elsewhere. It seems unlikely that there was no resource limitation in the plants, as some seeds and fruit aborted (although levels of seed abortion were low; see Figure 3.3) and there were differences between fruit development and ovule expansion between sites. However, there could be effectively little resource limitation within the fruit as they are green, and hence probably capable of photosynthesis (Todd *et al.*, 1961; Byrne and Mazer, 1990). Therefore, developing fruit and seeds may not be strongly reliant on maternally-donated resources, but may be predominantly self-dependent. A further consideration is that the bulbs could act as a buffer (Corbet, 1998; Brookes and Jesson, 2007; Brookes *et al.*, 2008), supplying additional resources where necessary and pooling surplus resources for future requirements. After removing ovaries, some resources allotted to fruit production were not needed, so could have been allocated back to the bulb. Another viable possibility is related to defence. The ovary removal treatment could have been perceived by the plant as an herbivorous attack, leading the freed-up resources to be used in defensive mechanisms such as by producing toxins, defensive proteins, and volatiles (Avila-Sakar *et al.*, 2001; Brookes *et al.*, 2008; Howe and Jander, 2008). Unfortunately, these possibilities could not be explicitly tested in the current study.

The removal of ovaries had no effect on fruit development or ovule development, and it had a slight negative effect on ovule expansion. These results are in contrast to those from an *H. non-scripta* population in West Cornwall (Corbet, 1998). In that study, Corbet (1998) stated that the proportion of seeds going on to mature after initiation in hand cross-pollinated plants and unpollinated plants was higher after the bud pruning treatments. The difference between that and the present study may possibly be explained by the contrasting experimental procedures. Corbet (1998) removed the flowers as buds from the plants, whereas in the present study, flower ovaries were removed after the flower had opened, in order to preserve the petals. Earlier bud removal could have given the plant time to re-adjust resource allocation to remaining buds compared to the later removal reported here. This is an interesting result as it suggests some possible time restraint to resource allocation in *H. non-scripta*.

However, in Corbet (1998), the removal of buds under open pollination, relatable to our study, did not show any effect of bud removal on fruit maturation (and fruit numbers were too low to evaluate seed initiation and maturation), although that might be due to reduced pollinator attraction. Furthermore, the generalised linear mixed models performed showed that bud removal had no significant effect on fruit initiation and maturation or on ovule initiation and maturation under any of the pollination treatments, which echoes the results from the present experiment. Studies focusing on a range of other plants do clearly show removing some flowers on those plant species increased seed production on the remaining flowers (see Lee and Bazzaz, 1986; Gorchov, 1988; Ehrlén, 1992; Yang, 2005). However, other papers indicate that resource manipulation may not have much of an effect on fruit or seed set on a bulbous plant such as *H. non-scripta* within a single season, but may have more of an effect in the following fruiting season due to the additional resources able to be stored in its bulb (Vaughton, 1991; Corbet, 1998; Brookes *et al.*, 2008).

It is predicted that the treatment may affect flower number, fruit development, and ovule development if these were tested in the following year. Ovule initiation may not be affected the following year as it is a stage that is dependent upon fertilisation quantity and quality, whereas the fruit and seed development stage is a very strong resource sink (Brookes *et al.*, 2010). As ovule development decreased with flower position up the raceme, it suggests an apical decrease in resources. There was found to be an interaction between flower position and treatment, as flower position had no effect on ovule expansion in untreated plants, but more basal flower positions had a negative effect on ovule expansion in treated plants (see Figure 2). This is odd as resources were only expected to affect ovule development, not expansion. It could therefore be assumed that the treatment may have resulted in altering pollinator behaviour, reducing the pollination levels of lower flowers (lower flowers were more likely manipulated if not all flowers were open when the plant was treated). For example, the manipulation may have led to a reduction in nectar production, resulting in reduced pollinator attraction.

Inflorescence size and plant density

Larger inflorescences were associated with a higher proportion of fruit developing, and plant density had a positive effect on ovule expansion. Increased flowers on a raceme, or display size, generally increases the number of plant visits by pollinators (Grindeland *et al.*, 2005; Ishii *et al.*, 2008). Similarly, insect plant visitation rate has been seen to increase with local plant density (Kunin, 1993; Mustajärvi *et al.*, 2001). Therefore, although inflorescence size and plant density may influence pollinator visitation in the same way, it is odd that the first has been found to affect fruit set and the second has been found to affect ovule expansion. Further experimentation between years may help to identify reasons for these contrasting effects, but it is clear both are related to pollination, and perhaps the proportion of ovules fertilised or fertilisation quality.

Site

Sites 1 and 2 had higher fruit set and ovule expansion than site 3. The sites differed in time of flowering as site 1 flowered slightly earlier than site 2, and site 3 flowered last (for an indication of the flowering time period between sites, see the dates that inflorescences were collected in the Methods section). The sites also differed spatially, as the sites were between 10 and 15m apart (horizontally) in a small valley in the woodland.

Temporal differences in flowering will be important for plant fitness as pollinator visitation typically varies within the season, which could be exacerbated by changing weather conditions. Furthermore, resource production could vary over time as light levels change with weather, altering photosynthesis in fruit or leaves. Habitat differences between sites could also affect pollination and resources. Site 3 was much more populated with heterospecifics, most noticeably by *Urtica dioica* (Stinging nettle; Urticaceae). The high density of *U. dioica* may influence resources available to *H. non-scripta. U. dioica* were seen to significantly shade *H. non-scripta* infructescences, possibly reducing photosynthesis*. H. non-scripta* plants did appear to be taller in site 3, however measurements were not taken. Although not tested, *H. non-scripta* stems may have grown through etiolation when shaded (Huber *et al.*, 2004). Thus, there are potentially a number of factors causing variation in resource availability and production for *H. non-scripta*, suggesting that the microenvironment may be an important source of variation in fruit and seed maturation. This is the case for all plants, as their sessile nature mean they experience environmental heterogeneity on a very fine scale (Huber *et al.*, 2004).

Conclusions

Resources are critical for fruit and seed production, and it is therefore important that we better understand how plants allocate resources to their developing fruit and seeds. This study has highlighted the need to incorporate knowledge of pollination and fertilisation limitation when investigating resource limitation, as our seed production data could not be fully explained by the resource manipulations alone. Other researchers agree that resources and fertilisation should be investigated in conjunction in order to fully understand each (Haig and Westoby, 1988; Ehrlén, 1992; Casper and Niesenbaum, 1993; Corbet, 1998; Yang *et al.*, 2005; Brookes *et al.*, 2008). This study has additionally indicated the importance of phenology when investigating resource limitation. By comparing results from our later removal of ovaries, to the earlier removal of buds by Corbet (1998), it appears that resource allocation to various functions including fruit and seed development varies over time. Therefore, any future studies should consider the age of the flower or fruit developmental stage when manipulating resources.

The present study has highlighted how variable seed production is in *H. non-scripta* across its range. Firstly, when comparing our results to those from Corbet (1998), open pollinated plants in Durham had much higher fruit set than those in West-Cornwall, which may be attributed to a lower pollinator visitation rate during the experimental period in West Cornwall. Secondly, the present results suggest that the Durham population has minimal resource limitation relative to Cornwall. Finally, it appears from the results presented in this chapter that the first-fertilised first-served resource allocation mechanism is taking place, as predicted in Chapter 2. This can be seen in Figure 3.3, as the probability of seed set clearly declines towards the basal end of the fruit.

Further studies would be beneficial to understanding resource allocation in this system. Simply the act of repeating the experiments over many more years would be useful, as pollination and resource levels in natural environments can vary significantly over time. Furthermore, the resource manipulations could be expanded to include a reduction of resources by removal of leaves early in the season before flower buds emerge. Resources could also be reduced by reducing light levels on the whole plant or exclusively on the fruit. Reduction of resources may provoke more of a response than increasing resources in terms of fruit and seed set (Brookes *et al.*, 2008), particularly as the *H. non-scripta* population appears to have very little resource limitation already. Chapter 5 provides additional avenues for future research investigating the role of resource limitation on plant fitness.

As mentioned above, pollen limitation is the accompanying partner to resource limitation in determining patterns of seed production. Chapter 4 therefore investigates pollen quality and quantity effects on seed production, which allows further testing of the predictions made by the simulation model presented in Chapter 2.

Chapter 4

Manipulation of pollen quantity and quality: altering ratios of self- to outcross-pollen in hand pollination experiments in *H. non-scripta*.

Introduction

Wild plants often exhibit high levels of seed, ovule and fruit abortions, which may be caused by pollen limitation (Nakamura, 1988; Mena-Alí and Rocha, 2005a; Alonso *et al*., 2013). Through influencing fruit, ovule and seed abortions, pollen limitation can then affect plant abundance and population viability (Knight *et al.*, 2005; Silveira and Fuzessy, 2014). Pollen limitation acts through two mechanisms: limited pollen quantity (enabled by a set ovule number) and low pollen quality (see Chapter 1). Surprisingly, pollen quality has received limited investigation in wild populations (Alonso *et al.*, 2013). Pollen quality and quantity limitations need to be better understood, as they are predicted to escalate with increasing habitat fragmentation, climate warming and pollinator decline (Aguilar *et al.*, 2006; Memmott *et al.*, 2007; Hegland *et al.*, 2009; Potts *et al.*, 2010; Winfree *et al.*, 2011; Gilman *et al.*, 2012). In this Chapter pollen quality and quantity are investigated conjointly in order to better understand their combined effect on fruit and seed abortions.

