Chapter 1

General introduction

Many angiosperms produce hermaphroditic flowers. The presence of male and female reproductive organs within a single flower can provide both advantages and disadvantages. Hermaphrodism is cost effective because only one perianth per male and female organ needs to be produced and the floral attributes of attraction and reward (e.g., size, colour, reward) benefit both male and female function (Charnov *et al.*, 1976; Willson, 1979). Reproductive assurance may be provided in the absence of pollinators or mating partners. However, hermaphrodism can represent a cost, by increasing the incidence of self-fertilisation, resulting in the production of fewer seeds that are of inferior quality. Determining mechanisms that reduce self seed set and the consequences of reduced selfed progeny fitness can provide an important insight into ecological and evolutionary processes in plant populations.

In this study, I use *Bulbine bulbosa* (R. Br.) Haw. Asphodelaceae, a partially self-fertile perennial herb, to examine two possible post-pollination, self-infertility mechanisms, inbreeding depression and physiological self-incompatibility. I also examine the effects of biparental inbreeding and mating between individuals in close proximity. Finally, I examine pollen limitation, the effect of self pollen interference and inbreeding depression under field conditions.

Selfing entails two processes: self-pollination and self-fertilisation. Self-pollination can be reduced and cross-pollination promoted by floral traits such as the spatial (herkogamy) and temporal (dichogamy) separation of male and female structures. Recent interpretations also view these floral traits as mechanisms to reduce self pollen interference (Lloyd & Webb, 1986; Webb & Lloyd, 1986). The basis of this interpretation is that many angiosperms with such mechanisms also have a physiological self-incompatibility mechanism which prevents self-fertilisation (Seavey & Bawa, 1986; Charlesworth & Charlesworth, 1987; de Nettancourt, 2001; Barrett, 2002a, 2003; Cesaro *et al.*, 2004).

Self-fertilisation can be prevented by physiological self-incompatibility because the maternal plant can recognise and reject self or incompatible pollen on the stigma or within the style (de Nettancourt, 1997; Sage *et al.*, 2000; Barrett, 2002a). Self-incompatibility may also act at the time of fertilisation, just prior to (pre-zygotic) or immediately after (post-zygotic) syngamy, in the ovary or the ovule respectively (Kenrick *et al.*, 1986; Chichiricco, 1993; Gibbs *et al.*, 1999; Bianchi & Gibbs, 2000; Aguilar & Bernardello, 2001). This form of self-incompatibility is known as 'late-acting' or 'ovarian self-incompatibility' and is of current interest because some authors suggest the expression of deleterious alleles (i.e., inbreeding depression) can explain self-incompatibility whereas others believe it would take large numbers of recessive alleles to mimic self-incompatibility (Kenrick *et al.*, 1986; Seavey & Bawa, 1986; Klekowski, 1988; Nic Lughadha, 1988; Waser & Price, 1991a; Gibbs *et al.*, 1999; Bianchi *et al.*, 2005).

Self-pollination and self-fertilisation may be reduced or prevented by floral traits and self-incompatibility respectively, but self pollen interference may still be problematic even in the presence of such mechanisms. If self-pollination occurs, self pollen can interfere with cross pollen in a number of ways. Self pollen can clog the stigmatic surface or block the style, preventing the germination of cross-pollen grains or the growth of cross-pollen tubes (Shore & Barrett, 1984). As self-pollen tubes grow, in either self-compatible plants or plants with late-acting self-incompatibility ovules may be disabled or usurped rendering them unavailable for outcrossing (i.e., ovule discounting; Waser & Price, 1991a; Sage *et al.*, 1999; Herlihy & Eckert, 2002; Cesaro *et al.*, 2004). Additionally, self pollen deposited on the source flower or another flower from the same plant is a loss of pollen available for export to other plants (i.e., pollen discounting; Harder & Wilson, 1998). Collectively, the loss of available pollen for outcrossing after self-pollination and the loss of ovules after self-fertilisation represent a cost associated with selfing (i.e., gamete discounting; Lloyd & Schoen, 1992; Harder & Barrett, 1995; Schoen *et al.*, 1996; Barrett, 2003). Additionally, the loss of ovules and pre-emption of resources by developing selfed seeds can represent a cost when ovules and resources could have been utilised for the production of outcrossed seeds. This is particularly pertinent for perennial plants where resources utilised for selfed seed production might compromise development of crossed seeds in successive seasons (i.e., seed discounting; Morgan *et al.*, 1997; Barrett, 2002b; Herlihy & Eckert, 2002, 2004).

Inbreeding depression is the reduction in fitness of selfed progeny relative to outcrossed progeny and is a common feature of many flowering plant populations. An increase in homozygosity, resulting from inbreeding, can cause inbreeding depression via 'over dominance' or 'partial dominance'. If inbreeding depression is caused by 'over dominance' genotypes that are heterozygous at individual loci will have superior fitness, relative to both homozygotes. If reduced fitness is due to 'partial dominance' inbreeding depression is the result of the expression of recessive or partially recessive deleterious alleles, as homozygotes (Wright, 1977; Charlesworth & Charlesworth, 1987; Charlesworth *et al.*, 1990). Populations with a history of outcrossing are expected to experience higher levels of inbreeding depression compared to selfing populations because selfing results in a greater probability of selection against recessive deleterious alleles, effectively purging them from the population (Husband & Schemske, 1996).

Inbreeding depression, caused by the expression of deleterious recessive alleles, can be expressed at any time during a plant life-cycle (e.g., seed production, seed germination, juvenile survival, growth and reproduction; Husband & Schemske, 1996). A reduction in selfed progeny fitness compared to crossed progeny fitness may be evident, from immediately after fertilisation through to male and female functions during reproduction (Seavey & Bawa, 1986; Husband & Schemske, 1996). Inbreeding depression can cause low self-fertility if it is expressed early during seed development by causing self-fertilised ovules to abort (Stephenson, 1981; Seavey & Bawa, 1986). Since both inbreeding depression and late-acting self-incompatibility

can act at similar stages, determining which mechanism is responsible for reducing self seed set can be challenging but is important to do because inbreeding depression may have been the selective force behind the evolution of physiological self-incompatibility (Charlesworth & Charlesworth, 1987). Both mechanisms, however, ensure cross pollen will preferentially sire seeds (Seavey & Bawa, 1986; Krebs & Hancock, 1990; Manasse & Pinney, 1991).

Mating between related individuals (consanguinity) can mimic selfing by increasing homozygosity and causing biparental inbreeding depression (Uyenoyama, 1986; Waller, 1986; Charlesworth & Charlesworth, 1998; Kelly & Willis, 2002). Related individuals occur in close proximity in genetically structured populations. Populations with spatial genetic structure can be the result of limited pollen and seed dispersal, and/or asexual reproduction (Levin, 1984; Heywood, 1991; Waser, 1993b; Griffin & Eckert, 2003; Herlihy & Eckert, 2004). If mating occurs between genetically related individuals, the subsequent progeny may be less fit compared to progeny of matings between unrelated individuals (Price & Waser, 1979). Biparental inbreeding depression has complex implications for the evolution of mating systems, as it simultaneously reduces the cost of outcrossing (i.e., genetic advantage of selfing) but increases homozygosity and the probability of inbreeding depression (Uyenoyama, 1986; Waller, 1986; Kelly & Willis, 2002; Griffin & Eckert, 2003). Determining the effect of inbreeding via consanguineous mating, in addition to via selfing, is important to assessing inbreeding depression.

Inbreeding depression has a range of ecological and evolutionary impacts on individual plants and plant populations. Reduced selfed progeny fitness lowers fecundity, limiting recruitment to the next generation. Inbreeding depression can also produce less fit offspring with a lower probability of reaching reproductive maturity (Fisher, 1941; Lande & Schemske, 1985; Seavey & Bawa, 1986; Charlesworth & Charlesworth, 1987; Holsinger, 1992; Uyenoyama *et al.*, 1993; Husband & Schemske, 1996). For example, inbreeding depression caused reduced fecundity in *Burchardia umbellata* and produced less fit selfed progeny compared to outcross in *Blandfordia grandiflora* and *Schiedea membranacea* (Ramsey & Vaughton, 1996; Culley *et al.*, 1999; Ramsey & Vaughton, 2000). In natural populations of these species mature plants probably result from outcrossing rather then selfing because of the low probability that selfed individuals survive to maturity.

In populations of self-compatible plants, high levels of inbreeding depression would be expected to favour traits that promote inbreeding avoidance and outcrossing such as herkogamy and dichogamy. Populations that have been predominately outcrossing are likely to have accumulated a substantial genetic load and when selfing is increased either because of a population bottleneck or a loss of pollinators, inbreeding depression will be severe (Husband & Schemske, 1996). Further inbreeding, providing inbred off spring survive, may result in purging the genetic load from within the population and consequently a reduction in the level of inbreeding depression (Charlesworth & Charlesworth, 1987; Byers & Waller, 1999; Crnokrak & Barrett, 2002).

Study species

Bulbine bulbosa (R. Br.) Haw. is one of about 60 species in the genus *Bulbine* (Asphodelaceae). Most species are found in southern and tropical Africa and five species are endemics in Australia (Smith & Van Wyk, 1998). *Bulbine bulbosa* is distributed in temperate Australia and occurs in a variety of habitats including grasslands, woodlands, and open forest (Fig. 1.1, 1.2). Cytological studies indicate that *B. bulbosa* is polyploid and specimens surveyed from the area in which this research was under taken are tetraploid but functionally diploid ($2n = 24$, $x = 6$; Watson, 1986). Plants are geophytic, with a bulb-shaped tuber, thickened roots and succulent, basal leaves (Watson, 1987). The scapose raceme bears approximately 30 hermaphroditic flowers which open acropetally, on average, one per day over several weeks. Flowers do not open if the preceding day's weather is inclement but a number of flowers, corresponding to the number of inclement days, open after one fine day (K. Owen personal observations). The zygomorphic flowers have six tepals approximately 10 mm long. Ovule numbers can vary from 3-8 per loculus and fruits mature approximately 30 days after pollination. The style is decumbent, extending from the axis of symmetry, with anthers opposite style approximately 3-4 m away (Fig. 1.2). The stamens are clustered with hair-like structures at the apex of the filament, partially concealing the anthers. The stigma and anthers are separated by approximately 3-4 mm. (Fig. 1.2; Rowley, 1967; Owen *et al.*, 2007).

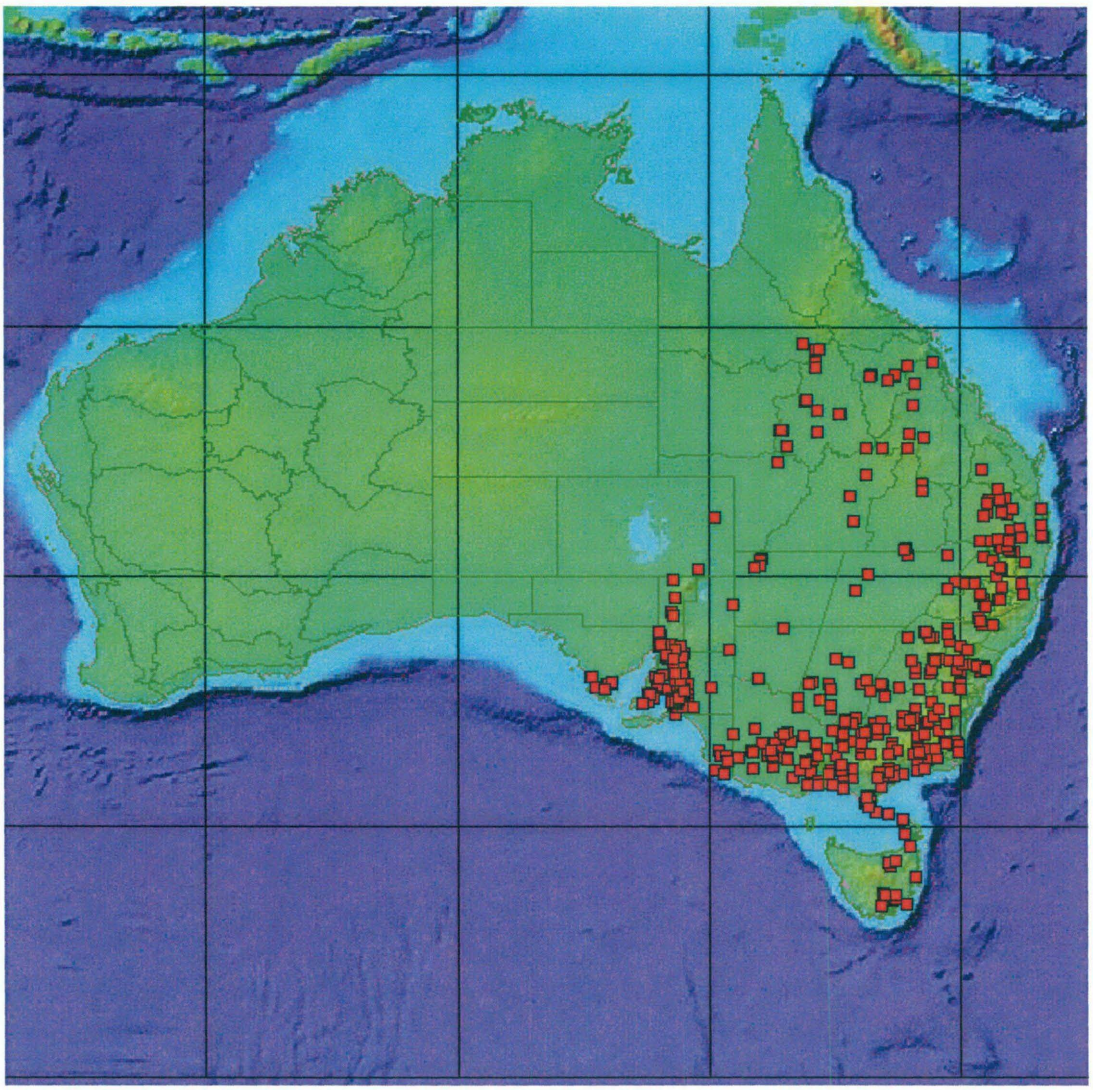

Figure 1.1. Recorded distribution of *Bulbine bulbosa* (R. Br.) Haw. Asphodelaceae (AVH, 2006). Red squares represent recorded collections held in Australian Herbaria.