As discussed in Chapter 1, pollen quantity may limit seed and fruit production when there are not enough pollen grains to fertilise all of the available ovules. Pollen quality may limit seed and fruit production when ovules are fertilised by self-pollen or pollen from a closely-related plant. Inbreeding depression leads to selfed ovules having reduced growth or being aborted, and the abortion of ovules fertilised by lower quality pollen is termed early-acting inbreeding depression.

Research on pollen quality and pollen quantity has found interesting effects on fruit and seed production. For example, Snow (1986) showed that seed production in *Epilobium canum* (Zauschneria; Onagraceae) was not affected by additional pollen on the stigma after a certain adequate amount of compatible pollen was added. This finding suggests that pollen quantity can only increase seed production up to a certain level in *E. canum*. This makes sense as there are a finite number of ovules in fruit and ovule number would then be limiting seed production. Fruit set in *Myrtus communis* (Myrtle; Ericaceae) has been found to be mostly constrained by pollen quantity, whereas the seed set was largely limited by pollen quality (González-Varo *et al.*, 2009; González-Varo and Traveset, 2010). It is interesting to note that fruit and seed set can be constrained by differing aspects of pollen limitation.

However, many past papers are restricted to utilising only three pollen qualities, full outcross, full self, and open pollination (e.g. Corbet, 1998; Yang *et al.*, 2005; Hegland and Totland, 2008; Brookes *et al.*, 2010; Shi *et al.*, 2010; Fulkerson *et al.*, 2012; Abdala-Roberts *et al.*, 2014). All of these studies found a similar result, namely that self-pollination produced fewer or smaller seeds and fruit than outcross-pollination, and open pollinated plants produced results in-between that of self- and outcross-pollen. Only Harder *et al.* (2011) consider a fuller-range of pollen qualities, by varying ratios of self- and outcross-pollen. Their results for two species of Orchidaceae indicated that the genetic costs of selfing is better estimated using a range of self- and outcross-pollen mixtures than simply complete self- and outcross-pollen because the shape of the relation between the outcross fraction and the fraction of fertilisations and seed development are informative with regards to inbreeding depression and resource limitation. For example, they discovered that using a range of self- and outcross-pollen mixtures allows detection of a plateau indicating ovule limitation, when measuring ovule expansion in higher proportions of outcross-pollen. This may also allow detection of a plateau in seed set arising from resource limitation in predominantly outcrossed fruits. It is important to indentify plateaus indicating limitations as the relation between ovule development (in Harder *et al.*, 2011 termed zygote survival) and pollen mixture ratio only provides the necessary information on the inbreeding depression measurement when under fertilisation limitation (ideally pollen limitation rather than ovule limitation), not resource limitation.

In Chapter 3, *H. non-scripta* showed the general seed maturation pattern of higher seed development towards the stylar end of the fruit, and higher ovule abortion and unfertilised ovules towards the basal end of the fruit. However, that result was exclusively under open pollination, and only Corbet (1998) has tested seed development under self-, outcross- and open pollination in *H. non-scripta*. She observed that outcross-pollination produced highest seed and fruit development, followed by open pollination, and lastly self-pollination produced the lowest seed and fruit set. However, none have looked at how the seed development pattern changes with different pollen qualities and quantities.

This investigation aimed to shed more light on how pollen limitation can affect the development of seeds in linear fruit, and sexual reproduction and mechanisms in *H. non-scripta*. Pollen quantity and quality limitation in *H. non-scripta* was investigated by hand-pollinating flowers with a range of ratios of self- and outcross-pollen.

I test the hypothesis that increasing both pollen quality and quantity (in a range from below to above natural open-pollination conditions) will increase fruit set and seed set in *H. non-scripta*. It is hypothesised that increasing pollen quantity affects the proportion of ovules initially expanded, but not the proportion of ovules developed because fertilisations are limited by pollen quantity. Furthermore, increasing pollen quality affects the proportion of ovules developed because development is limited by inbreeding depression. Thus, it is hypothesised that the highest fruit set and seed set will be achieved under the conditions of the highest pollen quality and the highest pollen quantity when combined.

Pollen limitation is thought to be stronger in environments where pollinators are uncommon or unreliable (Ashman *et al.*, 2004; Burd *et al.*, 2009; Fulkerson *et al.*, 2012). Pollen limitation may therefore be quite strong in *H. non-scripta* which is pollinated in the very changeable weather of April-June in Britain, which directly affects pollinator numbers. Furthermore, hybridising, decreasing pollinator numbers, increasing habitat fragmentation and climate change may mean *H. non-scripta* becomes rarer and more pollen-limited in the future (Aguilar *et al.*, 2006; Memmott *et al.*, 2007; Hegland *et al.*, 2009; Potts *et al.*, 2010; Winfree *et al.*, 2011; Gilman *et al.*, 2012). It is therefore of interest to investigate the role of pollen limitation in *H. non-scripta*.

Methods

Study species and site

A population of *H. non-scripta* was studied in Great High Wood and Little High Wood, County Durham, United Kingdom (54°45'42.8"N 1°34'13.1"W in Great High Wood, and 54°45'54.8"N 1°34'26.6"W in Little High Wood). Semi-ancient oak and beech woodland are present in both locations, but *H. non-scripta* plants appeared to grow in higher densities and be composed of larger inflorescences in Great High Wood, although data were not taken to support this.

Plant manipulations

Inflorescences were tagged in pairs in five separate areas that were at least 10m apart in a small valley in Great High Wood and one 10m^2 area in Little High Wood. In Great High Wood, both plants in the pairs chosen were neighbours with similar numbers of flower buds in a similar stage of development (e.g. see Figure 4.1). One plant in each pair was left as a control, while the other plant was treated by hand-pollination with self- and outcross-pollen. The control plants were left to be open pollinated. There were 96 plant pairs in total. Each ratio of self- to outcross-pollen was designated to eight plants, five in Great High Wood, and three in Little High Wood. The partner plants were not neighbours in Little High Wood as they were not abundant or dense enough. The treated plants were manipulated in two stages, firstly before any flowers opened, the stamens were removed from the bottom-most flower buds, dependent upon how many were present (usually three or four had stamens removed) with one lower flower bud left with its stamen to act as the self-pollen donor flower. As soon as this was performed, hand-made plastic mesh-netting bags were placed over individual racemes and secured below the raceme with a garden tie around the plant stalk (see Figure 4.1). The net bags were put in place to block pollinators from the flowers, and were left on until the flower buds matured and the anthers on the self-pollen donor flower had dehisced. At that point (usually at just over a week), the hand-pollinations were performed.

Figure 4.1: Pair of plants tagged for the experiment. Yellow dotted line indicates inflorescence bagged, to be hand pollinated. Yellow solid line indicates inflorescence not bagged, left out to be open pollinated. Both inflorescences are a similar size and in the same stage of development.

The hand pollinations composed of mixtures of self- and outcross-pollen. The hand pollinations were performed using short lengths of fishing line to collect a set amount of pollen, and touch the stigma of the flower once – this was counted as one prod. One prod was thought to be the amount required to cover the total tip of the fishing line in pollen, although pollen grains were not counted, and must have been quite variable. The number of prods could then be increased to reflect the ratios of self- to outcross-pollen stated below. Each prod was performed using a new, clean length of fishing line. There were twelve ratios of prods of self- to outcross-pollen that were performed. These were (self : outcross) low quantities of 0:1 and 1:0, middle quantities of 0:4, 1:3, 2:2, 3:1 and 4:0, and high quantities of 0:8, 2:6, 4:4, 6:2, and 8:0, which varied both the quantity and quality of pollen deposited. The order of self-pollen and outcross-pollen prods was random in order not to affect pollen set. The hand pollinations were performed only on flowers which were open and had had their stamen removed to prevent additional self-pollen touching the stigma. The self-pollen was sourced from the appointed flower on the raceme, and the outcross-pollen was sourced from a mixture of random flowers at least 20 m away from the focal areas. The self-pollen and mixed-source outcross-pollen were freshly gathered each day, and were placed into eppendorf tubes. After the hand-pollination had been performed, the bag was replaced on the treated plant until the fruit had reached its full size. The racemes of the treated and control plants were then collected.

In Great High Wood the plants in areas 1, 2 and 3 were hand pollinated between 24th April and 27th April 2015 and were harvested on 1st June 2015. The plants in area 4 in Great High Wood were hand pollinated on 1st May 2015 and harvested on 5th June 2015. The plants in area 5 in Great High Wood were hand pollinated between 5th and 8th May 2015 and were harvested on 13th June 2015. In Little High Wood the plants were hand pollinated between 6th and 9th May 2015 and were harvested on 13th June 2015.

Data collection and statistical analysis

From the racemes gathered, the fruit and ovules inside the fruit were counted, classified and their positions noted. The fruit were recorded as either developed fruit, which had significantly increased in size compared to an undeveloped ovary (these had at least one developed ovule inside), or undeveloped fruit, which appeared not to have developed much or at all, as gauged from the ovary size (these had no developed ovules inside). Flower positions corresponded to flower positions and were numbered from the base to the apex of the raceme, and included failed fruit. All three locules from the developed fruits had ovule number, position and fate recorded. The ovule positions were numbered from the stylar end (position 1) to the basal end of the fruit. The ovules fates were categorised in the same way as in Chapter 3, i.e. either:

1) undeveloped (these ovules appeared not to have grown at all)
2) partially expanded (these ovules appeared to have undergone an initial expansion but not grown as much as fully developed ovules, they were at least half as small as the fully developed ovules)
3) fully developed (these ovules had grown to the largest size).

The ovules that had partially expanded were assumed to have had the potential to become fully developed if they were not limited by some limiting factor, as they had increased in size from the undeveloped ovaries. Data from plants at all of the areas were combined as within-population variation was not a covariate that explicitly formed part of our hypothesis.

Generalised linear mixed models were fit by maximum likelihood using the binomial family of the GLMER framework (logistic regression was performed). Generalised linear mixed models were used to evaluate how the flower or ovule position (covariate), pollen quantity (discrete, fixed factor for fruit development, continuous fixed factor for ovule expansion and development), pollen quality (discrete, fixed factor for fruit development, continuous fixed factor for ovule expansion and development) and plant identity (random factor) impacted:

1) fruit development (probability of a flower growing into a fruit)
2) ovule expansion (probability of an undeveloped ovule growing into a partially developed ovule)
3) ovule development (probability of a partially developed ovule growing into a fully developed ovule).

Pollen quantity and pollen quality were treated as continuous fixed factors when analysing ovule expansion and development as the pre-analysis data plots (not shown) did not suggest they had discrete non-linear effects. In order to display results in graphs, measurements were used for:

1) Fruit set (proportion of flowers that matured into fruits)
2) Ovules expanded (proportion of undeveloped ovules that matured into partially developed ovules)
3) Ovules developed (proportion of partially developed ovules that matured into fully developed ovules)
4)Ovules aborted(proportion of partially developed ovules that did not mature into fully developed ovules)
5) Seed set (proportion of all ovules that matured into fully developed ovules).

The formulas of the models used in the analysis were as follows:

1) Fruit set = pollen quantity + pollen quality + pollen quantity*pollen quality + flower position + plant identity

2) Ovule expansion and ovule development in open pollinated flowers = ovule position + flower position + ovule position*flower position + plant identity

3) Ovule expansion and ovule development in hand pollinated flowers = ovule position + flower position + pollen quantity + pollen quality + plant identity

The interaction between fruit and ovule position for the fruit development, and ovule expansion and ovule development in open pollinated plants, were also considered. An interaction was not tested for with respect to ovule expansion and development in the hand pollinated plants as there were fewer data in those experiments and the pre-analysis data plots (not shown) did not indicate any complex interactions. An independent unit in this experiment was specified as a plant. All analyses were completed using R version 3.1.2 (R Core Team, 2014). The mixed models were fitted using “glmer” in the “lme4” package. Coefficients with a *p*-value < 0.05 were considered to be statistically significant.

For the hand-pollinated plants, inbreeding depression estimations were able to be calculated by using the equation:
Inbreeding depression = $(g_x - g_s)/g_x$,
where g_x is the mean proportion of seeds set under a pure outcross-pollen ratio and g_s is the mean proportion of seeds set under a pure self-pollen ratio. These were estimated by using the results of the seed set under each pollen quantity for the full outcross- and full self-pollen ratios. The differences between the estimations were then able to be compared.

Results

Fruit set

Pollen quality was the only factor that affected fruit set (Table 4.1). Pollen quantity and flower position did not affect fruit set, and there was no interaction between pollen quantity and pollen quality affecting fruit set. The probability of fruit set declined going up the raceme for open pollinated flowers (Figure 4.2A), however, the hand pollinated flowers were all towards the base of the raceme so the effect is not seen for hand pollinated flowers. The probability of fruit set was similar under all measured quantities of pollen, which lies around the same values as that for open pollinated flowers. In Figure 4.2B, all of the pollen qualities result in a high probability of fruit set, nearly all of which lie above the fruit set probabilities of open pollinated flowers (c.f. effect sizes, β, in Table 4.1). However, flowers pollinated exclusively with self-pollen resulted in much lower fruit set probabilities than open pollinated flowers. From the hand pollinations, the mean proportion of purely selfed flowers developing into fruit was 0.13 ±0.015 (±SE), and the mean proportion of plants pollinated with some amount of outcross-pollen developing into fruit was 0.63 ±0.021.

Table 4.1: Results of generalised linear mixed model of the effects of variables on the probability of fruit set. d.f. indicated degrees of freedom associated with the likelihood ratio test. β is the estimated effect size.

Variables	*G*	d.f	*P*	β
Pollen quality *pollen quantity	7.41	4	0.116	-
Pollen quality	42.77	4	<0.001	Quality fraction 0.25: 3.84 Quality fraction 0.50: 3.83 Quality fraction 0.75: 3.85 Quality fraction 1.00: 3.85
Pollen quantity	0.10	1	0.752	-
Flower position	1.98	1	0.160	-

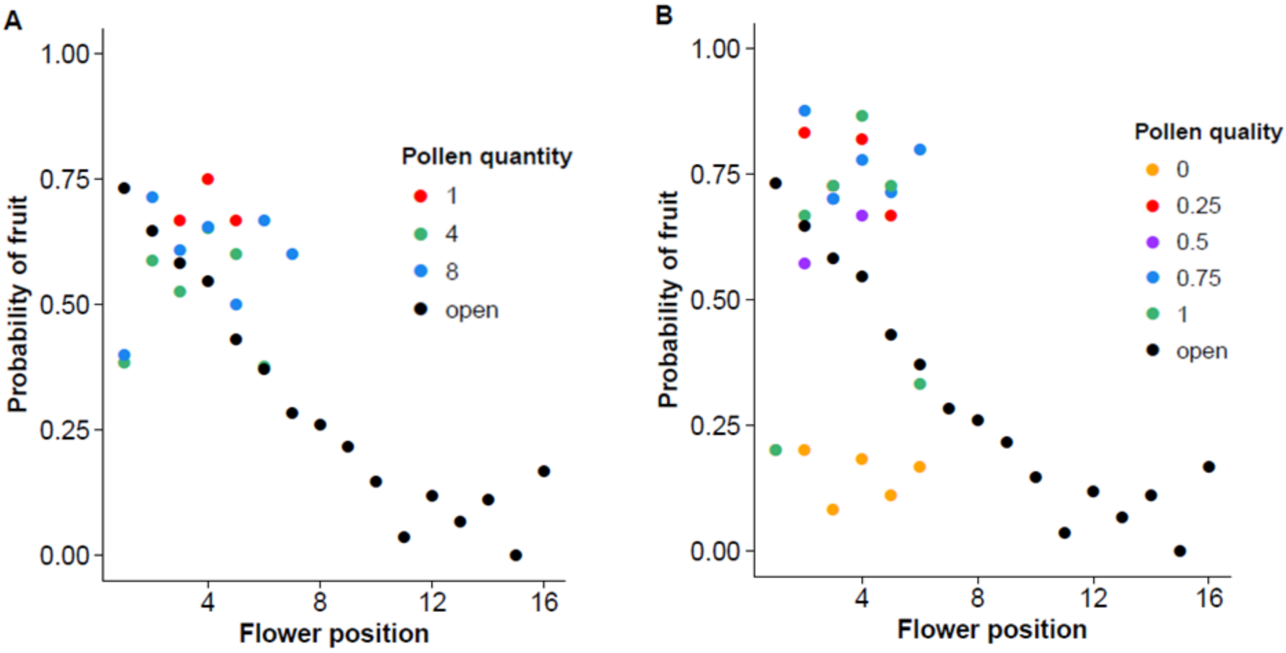


Figure 4.2: (A) Probability of fruit set versus flower position for hand pollinated *H. non-scripta* flowers under varying pollen quantities compared with open pollinated flowers, and (B) under varying pollen qualities compared with open pollinated flowers. Pollen qualities refer to the fraction of outcross-pollen compared to self-pollen in the hand pollinations. In A, for hand pollinated flowers, considering numbers of fruit tested, for pollen quantity 1 n=32, for quantity 4 n=98, for quantity 8 n=112. In B, considering pollen qualities, for pollen quality 0 n=54, for quality 0.25 n=45, for quality 0.5 n=41, for quality 0.75 n=45, for quality 1 n=60. For open pollinated plants in A and B, the number of fruits tested for open pollinated plants was n=671.