Figure 1.2. *Bulbine bulbosa*. Top, *Bulbine bulbosa* population on a moderate slope with a south-westerly aspect (Field site, Chapter 5). Bottom left, *B. bulbosa* inflorescence and bottom right, *B. bulbosa* developing fruits.

Previous work on *B. bulbosa* was undertaken during an Honours project at UNE (Owen, 2000; Owen *et al.*, 2007). This project provided insight into the mating system of *B. bulbosa.* We found that *B. bulbosa* plants were partially self-fertile but not autonomously self-pollinating. Plants produced fewer seeds following self-pollination than cross-pollination (self vs. cross: seed per fruit 2.4 ± 0.3 vs. 7.7 ± 0.4; $N = 16$). Selfed progeny were less fit than outcrossed progeny over a range of early life-cycle stages and cumulative inbreeding depression was severe (0.85; Owen *et al.*, 2007). Naturally pollinated plants experienced high pollen deposition but produced fewer fruits and seeds than cross-pollinated plants, indicating a lack of outcross pollen (Owen *et al.*, 2007).

Bulbine bulbosa is an excellent system to examine aspects of plant reproductive and evolutionary ecology. The flowering plant is easily transplanted from the field and responds well to glasshouse conditions where it can be grown all year round. Plants can also be grown from seed, reaching sexual maturity in 2-3 months, they are easily hand pollinated, seeds mature in approximately 1 month and unfertilised and aborted ovules can be counted as well as mature seeds. Populations are readily found in undisturbed grasslands and woodlands providing a system that can be used in both a natural context and under experimental glasshouse conditions. In this thesis I built upon the previous study broadening our understanding of inbreeding in *B. bulbosa* and further contributing to the knowledge of mating systems within the Asphodelaceae.

Thesis aim

The overall aim of this research is to investigate the causes of self-sterility and consequences of inbreeding on seed set and progeny fitness in *B. bulbosa* (Asphodelaceae). First, I investigate the cause of self-infertility in *B. bulbosa*. Second, I determine if mating between related individuals also caused infertility and reduced progeny fitness. Third, I examine if mating between individuals in close proximity reduced fecundity. Finally, I examine if self pollen or pollen from related individuals interfered with outcross pollen and caused or exacerbated pollen limitation of seed set under natural conditions. The specific aims and questions of the individual chapters are outlined below.

Chapter aims

In Chapter 2, I investigate the mechanism reducing self seed set in *B. bulbosa*. Infertility after self-pollination could be either due to inbreeding depression (ID) or physiological self-incompatibility (SI). For *B. bulbosa* and other species, selection against selfing can occur early in seed development because of either early-acting ID or late-acting SI. I complete a qualitative and quantitative histological investigation of post-pollination development to determine which one of these mechanisms was reducing self seed set in *B. bulbosa*.

To achieve the aim of this chapter I ask the following questions:

(1) Is the barrier to selfing pre- or post-zygotic?

(2) How does seed development differ following self- vs. cross-pollination?

(3) Is the mechanism reducing self seed set self-incompatibility or inbreeding depression?

In Chapter 3, I assess the effect on progeny fitness, of biparental inbreeding. Inbreeding depression can result from mating between related individuals as well as selfing (Uyenoyama, 1986). Additionally, outcrossing populations of perennials are likely to harbour a high genetic load and inbreeding depression may affect later life-cycle stages including male and female functions (Barrett & Eckert, 1990; Lande *et al.*, 1994; Husband & Schemske, 1995). A full investigation of inbreeding depression is important when assessing ecological and evolutional processes in plant populations. This assessment should include the degree of inbreeding depression over an extensive range of life-cycle stages and from sources of inbreeding other than pure selfing (Nason & Ellstrand, 1995). To achieve the aim of this chapter I specifically ask the following question:

(1) Do biparentally inbred progeny exhibit inbreeding depression during seed development and at later life-cycle stages?

In Chapter 4, I examine the effect, on fecundity, of mating individuals at varying distances. *Bulbine bulbosa* has limited seed dispersal and limited pollen transfer due to small insect pollination, probably resulting in genetically structured populations. Therefore, biparental inbreeding, in addition to selfing may be costly. To achieve the aim of Chapter 4, I specifically ask the following questions:

(1) Does mating between individuals in close proximity lower seed set due to

inbreeding depression?

(2) Does mating between populations also adversely affect seed set due to outbreeding depression?

(3) Is there an optimal mating distance for *B. bulbosa*?

Finally, in Chapter 5, I investigate the reproductive ecology of *B. bulbosa* under natural conditions. I assess pollen limitation, self pollen interference and inbreeding depression. It important to collect ecological data over an extended period as conditions might vary within and between seasons. I conducted a field experiment in a natural population of *B. bulbosa* over three flowering seasons. I also examine self pollen interference, in a glasshouse pollen chase experiment. In Chapter 5, I ask the following questions:

(1) Is seed set limited by the amount of pollen deposition?

(2) Is seed set limited by the quality of pollen deposition?

(3) Does self pollen interfere with cross pollen?

Chapter 2

The mechanism of self-infertility in *Bulbine bulbosa*: self-incompatibility or inbreeding depression?

Introduction

Flowering plants often produce more ovules than seeds. Adjustment of maternal investment can occur at fertilisation or during seed development. Self-infertility is the reduction in seed set following self-pollination compared with cross-pollination, and is widespread among flowering plants. There are two mechanisms that may cause self-infertility among hermaphroditic flowering plants, physiological self-incompatibility and inbreeding depression (Stephenson, 1981; Seavey & Bawa, 1986).

Physiological self-incompatibility is a genetically controlled mechanism that allows the recognition and rejection of self or incompatible pollen by the female reproductive tissue, consequently promoting out-breeding and heterozygosity (de Nettancourt, 1977, 1997; Sage *et al.*, 2000). The incompatibility is determined by a match between alleles of a single polymorphic gene usually referred to as the S-locus (Sage *et al.*, 1994). Physiological self-incompatibility can be present in angiosperms with hermaphroditic flowers that have the pistil and anthers in close proximity, an

arrangement which would otherwise easily facilitate selfing. Self-incompatibility is well documented and has been found to act in one-half of all angiosperms (Sage *et al.*, 2000).

Two distinct forms of physiological self-incompatibility (SI) have been characterised, based upon whether it is the sporophytic or gametophytic genome that expresses the incompatibility allele in the male gametophyte (de Nettancourt, 1977). Sporophytic SI, usually rejects pollen on the stigmatic surface and is determined by a match in alleles, at the *S*-locus, between the diploid sporophyte and the *diploid* genotype of the pollen grain (Van Den Ende, 1976; Sage *et al.*, 2000). Gametophytic SI is determined by a match between the diploid sporophyte and the *haploid* genome of the pollen grain (Sage *et al.*, 2000). The barrier here occurs in the style (Van Den Ende, 1976). Sporophytic and gametophytic SI provide a barrier to selfing that is pre-zygotic, so, even if self-pollination occurs, self-fertilisation is prevented. Furthermore, assuming self pollen or self-pollen tubes do not cause direct interference to cross pollen (Chapter 4), ovules are still available for cross-fertilisation.