Open pollinated flowers

Both ovule expansion and ovule development (ovule development as a proportion of ovules expanded) in open pollinated control plants varied with ovule position and flower position (see Table 4.2). However, the effect of flower and ovule position on the probability of ovules developing was statistically less significant than their effect on the probability of ovules expanding. Ovules towards the basal end of the fruit were less likely to expand, and were slightly less likely to develop (Figure 4.3). Furthermore, ovules were more likely to expand at higher flower positions, but were more likely to develop at lower flower positions (Table 4.2). There was no evidence of an interaction between ovule position and flower position in regards to either ovule expansion or ovule development (Table 4.2). The mean proportion of all ovules developed into seeds in open pollinated plants between flower positions 1 and 5 was 0.34 ±0.0012 (±SE).

Figure 4.3 shows low probabilities of ovules aborting at all ovule and flower positions. It also shows a clear overall decrease in the probability of ovule expansion towards the basal end of the fruit, however there does seem to be an initial rise in probability of ovule expansion through the first three stylar positions. The probability of ovule development very slightly decreased with ovule position at most flower positions.

Table 4.2: Results of generalised linear mixed model on open pollinated control plants of the effects of covariates on the probability of ovule expansion and ovule development given expansion.

	Ovules expanded				**Ovules developed**			
Covariates	***G***	**d.f.**	***P***	**β**	***G***	**d.f.**	***P***	**β**
Ovule position *Flower position	0.10	1	0.750	-	0.003	1	0.955	-
Ovule position	43.10	1	<0.001	-0.042	7.54	1	0.006	-0.039
Flower position	54.21	1	<0.001	0.066	8.07	1	0.005	-0.052

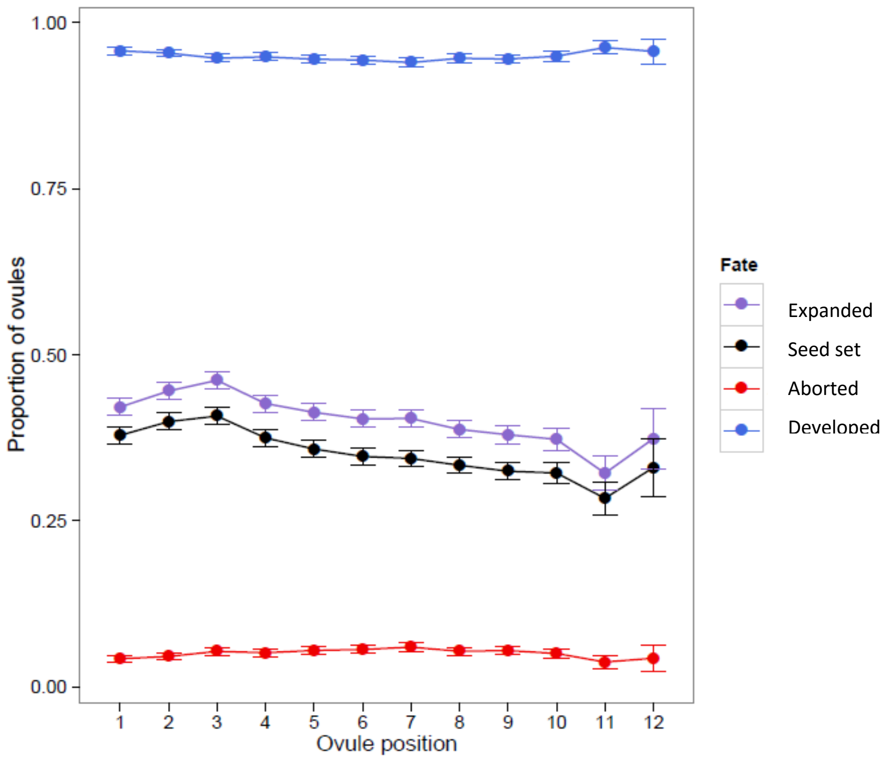


Figure 4.3: In open pollinated flowers, mean proportion of ovules expanded, developed, aborted, and seed set at ovule positions 1 to 12 inside the fruit, from the stylar end (from position 1) to the basal end (at position 12). Points represent the mean ±SE. n=14,038 ovules.

Hand pollinated flowers

Ovule expansion varied with ovule position, pollen quantity and pollen quality, but flower position had no effect (see Table 4.3). However, none of the variables affected the proportion of ovules developed. The proportion of ovules expanding decreased towards the basal end of the fruit. Higher levels of pollen quantity and pollen quality both increased the proportion of ovules expanding (Figures 4.4 and 4.5). The pattern of ovule maturation at different ovule positions under hand pollination is similar to that under open pollination (c.f. Figures 4.4 and 4.3). By comparing the seed maturation pattern in open pollinated flowers from Figure 4.3 to that of hand pollinated flowers from Figure 4.4, we may estimate that open pollination results in a pollen quality deposited somewhere around 0.5, and a high pollen quantity. This corresponds to approximately 50% of the pollen deposited being outcross in open pollination of *H. non-scripta*.

Interestingly, as seed development escalated with both pollen quality and quantity, this leads to the proportion of seeds developed under pure self-pollen and pure outcross-pollen increasing with increasing amounts of pollen (Figure 4.5). This means estimates of g_s and g_x increase with pollen quantity and estimates of inbreeding depression decrease with pollen quantity (Table 4.4).

Table 4.3: Results of generalised linear mixed model on hand pollinated plants of the effects of variables on the probability of ovule expansion and ovule development. Pollen quantity and quality were treated as continuous variables as pre-analysis data plots (not shown) did not suggest they had discrete non-linear effects. An interaction was not tested for because there were fewer data for the hand pollination experiments and the pre-analysis data plots (not shown) did not indicate any complex interactions.

	Ovules expanded				**Ovules developed**			
Covariates	***G***	**d.f**	***P***	**β**	***G***	**d.f**	***P***	**β**
Ovule position	24.65	1	<0.001	-0.043	0.48	1	0.488	-
Flower position	1.029	1	0.310	-	0.68	1	0.409	-
Pollen quantity	13.32	1	<0.001	0.172	0.49	1	0.485	-
Pollen quality	18.08	1	<0.001	1.352	0.58	1	0.448	-

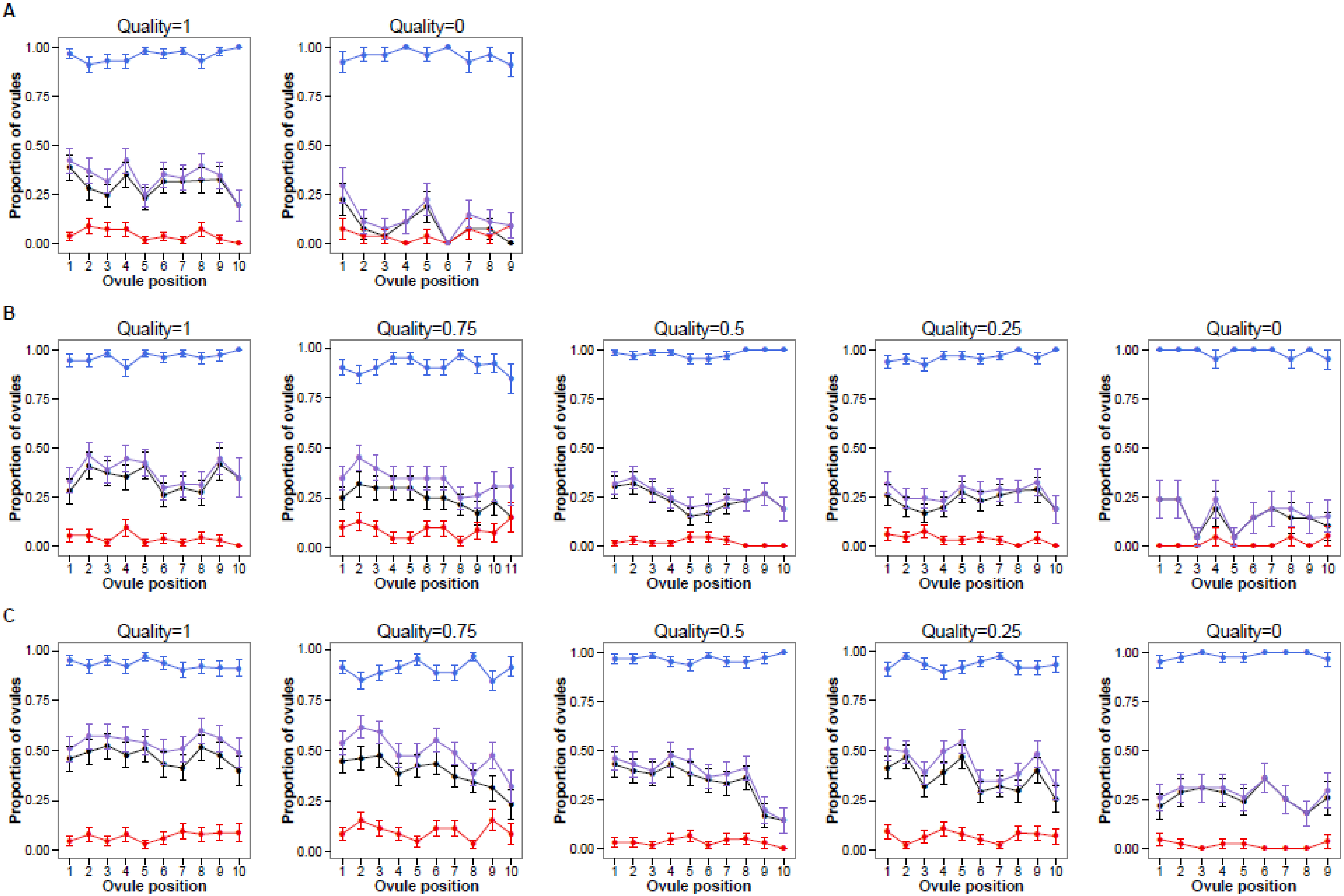


Figure 4.4: The effect on mean probability of seed fates occurring by ovule position. Pollen quantity is 1 for all panels in row A, is 4 for all panels in row B and is 8 for all panels in row C. Pollen qualities decrease along the rows. Purple represents ovules expanded, black represents seed set. Blue represents ovules developed, and red represents ovules aborted. Error bars represent ±1SE, n=6473 ovules.

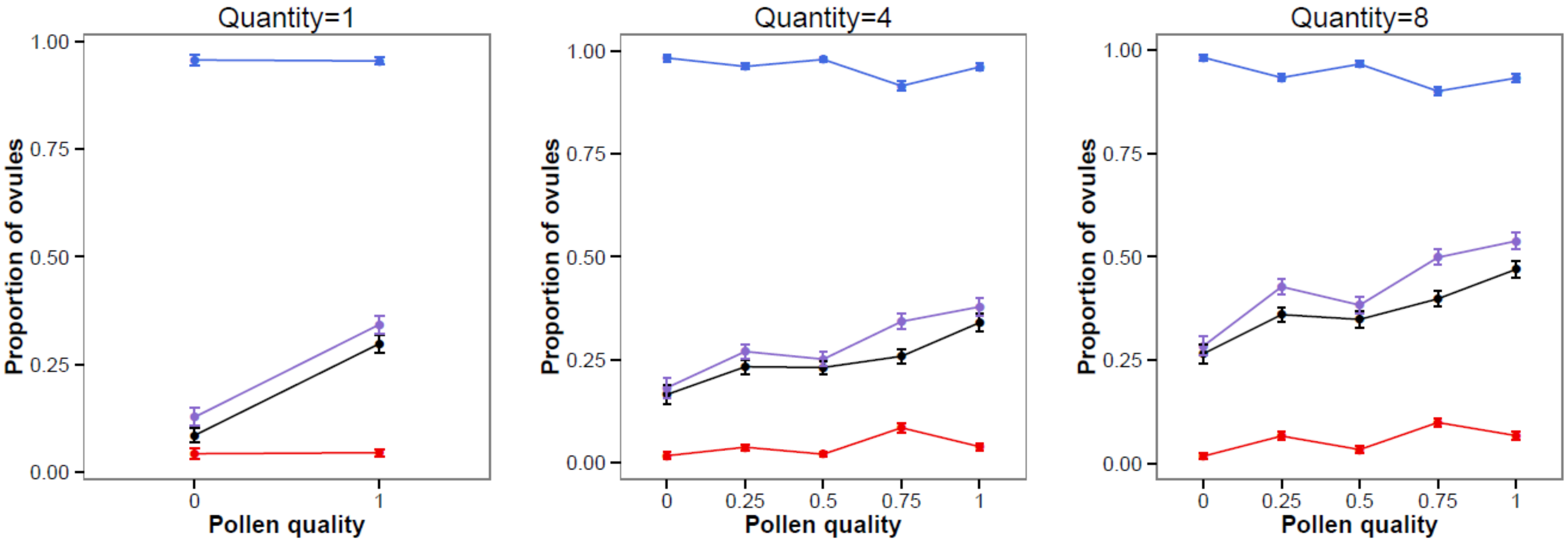


Figure 4.5: The effect of pollen quality on the mean proportion of ovules aborted (red), seed set (black), ovules expanded (purple) and ovules developed (blue), for three different pollen quantities. Error bars represent ±SE, n=6473 ovules.

Table 4.4: Estimates of g_s, g_x and inbreeding depression drawn from the mean proportion of seed development under pure self- (g_s) and pure outcross-pollen (g_x), under differing pollen quantities (corresponds to Figure 4.5).

Pollen quantity	g_s (mean ±SE)	g_x (mean ±SE)	Inbreeding depression ((g_x-g_s)/g_x) (SE range)
1	0.085 ±0.017	0.30 ±0.020	0.72 (0.56 - 0.90)
4	0.17 ±0.024	0.34 ±0.021	0.50 (0.35 - 0.67)
8	0.27 ±0.023	0.47 ±0.020	0.43 (0.32 - 0.54)

Discussion

Open pollinated flowers

In open pollinated plants, ovule expansion, and ovule development, diminished towards the base of the fruit. However, the decrease in ovule development with ovule position was less statistically significant than that of ovule expansion. These results very accurately echo those from Chapter 3, and agree with ovule development patterns found by Corbet (1998). However, an additional pattern was clear in the open pollinated plants. There was an initial increase in ovule expansion at the first three ovule positions at the stylar end of the fruit, before the general decrease from position 3 to the base of the fruit (see Figure 4.3). This pattern has not previously been recorded in *H. non-scripta*, and it may support top-limited resource allocation, which was suggested in Chapter 2. Top-limited resource allocation consists principally of the first-fertilised first-served resource allocation mechanism occurring in the whole fruit, but the most stylar ovules experience more significant resource limitation, leading to the most stylar ovules portraying decreased expansion.

Pollen quantity and quality

Pollen quality and quantity had different effects on fruit and ovule maturation in *H. non-scripta*. Pollen quantity did not affect fruit development, but it did affect ovule expansion. Moreover, pollen quality affected both fruit development and ovule expansion. Neither quantity nor quality affected ovule development, and therefore they also had no effect on ovule abortion. These outcomes suggest that seed set in the *H. non-scripta* plants tested is limited by pollen quality and quantity, but fruit set has much lower levels of pollen limitation. These results are similar to many from other studies. For example, papers commonly find that selfed ovules and/or fruit are less likely to expand and/or develop than outcrossed ovules or fruit (e.g. Lloyd and Schoen, 1992; Montalvo, 1992; Husband and

Schemske, 1996; Corbet, 1998; Aizen and Harder, 2007), which is mirrored in this population of *H. non-scripta*, and in a population in West Cornwall (Corbet, 1998).

The outcome reported here differs to *Epilobium canum* (Zauschneria; Onagraceae), for which additional pollen did not affect seed production after adequate outcross pollen was deposited (Snow, 1986). In our results, seed set was increased by all additional pollen and did not seem to level off (see figure 4.5, although further experimentation could reveal a limit to seed production with outcross pollen quantity. However, fruit set in *H. non-scripta* was not affected by additional pollen quantity, or by increasing pollen quality. Perhaps this shows that pollen limitations can affect differing aspects of fruit and seed maturation in different plants species. In terms of seed production, our study found that both quantity and quality had to increase to produce the highest seed development, simply having the highest possible value of one variable was not enough. The limited effect of additional pollen quality has also been observed n *Vaccinium corymbosum* (Highbush blueberry; Ericaceae) in which seed production, fruit mass and fruit set were unaffected by the outcross fraction (Dogterom *et al.*, 2000).

Figure 4.5 provides similar outcomes to the two Orchidaceae species investigated in Harder *et al.* (2011); we also did not observe a straight line relationship between the developmental fractions and pollen quality. Furthermore, as none of the curves plateau off, this could imply that ovule limitation and resource limitation are not significant in these plants (Harder *et al.*, 2011). Although the mechanism leading to the unexpected relationship between developmental fraction and pollen quality is unclear, it may involve differences in competition between pollen tubes and between zygotes, and also resulting diversity in inbreeding depression. For example, under high pollen import plants may experience intense competition between pollen tubes. More research is required to understand competition within the style and how it may affect seed development and abortion.

Figure 4.5 and Table 4.4 illustrate another interesting result, estimates of g_s and g_x increase with pollen quantity and estimates of inbreeding depression decrease with pollen quantity. This result suggests that not all self-pollen grains or outcross-pollen grains are the same, that some are better able to fertilise ovules that others. Furthermore, increasing amounts of pure self-pollen for example, could enable a sifting effect, allowing increased numbers of viable self-pollen grains to fertilise ovules. This sifting effect may be able to occur through increased competition between self-pollen grains under higher pollen loads resulting in increased g_s, and similarly increased competition between outcross-pollen grains under higher outcross-pollen loads resulting in increased g_x. Lankinen and Armbruster (2007) also found decreasing inbreeding depression with increasing pollen load in *Collinsia heterophylla* (Purple Chinese houses; Plantaginaceae), and propose this results from increased competition between pollen grains under higher pollen quantities. Pollen competition may be of great importance in plants with mixed-mating systems such as *H. non-scripta*, as it may allow for screening of recessive deleterious alleles, and so should mitigate the negative effects of selfing (Armbruster and Rogers, 2004; Lankinen and Armbruster, 2007).