Late-acting self-incompatibility (LASI) is a third and lesser known type of SI and manifests in an extremely wide range of ways (Seavey & Bawa, 1986). For example, LASI was used to describe the rejection of self-pollen tubes before fertilisation, within the ovary or ovular tissue (pre-zygotic) of *Acacia retinodes, Crocus thomasii, Lycium cestroides*, and the arrest of development of self-pollen

tubes after they penetrated the ovules (post-zygotic), in *Hymenaea stigonocarpa* and *Capparis retusa* (Kenrick *et al.*, 1986; Chichiricco, 1993; Gibbs *et al.*, 1999; Bianchi & Gibbs, 2000; Aguilar & Bernardello, 2001). The failure of the embryo sac to mature in the presence of self-pollen tubes in *Narcissus triandrus* and the degeneration of selfed ovules in *Ipomopsis aggregatais* has also been classified as a LASI-type phenomenon (Waser & Price, 1991a; Sage *et al.*, 1999). For *Narcissus triandrus*, Sage (1999) suggested that the failure of ovules to develop to maturity was due to the absence of a stimulus from compatible pollen tubes, inferring a 'signalling process' between pollen grains and the female gametophyte. The presence of a slower rate of growth and/or ovule penetration by self-pollen tubes compared to cross in bignoniaceous species has also been attributed to a LASI system (Gibbs & Bianchi, 1999; Bittencourt Jr. *et al.*, 2003; Bittencourt Jr. & Semir, 2004, 2005). Evidence for single gene control of a late-acting self-incompatibility system has been provided by some authors (Cope, 1962; Jacob, 1980; Lipow & Wyatt, 2000). Late-acting self-incompatibility encompasses all phenomena not covered by conventional SI mechanisms.

Seavey and Bawa (1986) reviewed 20 species with a putative LASI mechanism, however, Bianchi *et al.* (2005) suggested that insufficient post-pollination details were available for many of these species to determine accurately if the self-sterility mechanism is LASI in each case. Gibbs and Bianchi (1999) provided an update of Seavey and Bawas' list by including all later reports, raising the number of species with a type of LASI to 35, from a wide range of family groups. However,

the research on the additional species does not conclusively support LASI in all cases. For example, *Blandfordia grandiflora* is listed by Gibbs and Bianchi, yet Ramsey *et al.* (1993), in their work on *Blandfordia grandiflora*, concluded cytological studies are needed for an accurate determination even though they provided an argument for LASI. Similarly, for *Dalbergia miscolobium*, Gibbs and Sassaki (1998) discussed LASI in this species, but they did not favour it as the likely explanation for low self-fruit set. Despite the current research interest in late-acting physiological self-incompatibility, the characterisation of this phenomenon remains unresolved.

Inbreeding depression (ID) is an alternative mechanism causing self-infertility and results from the expression of deleterious recessive alleles in homozygotes (Charlesworth & Charlesworth, 1987). Inbreeding depression can act at any stage of development, depending upon the genotypic effect of the locus in question (Seavey & Bawa, 1986; Husband & Schemske, 1996). Early-acting inbreeding depression (EAID) acts post-zygotically and can cause seed abortion very soon after zygote formation, as well as later in development (Seavey & Bawa, 1986; Krebs & Hancock, 1990; Manasse & Pinney, 1991; Husband & Schemske, 1996). Seavey and Bawa (1986) acknowledged the difficulty in distinguishing between EAID and LASI and provided a set of criteria to determine which phenomenon was responsible for low self seed set comparative to cross. These criteria have been challenged, suggesting that EAID, caused by the expression of recessive lethals, could explain most LASI phenomena (Klekowski, 1988; Nic Lughadha, 1988). In contrast,

arguments have suggested that it would take large numbers of deleterious recessive alleles, in very early seed development, to mimic SI (Kenrick & Knox, 1989; Waser & Price, 1991a). Nevertheless, lethal recessive genes causing seed abortion are thought to be common in natural plant populations (Wiens *et al.*, 1987; Krebs & Hancock, 1990; Seavey & Carter, 1994).

Inbreeding depression was determined as the cause of self-sterility in *Crinum erubescens*, using the criteria outlined by Seavey and Bawa (1986), namely that both pre- and post-zygotic effects of selfing were exhibited. There was a variation in self-compatibility and the genetic load was expressed in other life-cycle stages. Similarly, using theoretical criteria, Ramsey (1993) also determined EAID to be the most likely cause of reduced self seed set in *Blanfordia grandiflora.* More convincingly, however, observed embryo failures at various stages in *Epilobium obcordatum* also favoured EAID as the explanation (Seavey & Carter, 1996). On the other hand, microscopic observation of synchronous selfed-embryo failure in *Vaccinium corymbosum* provided support for a LASI explanation (Seavey & Bawa, 1986; Vander Kloet, 1991). Some authors have chosen to be cautious, e.g., Gribel and Gibbs(2002), who favour ID for an explanation to low self-fruit set in *Pseudobombax munguba* rather than invoke a 'as yet hypothetical post-zygotic self-incompatibility mechanism'.

Distinguishing between SI and ID is a central question in evolutionary ecology. The two mechanisms have the similar effect of reducing the number of selfed

compared to crossed progeny, but they have different fitness consequences for the maternal plant. Inbreeding depression can be extremely costly, reducing fecundity to very low levels if ovules are self-fertilised and lost through EAID. These ovules are wasted and unavailable for crossing. Self-incompatibility can be less costly depending upon the stage of rejection. If the SI mechanism is pre-zygotic, as with sporophytic and gametophytic SI, and the rejection of the male gametophyte is sufficiently early, then ovules may still be available for cross-fertilisation. However, if the rejection is post-zygotic, ovules are also wasted and unavailable for out-crossing (Lewis, 1979; Waser & Price, 1991a). In this case the cost to the maternal plant can be similar to ID. Many studies, since Seavey and Bawas' review (1986) on species with low self compared to cross fruit and/or seed set despite apparent successful self-pollen tube growth, have been set in the context of investigating the cause of self-sterility, either EAID or LASI (e.g, Krebs & Hancock, 1990; Manasse & Pinney, 1991; Mahy & Jacquemart, 1999). Although genetic analysis may be required to clearly characterise such a mechanism (Charlesworth, 1985; Seavey & Bawa, 1986), theoretical criteria and histological investigation can provide support for a LASI. After three decades, however, the subject remains challenging.

Earlier studies on *B. bulbosa* showed that it is partially self-fertile with cross seed set three-fold greater than self seed set (self vs. cross: mean number of seeds per fruit 2.4 ± 0.3 vs. 7.7 ± 0.4; Owen *et al.*, 2007). Although self seed set was reduced, fertilisation frequencies were similar after self- and cross-pollinations, leading to the hypothesis that either EAID or a LASI-type mechanism was responsible for reducing

self seed set. As far as I am aware there has been no other comprehensive reproductive ecological study on any species in the Asphodelaceae, with the exception of *Gasteria verrucosa* (*Gasteria verrucosa* Sears, 1937; also see *Ananas* and *Gasteria* Brewbaker & Gorrrez, 1967; *Asphodelus aestivus* Lifante, 1996). Interestingly, *G. verrucosa* was one of the earlier species suggested to have some sort of LASI mechanism.