Effect on seed development pattern

The seed development pattern in the hand pollinated plants appears to be influenced by pollen quality. Figures 4.4B, C, show that the proportions of ovules expanded, seed set, and ovules aborted, are more constant between ovule positions under pure outcross- or pure self-pollen than under mixed pollen loads. This outcome is in accord with our understanding of seed development under varying pollen qualities. Under mixed pollen loads, higher quality outcross-pollen tubes grow faster, and therefore reach and fertilise most stylar ovules first, leaving the basal ovules for slower self-pollen tubes to fertilise (see Chapter 1). This results in segregation of ovule quality between the two ends of the fruit due to their fertilisations, which in turn leads to differences in resource garnering ability. Pure pollen loads would produce significantly less difference in capability between pollen tubes for fertilisation of ovules, and also between fertilised ovules for resources, resulting in much diminished segregation of ovule quality. Therefore, there is more consistent ovule expansion, seed set and ovule abortion between ovule positions under pure outcross- or self-pollen than under mixed pollen loads.

Conclusions

This study provides additional insights regarding pollen limitation in *H. non-scripta*. Due to *H. non-scripta* flowering in spring, early in the year, previous papers have led to the expectation that they should experience relatively high pollen limitation due to lack or variability of pollinator abundance (Ashman *et al.*, 2004; Burd *et al.*, 2009; Fulkerson *et al.*, 2012). Our result showed that as long as a very small amount of outcross-pollen is deposited on the stigma of the flowers, *H. non-scripta* fruit production will be at or near the maximum level. However, the maximum seed production can only be achieved by depositing large proportions of high quality pollen on the stigma, which ensures high levels of ovule expansion, and very low levels of ovule abortion. As pollen quality and quantity appear much more limiting for seed production than for fruit production, it suggests fruit are less likely to be aborted when pollen import is low in order to minimise seed losses.

Our results suggest that the *H. non-scripta* plants in Great High Wood and Little High Wood, Durham, are only slightly limited by pollen quantity and pollen quality. Although the number of seeds produced is highest under the largest quantity of full outcross-pollen, and reducing either the pollen quantity or quality reduces the number of seeds produced, the reduction was usually very small. For example, the seed set under open pollination was 0.34 ±0.0012 (mean ±SE), which was slightly less than 0.36 ±0.0060 under full outcross-pollination. The results from our population of *H. non-scripta* are comparable to a West Cornwall population, as the mean proportion of seeds developed out of all ovules for self-pollinated plants was also close to 0.17, and for outcross-pollinated plants it was also around 0.36 (see Figure 4.6) (Corbet, 1998). This suggests that the variables involved in the self-incompatibility systems and inbreeding depression systems in *H. non-scripta* are consistent across populations or plants have evolved strategies to buffer environmental variation.

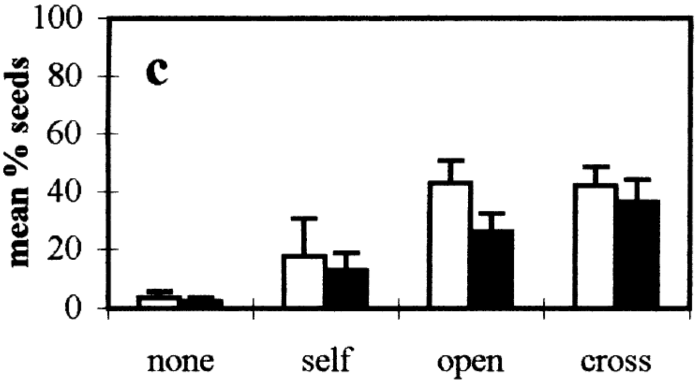


Figure 4.6: Effects of pollination treatment in field experiment subset on seeds initiated (white) and matured (black) as percent of all ovules in surviving fruits, bars show the mean with SE (Figure 1c from Corbet, 1998).

Comparing seed production between open pollinated plants and hand pollinated plants suggests a 4:4 pollen ratio is occurring under natural conditions (see Figure 4.3 and 4.4).

Thus it appears that open pollination by insect pollinators over the dates tested was composed of around 50% outcross-pollen, and the amount of pollen deposited was relatively high. However, further experiments need to be conducted to get closer to the true pollen quality and quantity deposited under open pollination from this first estimate.

Much more could be learnt about seed development in *H. non-scripta* if a few procedures were changed. For example, lower pollen quantities could be applied to estimate the minimum pollen import for producing fruit. It should also be determined how high pollen import must be before ovule expansion is unaffected by additional import. Furthermore, hand pollinations were mainly within the five most basal flower positions, but should be extended to all positions, allowing results to incorporate true effects of flower position. Chapter 5 continues the discussion of improved future experimental design and also makes overall conclusions from the results of the present thesis.

Chapter 5

Thesis Discussion and Conclusions.

The preceding chapters have reported studies on pollen and resource limitation in order to better understand the non-random seed development pattern in linear fruit such as *H. non-scripta*. In the recent past it has been discovered that increased resources such as nutrients and water available to the focal plant (either through addition of resources or thinning of flowers) may increase the seed set and/or fruit set (Gorchov, 1988; Ehrlén, 1992; Yang *et al.*, 2005). Furthermore, discoveries have shown that increasing pollen quantity, and pollination using outcross-pollen compared to self-pollen increased seed set and/or fruit set (Corbet, 1998; Yang *et al.*, 2005; Fulkerson *et al.*, 2012; Abdala-Roberts *et al.*, 2014). This thesis was able to build upon this understanding by creating a stochastic model, and performing both resource addition by ovary removal and hand pollinations using a small spectrum of pollen qualities and quantities. Through these methods, it sought to answer the question how do resources and pollen quality and quantity influence fruit and seed development, and the non-random seed maturation pattern seen in linear fruit such as *H. non-scripta*. This chapter summarises the findings of the present study and suggests future directions for studies on the non-random seed maturation pattern in linear fruit which this study has highlighted.

The stochastic simulation model suggested that for plants experiencing mixed pollen quality loads, the best strategy to produce highest seed set is to have resources allocated to ovules via the first-fertilised first-served mechanism and to have a high q value (the strength of the attraction between pollen tubes and an unfertilised ovule). This ensures any resource limitation would lead to the abortion of a very high proportion of self-fertilised ovules, and a very high development of outcross-fertilised ovules. This is due to the high segregation created between them from the stylar end to the basal end of the fruit. Open-pollinated inflorescences of *H. non-scripta* from field experiments showed that there was first-fertilised first-served resource allocation taking place in the fruit, as the non-random seed development pattern did occur. However, it was not as strong as was tested in the stochastic simulation model, as the proportion of seed development at the stylar end was only about 10 percent higher than at the basal end of the fruit and not *ca.* 50 percent higher as in the model. This may be due to a much lower q value in *H. non-scripta* plants than was tested in the model.

A high q value was expected as it was thought to increase seed set. However, as it was much lower than expected in the plants studied, this suggests there may be a cost to a large q value which was unanticipated. The attraction between pollen tubes and unfertilised ovules is due to a chemical signal produced by two synergid cells inside an ovule (Okuda *et al.*, 2009; Okuda and Higashiyama, 2010; Kessler and Grossniklaus, 2011; Lu *et al.*, 2011). Therefore, there must be some cost for an ovule to produce too much chemical signal. For example, the cost of producing the chemical signal may have to stay below a certain level in order for the ovule to have sufficient energy remaining to commence development once fertilised. Alternatively, the chemical signal may diffuse too far from the unfertilised ovule source if the level produced is too high, meaning the signal is spread around the whole ovary. This would confuse the signal to the extent that the pollen tube would not be attracted to an unfertilised ovule. These or other possible explanations could keep the q value reduced to that seen in the field plants.

The ovary removal treatment also produced unexpected results. It was thought that the treatment would increase resources available to remaining fruit, and therefore would increase the fruit and seed development. However, the removal of up to half of the ovaries of open flowers on racemes did not affect fruit development of the remaining flowers, or affect their seed development. It only had a slight negative effect upon ovule expansion, and the seed maturation pattern appeared unaffected (see Figure 3.3). It can therefore be postulated that the ovary removal treatment was ineffective, and several causes are possible, which were discussed in Chapter 3. However, it may equally be possible that the *H. non-scripta* plants tested were not resource limited. The simplest way to test this would be to remove resources from the plants, for example by removing leaves or shading, and if this reduces seed set, it is more likely that the *H. non-scripta* plants were not resource-limited, rather than the ovary removal treatment being ineffective.

On the other hand, manipulating pollen quantity and quality did produce a result which was similar to that expected. Pollen quantity did not affect fruit set or seed development given expansion, but had a positive effect on ovule expansion. Increasing the pollen quality, however, increased fruit set and ovule expansion, but had no effect on seed development given expansion as was predicted. Interestingly, when the relationship between pollen quality and ovule expansion was examined, the correlation appeared not to be a simple positive one. Rather, certain pollen qualities produce a slightly higher or lower ovule expansion than would be expected from a straight-line relationship (see Figure 4.5). Further statistical analyses would need to be conducted on the data in order to test this relationship thoroughly. It is, to the best of my knowledge, the first time it has been suggested for *H. non-scripta*, but a non-simple straight-line relationship has also been shown in two Orchidaceae species by Harder *et al.* (2011). The latter is the only other paper which studies the correlation between pollen quality increase and ovule maturation, but due to the interesting results in their paper and this thesis, it could make an interesting area to study in future projects.

A further intriguing outcome from the pollen manipulations was that inbreeding depression appeared to decrease with increasing pollen quantity (Table 4.4). Although this is thought to occur through increasing pollen competition with increasing pollen quantity, there is still no clear single estimate for inbreeding depression in the population of *H. non-scripta* studied. Harder *et al.* (2011) suggest that most accurate estimates of inbreeding depression are gained using the standard approach using g_s (proportion of seeds set under a pure self-pollen ratio) and g_x (proportion of seeds set under a pure outcross-pollen ratio) values, only if there is no pollen quantity limitation or limitation in maternal resources for developing ovules and seeds. Therefore, the most accurate estimate of inbreeding depression presented in Chapter 4 may be that under the highest pollen quantity 8 of 0.43, as this estimate would have lowest pollen quantity limitation. However, it is not certain whether this population is not resource limited, so further experimentation and statistical analyses such as that proposed by Harder *et al.* (2011) are likely to provide a more accurate estimate of inbreeding depression.

Although the results imply that this population of *H. non-scripta* may not be resource-limited, they could easily be interpreted to suggest that pollen limitation occurs. This is due to the fact that high levels of outcross-pollen produced higher ovule expansion and seed set than under open pollination. This finding of pollen limitation agrees with the only other study on seed and fruit development in *H. non-scripta* by Corbet (1998). However, what Corbet (1998) or any other literature does not consider is how pollen quality and quantity may affect the seed development pattern inside the fruit. The results of the present study revealed that the ovule positional effects become stronger under mixed pollen loads. For example, viewing rows B and C in Figure 4.3, ovule expansion and development appear to be more constant between ovule positions under pure outcross- or pure self-pollen than under mixed pollen loads, for which ovule expansion and development is significantly higher at the stylar end of the fruit than the basal end. This implies that the non-random seed maturation pattern breaks down for pure outcross- and pure self-pollen loads. This is credible as under pure self- or outcross-pollen quality, ovules would be fertilised by pollen tubes of the same or more similar quality, so embryo fitness and ability to garner resources would be much more equal rather than segregated.

There was, however, another intriguing finding relating to the non-random seed maturation pattern. A clear decline in ovule expansion and seed development was seen from the stylar end of the fruit to the basal end in open pollinated flowers, illustrating the general non-random seed maturation pattern. However, a pattern of an initial increase in ovule expansion at the first three ovule positions at the stylar end of the fruit was also detected (see Figure 4.2). This indicated that, as well as first-fertilised first-served resource allocation appearing, there was a slight decline in resources at the very far stylar end of the fruit, probably due to the increased distance from the source of the resources. This pattern, here termed a top-limited resource allocation mechanism, was anticipated in Chapter 2, and is also noticeable in results from other studies on linear fruit (e.g. Bawa and Webb, 1984; Corbet, 1998). Top-limited resource allocation has not been identified so clearly in any other literature, so this thesis provides a good incentive to study this phenomenon in more detail in the future.

It is clear from the present study that further investigation into the non-random seed maturation pattern in *H. non-scripta* is necessary. Top-limited resource allocation and many other factors considered in this thesis could provide good material to investigate. However, the methods used here need to be improved upon in order to better understand future results. For example, setting up the experiments in a glasshouse rather than in the field may improve the accuracy of the experiment, as it would be possible to exclude pollinators to reduce unknown additional pollination, and could allow more straightforward control of resource manipulations. Furthermore, in future studies it would be useful to count pollen grains under a microscope when creating pollen quantities in order to ensure the pollen quantities are as accurate as possible.

The flow of resources through the plant would be extremely useful to know as it could give a definitive answer as to what resource allocation mechanism operates in fruits to create the non-random seed maturation pattern: is it first-fertilised first-served, bottom-up, or as suspected in this thesis, top-limited? One method which could enable this answer in the future is pulse-labelling with stable or radioactive carbon isotopes. Pulse labelling with labelled carbon dioxide of the aerial parts of a plant can allow the tracing of carbon through the plant, and can therefore allow the determination of how stored or recent assimilates can contribute to growth in sink organs such as developing fruit (Lundmark *et al.*, 2009; Epron *et al.*, 2012). Pulse labelling could therefore be very well suited to building the understanding of how resources are distributed to fruit and seeds and how they affect the seed development pattern in *H. non-scripta*. Pulse-labelling should be possible in *H. non-scripta*, as it has previously been performed in another bulbous perennial plant, *Crocus vernus* (Spring Crocus; Iridaceae) (Lundmark *et al.*, 2009).

There may also be a better method of determining the fitness and pollen donor quality of seeds at differing ovule positions. Future studies should use methods like those in Yuan *et al.* (2014), where they were able to determine the genetic donor of the pollen which fertilised ovules at each position along the fruits using paternity analysis. The paternity analysis involves performing DNA tests on both matured and aborted ovules and the potential parent plants, and comparing the genotypes of parents with the offspring. This method would make it possible to discover the pollen donor for seeds at every ovule position, and could provide much better evidence of a correlation between selfed ovules and abortion. This method would also provide us with more solid evidence of the theory of segregation between outcross-fertilised ovules at the stylar end of the fruit and self-fertilised ovules at the basal end of the fruit.

There was clearly one major factor related to the present study which needs to be acknowledged in future studies: the two field experiments highlighted the need to consider both pollen and resources together in future, even if just one of them is the focus of the study. For example, if a study is examining resources, pollen needs to be considered too and either controlled to be the same in all plants or at least recorded in great detail, even if it is under open pollination. This is in order to account for the fruit and seed production and the seed maturation pattern, as both pollen and resources greatly affect these. Other studies have found similar outcomes for the necessity to consider both pollen and resources (e.g. Corbet, 1998; Brookes *et al.*, 2008), after originally having been stated in the Haig-Westoby model (Haig and Westoby, 1988).

Many studies have considered how resources and pollen may affect seed abortion in plants, and many have noted a non-random seed maturation and abortion pattern in linear fruit. However, until now, none have recognised that both aspects may have to be studied together to fully understand seed maturation in linear fruit such as *H. non-scripta*. This study has begun the process, and found some initial results. It has been discovered that *H. non-scripta* plants in the experiments were slightly pollen quality limited, but did not appear to be limited by resources or pollen quantity. Furthermore, top-limited resource allocation may be the resource distribution strategy adopted in *H. non-scripta*. Hence, top-limited resource allocation may affect the non-random seed maturation pattern, which has not been considered in other literature before. In addition, this thesis has identified that mixed pollen qualities make the non-random seed maturation pattern stronger, and estimates of inbreeding depression decrease with pollen quantity. These discoveries certainly need further investigation in the future, and better understanding of them may be achieved by adopting the methods discussed above.