Aim

In this chapter I investigate the mechanism reducing self seed set in *Bulbine bulbosa.* To achieve this I have asked the following questions:

1. Is the barrier to selfing pre- or post-zygotic?
2. How does seed development differ following self- vs. cross-pollination?
3. Is the mechanism reducing self seed set self-incompatibility or inbreeding depression?

Methods

Plant material

Experimental plants were collected from Tea Tree Gully, 20 km west of Armidale on the New England Tablelands, NSW, Australia (30°30'S, 151°40'E , 850m a.s.l.) and are hereafter referred to as the 'glasshouse collection'. Individuals were collected randomly from an area of about 1 ha, sampling no closer than 5m apart, to reduce the probability of selecting genetically related individuals. Plants were potted in potting mix of equal parts loam, sand and peat and kept in glasshouse conditions. Pollinating

insects were excluded. Pots were fertilised at fortnightly intervals with half strength soluble fertilizer (Aquasol ^{TM} 0.8g/L) and watered regularly.

Pollinations

To determine if the barrier to selfing is pre- or post-zygotic, I compared the probability of fertilisation following self- vs. cross-pollination by conducting controlled pollinations on arbitrarily chosen flowering plants from the glasshouse collection ($N = 13$). For cross-pollinations, flowers were emasculated. Pollen from two to four donor plants, within the glasshouse collection, was placed in small plastic vials and mixed thoroughly. Pollen was applied to the stigma of the target flower with a small metal probe. Self-pollinations were performed in a similar manner, except that pollen from the target flower was used. Three flowers were pollinated for each treatment on each plant. Treatments were administered, in a random order, within the first 15 flowers of an inflorescence. Pollinations were undertaken between 10.00 and 11.00 am eastern Australia daylight savings time (EADST). Flowers were harvested 2-days post-pollination (PP), at approximately the same time as hand pollinations were done, and placed in FPA fixative (Formalin: Propionic acid: Ethanol) for at least 48 hours and then transferred to 70 % ethanol for storage.

To assess differences in ovule development following self- vs. cross-pollination, controlled pollinations were undertaken on randomly chosen flowering plants from the glasshouse collection ($N = 8$). Pollinations were undertaken as described above except that six flowers were cross-pollinated and six or more

flowers were self-pollinated. To detect developmental differences over time, three self- and three cross-pollinated flowers were harvested from each plant at each 5- and 7-days PP. All cross-pollinated flowers initiated fruit development however, some self-pollinated flowers either failed to initiate fruit or abscised before 5- and 7-days PP. Extra pollinations were performed in order to obtain the correct sample sizes. Flowers were fixed and stored as above.

Clearing

To detect double fertilisation, material harvested at 2-days PP was prepared by dissecting ovules from the ovary and clearing in Herr's clearing medium (lactic acid, choral hydrate and clove oil) for approximately 10 minutes (Herr, 1971). Whole mounts of cleared ovules from each ovary were positioned on a 'raj' slide (Prakash, 1986) in a few drops of the clearing medium. Optical sagittal sections were observed using Nomarski interference optics at 20x magnification. All ovules in each ovary were examined. In total 626 selfed and 607 crossed ovules were examined. Ovules were scored as fertilised, unfertilised, undeveloped without embryo sac or undetermined. A detailed description of these categories, with images, is outlined in the results section (pages 25-28).

As a control, permanent slides of the following ovaries were prepared (see sectioning and staining methods below): harvested at maturity on the day flowers opened (un-pollinated), 2 days after flowers opened (un-pollinated), and self- and cross-pollinated harvested at 2-days PP. These permanent serial sections provided

material to compare and verify structures observed in the cleared optical sections. Cleared unfertilised ovules harvested on the day flowers opened and 2 days later where also observed.

Sectioning and staining

To examine ovule development following self- and cross-pollination at 5- and 7-days PP, permanent serial sections of ovaries were prepared for observation by sectioning and staining following Johansen (1940). Each ovary was processed by dehydration through a graded series of Tertiary Butyl Alcohol (TBA) to pure TBA, then infiltrated with, and embedded in, paraffin wax. The wax block was cut, into longitudinal serial sections 12μm thick, using an American Optical 820 Rotary Microtome. Sections were mounted and stained with Safranin and Fast Green. Slides were made permanent using Eukitt™ permanent mounting medium. In total, 677 ovules in 46 selfed ovaries and 668 ovules in 44 crossed ovaries were examined. Ovules were scored as fertilised, unfertilised, aborted, undeveloped without embryo sac, or undetermined. The defining features of these categories are explained and illustrated within the results section.

Statistical analysis

All data were checked for homoscedasticity and normality using Levene's and Ryan-Joiner's tests respectively (Minitab™ 13.1). Data satisfied the assumptions of homoscedasticity and normality for analysis of variance and were not transformed. The General Linear Model (GLM) feature of Minitab™ 13.1 was used for analyses

because there were small differences in sample sizes because some ovaries were damaged during the microtechnique process. The difference in fertilisation frequency after self- and cross-pollination at 2-days PP was investigated using a random block design. Pollination treatment was considered a fixed factor and plant as a random factor. The plant × pollination interaction was nonsignificant ($P = 0.896$) and was omitted from the final analysis to improve degrees of freedom for testing the main effects (Sokal and Rohlf 1995). Comparisons of self and cross seed development at 5- and 7-days PP were made using a factorial randomised block design with replication. Treatment and days since pollination were fixed factors and plant was a random factor.

Results

Mature ovules

Ovules are mature when flower opens. Mature ovules are hemitropous and bitegmic with the presence of an aril (Fig. 2.1a). The inner integument is three cells thick and forms the micropyle (Fig. 2.1b & c). At ovule maturity, the aril remains small, extending only slightly beyond the funicle (Fig. 2.1a). The embryo sac is the polygonum type. The two polar nuclei are fused forming a conspicuous secondary nucleus within the central cell (CC; Fig. 2.1b & c; Maheshwari & Singh, 1930). The CC is often surrounded by starch granules, presenting as shiny circles in cleared material (Fig. 2.1a & b). The antipodal cells are obscured by tannin containing nucellar cells, at the base of a crassinucellate nucellus.

Fertilised ovules 2-days PP

Fertilised ovules were observed in cleared material 2-days PP in approximately 70% of all ovules, regardless of pollination treatment. The following characteristics signified double fertilisation. Following syngamy, the zygote was observed easily, in cleared specimens with one or both synergids less obvious than in an unfertilised ovule (Fig. 2.2a & b). The primary endosperm nucleus (PEN) was conspicuous and with little or no obvious starch present (see Fig. 2.1b unfertilised vs. Fig. 2.2a fertilised). The PEN moved to the chalazal end of the embryo sac (Fig. 2.2b). The first mitotic division of the primary endosperm nucleus formed two chambers, the first stage of a helobial endosperm. The nucleus in the chalazal chamber divided only once or twice more (Fig. 2.2c & d). A free nuclear endosperm formed in the micropylar chamber becoming the functional endosperm. In a few specimens, a pollen tail was observed extending from the micropyle (Fig. 2.2e). After fertilisation the aril becomes enlarged, growing over the micropyle (Fig. 2.2c). Although the aril appears to become an outer seed coat (K. Owen personnel observation), aril growth was not taken as a sign of fertilisation. Continued growth of the aril was also observed in unfertilised ovules.

Fertilised ovules 5- and 7-days PP

Fertilised ovules observed in permanent sections 5- and 7-days PP were well stained and characterised in the same manner as fertilised ovules in cleared material. This involved the observation of a zygote (Fig. 2.3a & b) or the beginning of embryo formation (2- or rarely 3-celled, Fig. 2.3c), two chambers forming a helobial

endosperm (Fig. 2.3d) and/or free nuclear endosperm in the micropylar chamber (Fig. 2.3a, b, d & e). In most specimens, the zygote had not divided but the endosperm was multi-cellular (Fig. 2.3e).

Unfertilised ovules

Unfertilised ovules in cleared material and permanent sections were characterised by the presence of an organised embryo sac (Fig. 2.4a & d) and/or clearly visible intact synergids (Fig. 2.4b & e). In cleared material, the observed presence of starch granules around the polar nuclei or central cell also signified an absence of fertilisation (Fig. 2.4a, b & c).

Aborted ovules

Aborted ovules were shrivelled and/or showed signs of cellular degeneration in the nucellar tissue and/or embryo sac, but had a zygote and/or endosperm development (Fig. 2.5a). In comparison to fertilised ovules, aborted ovules stained poorly (Fig. 2.5b). They were usually larger than unfertilised ovules and mostly smaller than fertilised ovules at the same time PP. As viable ovules develop, the nucellar tissue becomes disorganised and breaks down to be re-absorbed by the endosperm (Norstog, 1974). Care was taken not to confuse the process of, re-absorption of nucellar tissue, with ovular degeneration (Fig. 2.5c). Some ovules were observed in a state of advanced degeneration and others were observed in the initial stages of degeneration (Fig. 2.5a vs. Fig. 2.6a). Ovules in the initial stage of abortion had nucellar tissue that was poorly stained and endosperm nuclei and zygote stained as if

they were still developing, suggesting that nucellus failure occurred first (Fig. 2.6a & c). Although this was not quantified, ovule abortion apparently occurred at various stages of development following self-pollination. Degenerating ovules were observed where abortion had occurred early, with a few endosperm nuclei present. Others appeared to have aborted later, with a relatively large endosperm cavity containing numerous free nuclei (Fig. 2.6a, b & c). Some aborted self-fertilised ovules were observed with a zygote and others had a two-celled embryo (Fig. 2.6a vs. 2.6b).

Undeveloped ovules

Undeveloped ovules were observed in cleared preparations and permanent sections and were characterised by the absence of an organised 7-celled embryo sac. These ovules showed no signs of an obvious megaspore or any stage of embryo sac development and may represent inherent early developmental failure in ovule differentiation (Seavey & Carter, 1996). Undeveloped ovules were not investigated further and were not included in statistical analyses (see below), as they represented only a small percentage of total ovules (less than 1%, 7% and 1% for 2-, 5- and 7-days PP respectively).

Undetermined ovules

It was not always possible to determine if fertilisation had occurred. For cleared material, ovules were placed in this category if a zygote was not clearly visible. In very early development it can be difficult to distinguish between an unfertilised egg and a single-celled zygote (Prakash pers. comm. 2002) In addition, it can sometimes

difficult to tell if the central structure was an unfertilised CC or the PEN (Prakash pers. comm. 2002). In the absence of a clear zygote or embryo and/or endosperm development, ovules were classed as undetermined. In material that was examined at 5- and 7-days PP, structures that were shrivelled could have been either unfertilised or aborted ovules, such structures were placed in the undetermined class (Fig. 2.7a). In a few instances, where the ovule was shrivelled, but both synergids were still recognisable, this indicated that fertilisation had not occurred (Fig. 2.4e and Fig. 2.7b).

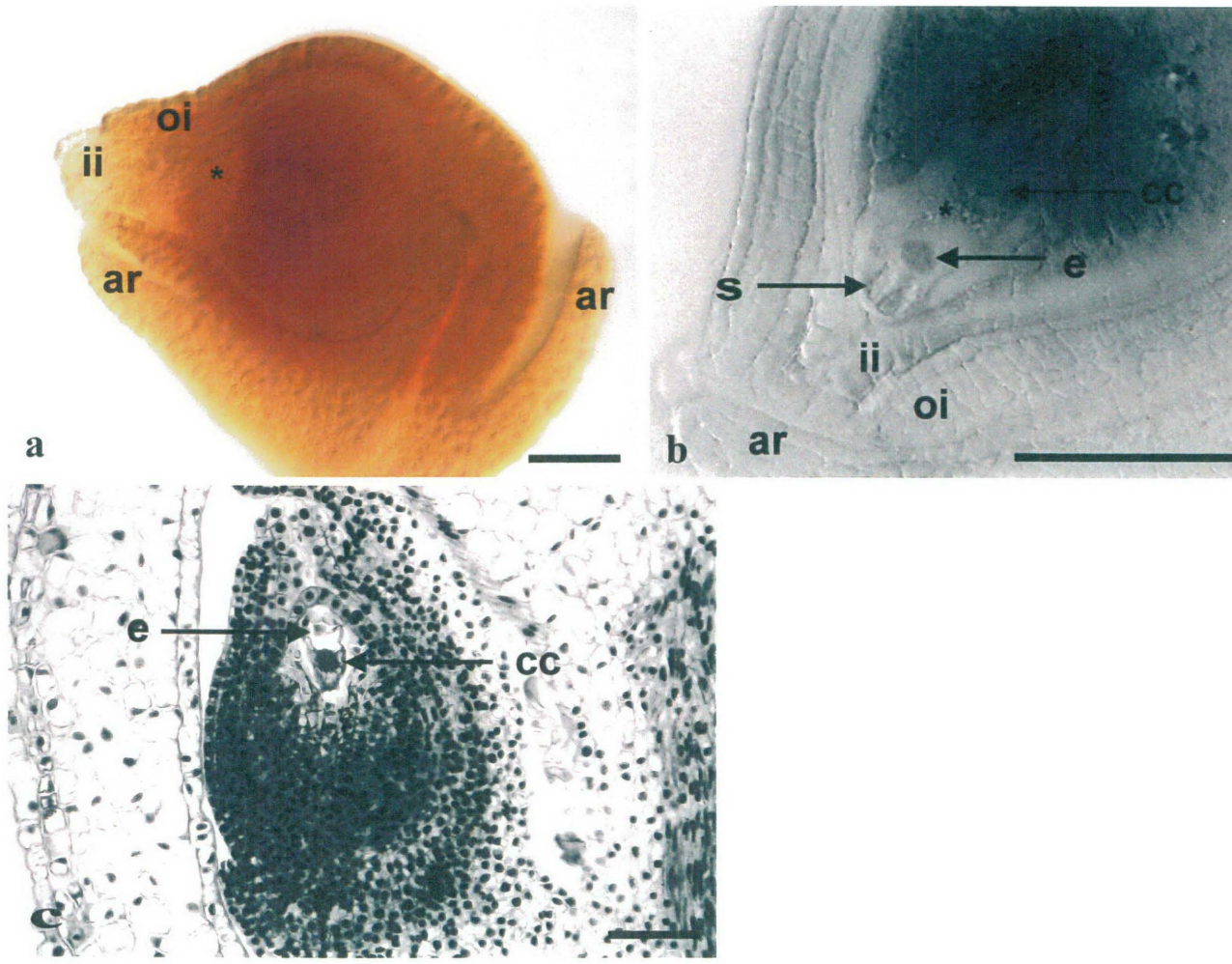

Figure 2.1. Mature unfertilised ovules. (a) Cleared ovule harvested at maturity. (b) Cleared ovule harvested at 2-days PP. (c) Ovule harvested at maturity. Longitudinal section (12µm) stained with Safranin and Fast Green. Abbreviations: ii, inner integument; cc, central cell; *, starch deposits; e, egg; s, synergids and ar, aril. Scale bar = 500µm.