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Appendix A

A copy of the code used to create the stochastic simulation model in R Studio from Chapter 2.

```
# Chapter 2 Model.R
# 23/01/2015
rm(list = ls()) # clear memory

install.packages("dplyr")
install.packages("ggplot2", dependencies=TRUE)

library(dplyr) # data frame manipulation install.packages("dplyr")
library("ggplot2") # plotting functions

par(mar = c(5,4,1,1) + 0.1) # set graph boundaries

# setwd

# Model parameters (these are some defaults)
N.flowers <- 1000  # number of flowers sampled
N.ovules <- 12     # number of ovules per locule (1 = top, Rmax = bottom)

# N.outcross <- 8   # number of outcross pollen grains that successfully germinate
# g.outcross <- 0.85 # probability an outcross-fertilised ovule survives fertilisation

# N.self <- 8       # number of self pollen grains that successfully germinate
# g.self <- 0.25     # probability a self-fertilised ovule survives fertilisation

# q <- 0.5         # probability a pollen grain accepts each free ovule's signal
```

```
# sd.seq <- 0.1      # standard deviation of timing of resource allocation for first-served (0.1 =
        sequential, 100 = random)

# max.develop <- 9  # number of ovules that could be developed


# First.served <- TRUE # are resources allocated to first fertilised?




# ==================================

# =     SIMULATION CODE STARTS     =

# ==================================


simulate.development <- function(N.outcross, N.self, g.outcross, g.self,

  max.develop, sd.seq, q, First.served) {


if (max.develop > N.ovules) { # make sure max.develop makes sense

  max.develop <- N.ovules

}


# set up the pollen grains data frame

N.total <- N.outcross + N.self # total number of pollen tubes

Location <- 1:N.total

Outcross  <- c(rep(TRUE, N.outcross), rep(FALSE, N.self))

Fert.time <- rep(0, N.total)

Rank.time <- rep(0, N.total)

Fertilized <- rep(FALSE, N.total)

Ovule <- rep(0, N.total)

Fert.success <- rep(FALSE, N.total)

Develop <- rep(FALSE, N.total)

Pollen <- data.frame(Location, Outcross, Fert.time, Rank.time, Fertilized, Ovule,

  Fert.success, Develop)
```

```
Pollen <- tbl_df(Pollen)

# set up the ovule data frame
Fertilized <- rep(FALSE, N.ovules)
Grain <- rep(0, N.ovules)
Outcross <- rep(FALSE, N.ovules)
Fert.success <- rep(FALSE, N.ovules)
Order <- rep(0, N.ovules)
Location <- N.ovules:1
Develop <- rep(FALSE, N.ovules)
Ovule <- data.frame(Location, Fertilized, Grain, Outcross, Fert.success, Order, Develop)
Ovule <- tbl_df(Ovule)

# set up summary data.frames
Position <- 1:N.ovules
Summaries <- data.frame(Position, Fertilized, Fert.success, Develop)
Summaries <- tbl_df(Summaries)

for (f in 1:N.flowers) { # for each flower
  # 1. Determine the ordering of pollen entering the ovary
  Pollen$Fertilized <- rep(FALSE, N.total)
  for (i in 1:N.total) {
    Pollen$Fert.time[i] <- rnorm(1, mean = i, sd = sd.seq)
  }
  Pollen$Rank.time <- rank(Pollen$Fert.time) # order pollen tube arrivals
  Pollen <- arrange(Pollen, Rank.time)

  # 2. perform fertilizations
  successes <- 0
```

```
Ovule$Fertilized <- rep(FALSE,N.ovules) # reset the fate sequence of each ovule to
    unfertilised

Ovule$Fert.success <- rep(FALSE, N.ovules)

Ovule$Order <- rep(N.ovules+1, N.ovules)

for (i in 1:N.total) { # for each outcross pollen grain

  current.row <- 1

  found.ovule <- FALSE

  while (!found.ovule & (current.row <= N.ovules)) {

    if ((Ovule$Fertilized[current.row] == FALSE) & !found.ovule) {

      if (runif(1) < q) {

        # ovule atracts the pollen tube

        found.ovule <- TRUE

        Ovule$Fertilized[current.row] <- TRUE

        Ovule$Outcross[current.row] <- Pollen$Outcross[i]

        Ovule$Grain[current.row] <- i

        Pollen$Ovule[i] <- current.row

        if (Ovule$Outcross[current.row] == FALSE) { # self grain

          Ovule$Fert.success[current.row] <- (runif(1) <= g.self)

          Pollen$Fertilized[i] <- TRUE

          Pollen$Fert.success[i] <- Ovule$Fert.success[current.row]

        } else {

          Ovule$Fert.success[current.row] <- (runif(1) <= g.outcross)

          Pollen$Fertilized[i] <- TRUE

          Pollen$Fert.success[i] <- Ovule$Fert.success[current.row]

        }

        if (Pollen$Fert.success[i]) {

          successes <- successes + 1

          Ovule$Order[current.row] <- successes

        }

      }
```

```
   }
   current.row <- current.row + 1 # no ovules accepted on current row so drop down
  }
 }
 Pollen <- arrange(Pollen, Location)


 # 3. Allocate resources
 Ovule$Develop <- rep(FALSE,N.ovules) # reset the fate sequence of each ovule to unfertilised
 if (First.served) {
  Ovule <- arrange(Ovule, desc(Fert.success), Order) # allocate resources according to fertilisation order
 } else {
  Ovule <- arrange(Ovule, desc(Fert.success), Location) # allocate resources according to ovule location
 }
 seeds <- min(max.develop, successes)
 Ovule$Develop <- c(rep(TRUE, seeds), rep(FALSE, N.ovules - seeds))
 Ovule <- arrange(Ovule, desc(Location))


 # 4. Summarise findings
 Summaries$Fertilized <- Summaries$Fertilized + Ovule$Fertilized
 Summaries$Fert.success <- Summaries$Fert.success + Ovule$Fert.success
 Summaries$Develop <- Summaries$Develop + Ovule$Develop
}


# set up plotting data.frames
Position <- factor(rep(1:N.ovules,4))
Unfertilized <- 1 - Summaries$Fertilized/N.flowers
Inbreeding <- (Summaries$Fertilized - Summaries$Fert.succ)/N.flowers
Developed <- Summaries$Develop/N.flowers
```

```
Resource.limited <- 1 - Unfertilized - Developed - Inbreeding

Fate <- c(rep("U", N.ovules), rep("ID", N.ovules), rep("RL", N.ovules), rep("D", N.ovules))
Fate <- factor(Fate, levels = c("U", "ID", "RL", "D"))
Frac <- c(Unfertilized, Inbreeding, Resource.limited, Developed)

Plot.df <- data.frame(Position, Fate, Frac)
Plot.df <- tbl_df(Plot.df)

return(Plot.df)
}

# ============================
# =   SIMULATION CODE ENDS   =
# ============================

# =================================
# =     multiplot CODE STARTS     =
# =================================

multiplot <- function(..., plotlist=NULL, file, cols=1, layout=NULL) {
  require(grid)

  # Make a list from the ... arguments and plotlist
  plots <- c(list(...), plotlist)

  numPlots = length(plots)

  # If layout is NULL, then use 'cols' to determine layout
  if (is.null(layout)) {
```

```
    # Make the panel
    # ncol: Number of columns of plots
    # nrow: Number of rows needed, calculated from # of cols
    layout <- matrix(seq(1, cols * ceiling(numPlots/cols)),
                     ncol = cols, nrow = ceiling(numPlots/cols))
  }

  if (numPlots==1) {
    print(plots[[1]])

  } else {
    # Set up the page
    grid.newpage()
    pushViewport(viewport(layout = grid.layout(nrow(layout), ncol(layout))))

    # Make each plot, in the correct location
    for (i in 1:numPlots) {
      # Get the i,j matrix positions of the regions that contain this subplot
      matchidx <- as.data.frame(which(layout == i, arr.ind = TRUE))

      print(plots[[i]], vp = viewport(layout.pos.row = matchidx$row,
                                      layout.pos.col = matchidx$col))
    }
  }
}

# ===========================
# =   multiplot CODE ENDS   =
# ===========================
```

```
# == Investigate single simulations

# order of parameter: N.outcross, N.self, g.outcross, g.self, max.develop,

# sd.seq (0.01 = outcross first, 100 = random), q, First.served (TRUE/FALSE)


Plot.df <- simulate.development(10, 10, 0.85, 0.25, 12, 0.01, 0.35, TRUE)

ggplot(Plot.df, aes(x=Position, y=Frac, fill=Fate, order=desc(Fate))) +

  geom_bar(stat="identity", colour="black") +

  ylab("Probability") +

  scale_fill_manual(values = c("blue", "red", "orange", "green"))


# === RUN SCENARIOS OF INTEREST AND PRODUCE MULTI-PANEL FIGURE


Plot.df.1 <- simulate.development(10, 10, 0.85, 0.25, 10, 0.01, 0.35, TRUE)

p1 <- ggplot(Plot.df.1, aes(x=Position, y=Frac, fill=Fate, order=desc(Fate))) +

  geom_bar(stat="identity", colour="black") +

  ylab("Probability") +

  guides(fill=FALSE) + # suppress the colour guide: mention colours in figure legend

  annotate("text", x=6.5, y=1.05, label="(A) Outcross first", size = 4) +

  theme_bw() +

  theme(panel.grid.major.x = element_blank(),

        panel.grid.minor.x = element_blank(),

        panel.grid.major.y = element_blank(),

        panel.grid.minor.y = element_blank()) +

  scale_fill_manual(values = c("blue", "red", "orange", "green"))


Plot.df.2 <- simulate.development(20, 0, 0.85, 0.25, 12, 0.01, 0.35, TRUE)

p2 <- ggplot(Plot.df.2, aes(x=Position, y=Frac, fill=Fate, order=desc(Fate))) +

  geom_bar(stat="identity", colour="black") +
```

```
  ylab("Probability") +
  guides(fill=FALSE) +
  annotate("text", x=6.5, y=1.05, label="(B) Random quality", size = 4) +
  theme_bw() +
  theme(panel.grid.major.x = element_blank(),
        panel.grid.minor.x = element_blank(),
        panel.grid.major.y = element_blank(),
        panel.grid.minor.y = element_blank()) +
  scale_fill_manual(values = c("blue", "red", "orange", "green"))

Plot.df.3 <- simulate.development(10, 10, 0.85, 0.25, 12, 100.0, 0.35, TRUE)
p3 <- ggplot(Plot.df.3, aes(x=Position, y=Frac, fill=Fate, order=desc(Fate))) +
  geom_bar(stat="identity", colour="black") +
  ylab("Probability") +
  guides(fill=FALSE) +
  annotate("text", x=6.5, y=1.05, label="(B) Random quality", size = 4) +
  theme_bw() +
  theme(panel.grid.major.x = element_blank(),
        panel.grid.minor.x = element_blank(),
        panel.grid.major.y = element_blank(),
        panel.grid.minor.y = element_blank()) +
  scale_fill_manual(values = c("blue", "red", "orange", "green"))

multiplot(p1, p2, p3, cols = 3)
dev.copy2pdf(file = "Test figure.pdf", height = 4, width = 10)
dev.off
```