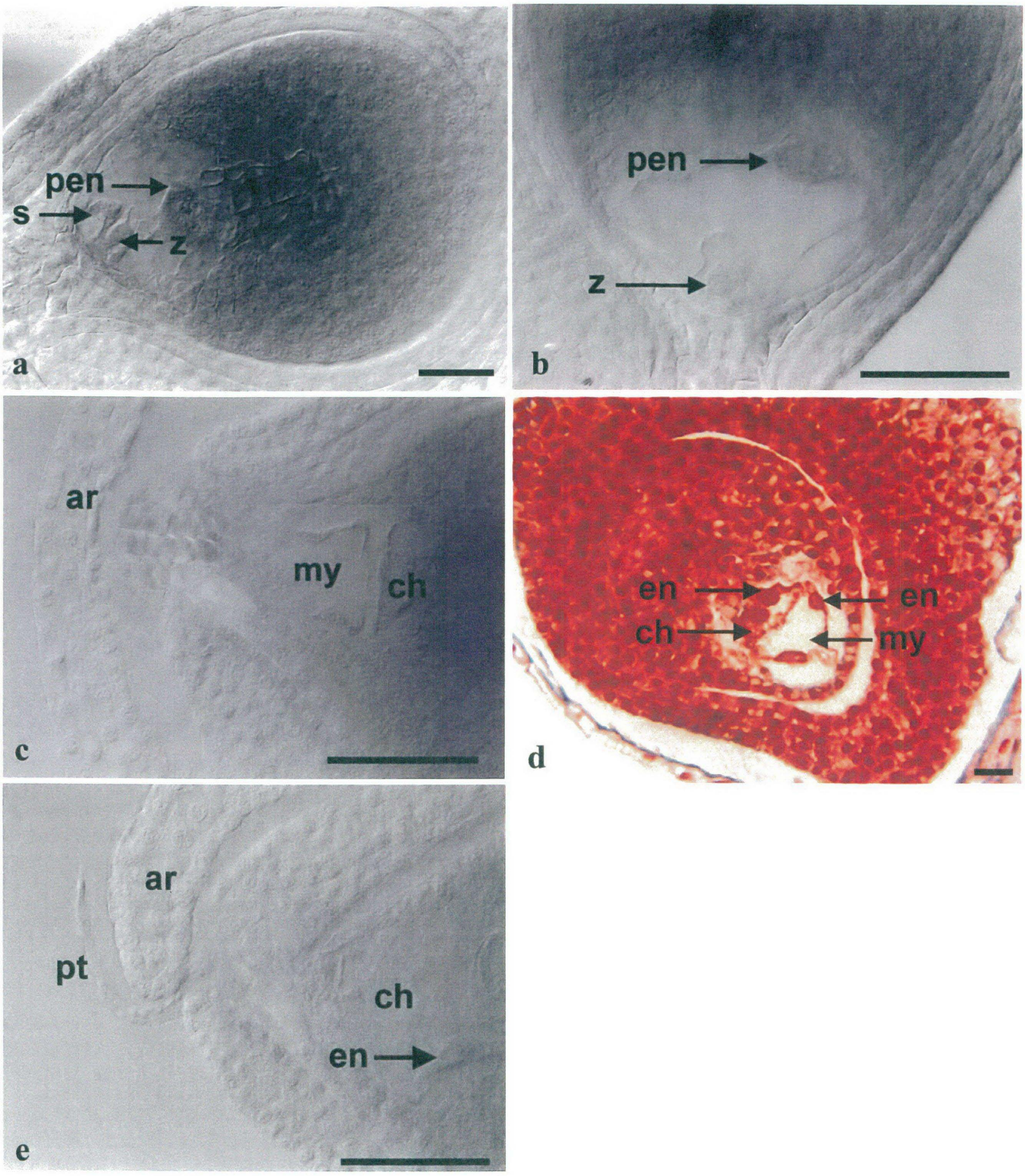

Figure 2.2. Fertilised ovules 2-days PP. (a) Cleared self-fertilised ovule with zygote, one visible synergid and the primary endosperm nucleus contains starch granules. (b) Cleared cross-fertilised ovule showing a distinctive zygote and primary endosperm nucleus at the chalazal end of the embryo sac. (c) Cleared cross-fertilised ovule with the helobial endosperm and two free nuclei in the micropylar chamber and the aril covering the micropyle. (d) Longitudinal section (12µm) of a self-fertilised ovule stained with Safranin and Fast Green at the same stage of development as c. (e) Cleared self-fertilised ovule with a visible pollen tail. Abbreviations: ar, aril; ch, chalazal chamber; en, endosperm nuclei; my, micropyle chamber; pen, primary endosperm nuclei; pt, pollen tail and z, zygote. Scale Bar = 100µm.

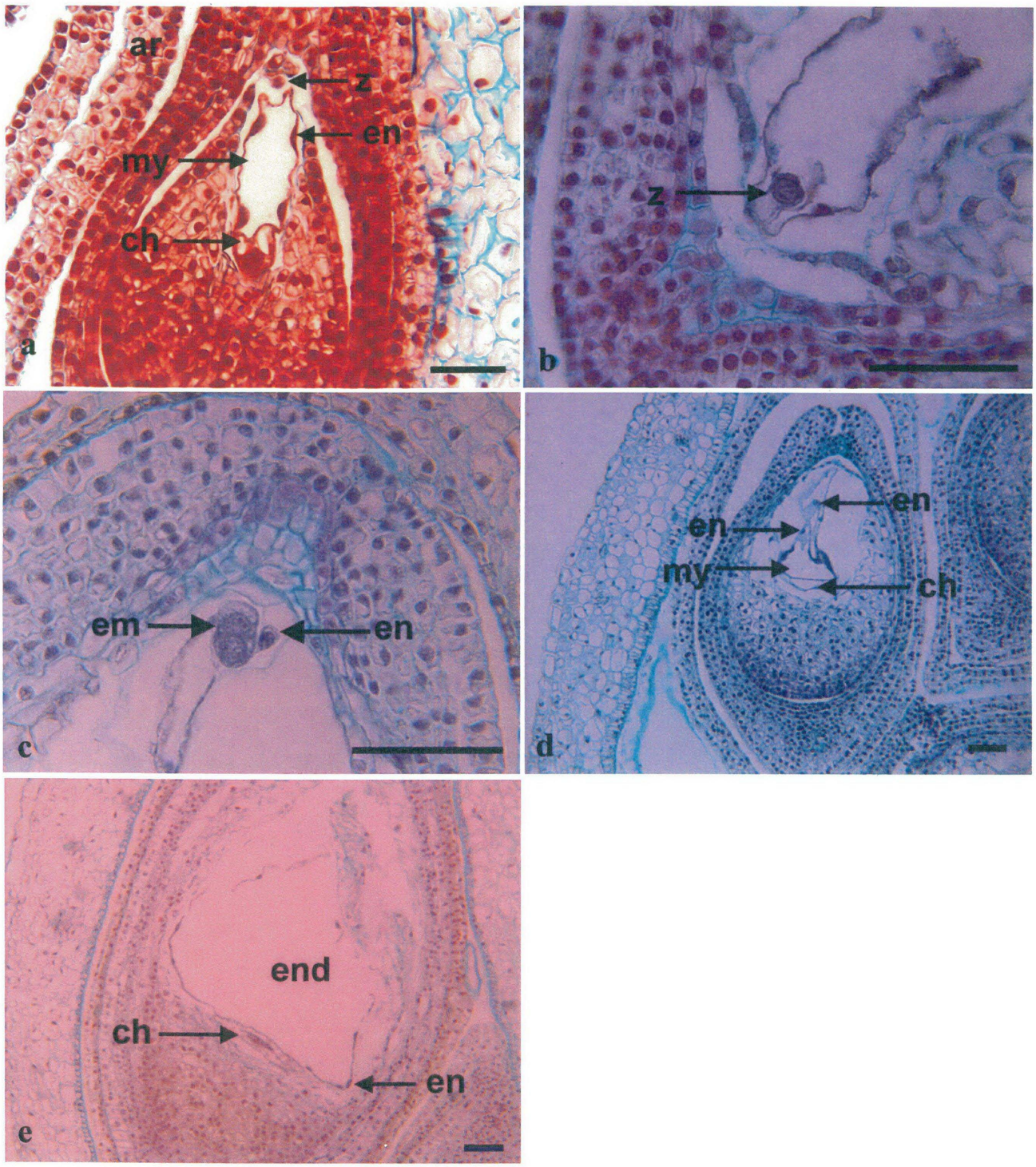

Figure 2.3. Fertilised ovules. Longitudinal sections (12μm) stained with Safranin and Fast Green. (a) Cross-fertilised ovule at 5-days PP. (b) Self-fertilised ovule at 5-days PP with a zygote. (c) Cross-fertilised ovule at 7-days PP with a 2-celled embryo. (d) Cross-fertilised ovule at 5-days PP. (e) Cross-fertilised ovule at 7-days PP with a free nucleate developing endosperm compressing the chalazal chamber. Abbreviations: ar, aril; ch, chalazal chamber; em, embryo; en, endosperm nuclei; end, endosperm; my, micropyle chamber; z, zygote. Scale Bar = 100μm.

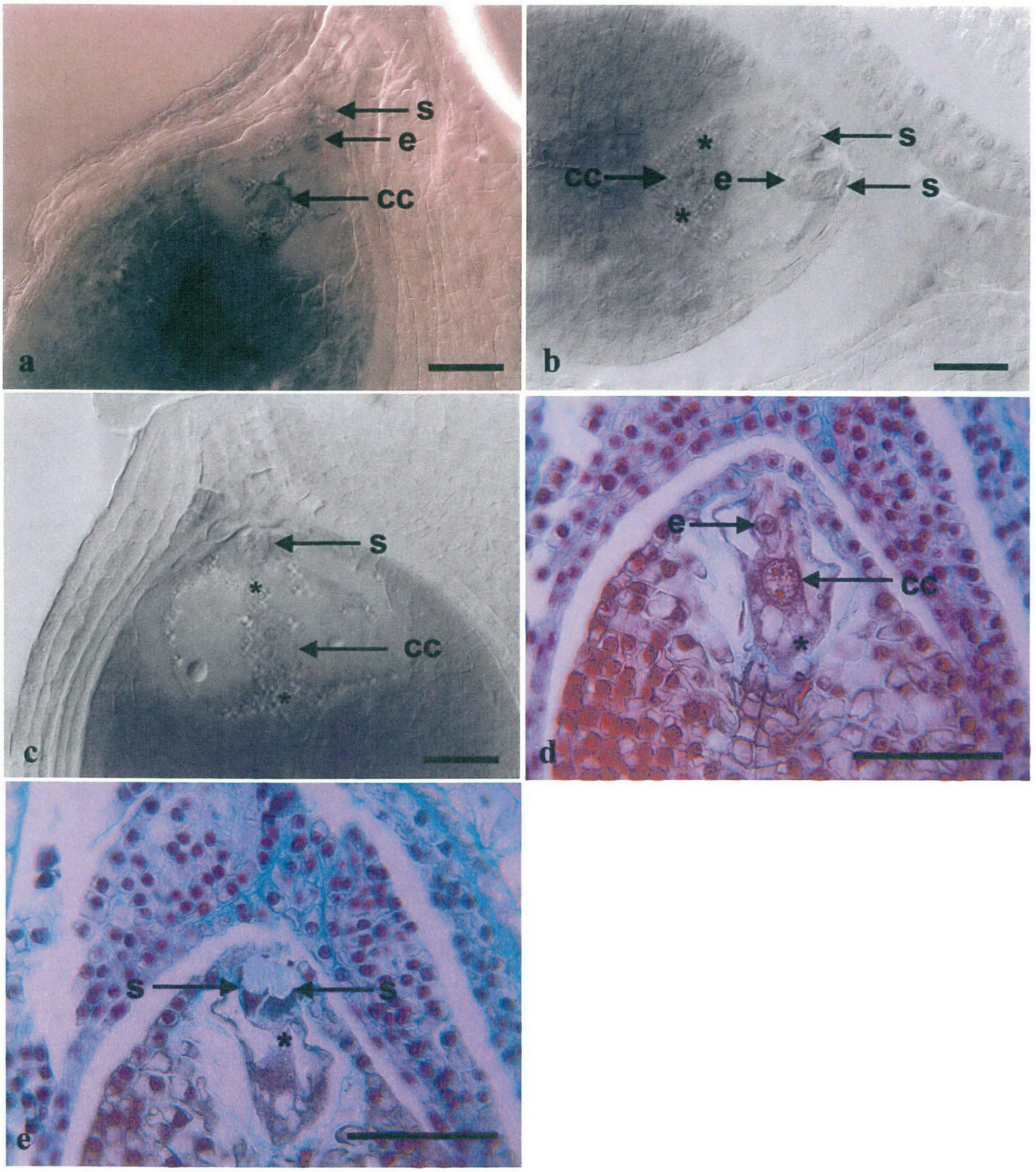

Figure 2.4. Unfertilised ovules. (a) – (c) Cleared, (d) – (e) longitudinal sections (12µm) stained with Safranin and Fast Green. (a) Ovule at 2-days PP from a self-pollinated flower with an organised embryo sac. (b) Ovule at 2-days PP from a cross-pollinated flower with starch deposits and synergids still obvious. (c) Ovule at 2-days PP from a self-pollinated flower with starch deposits and synergids still obvious. Two polar nuclei have not fused. (d) Ovule at 5-days PP from a self-pollinated flower. The central cell, with two polar nuclei not fused, is surrounded by starch and the egg is clearly visible. (e) Ovule at 7-days PP from a cross-pollinated flower with two synergids intact. Abbreviations: *, starch deposits; e, egg; cc, central cell; pn, polar nuclei; s, synergids. Scale Bar = 100µm.

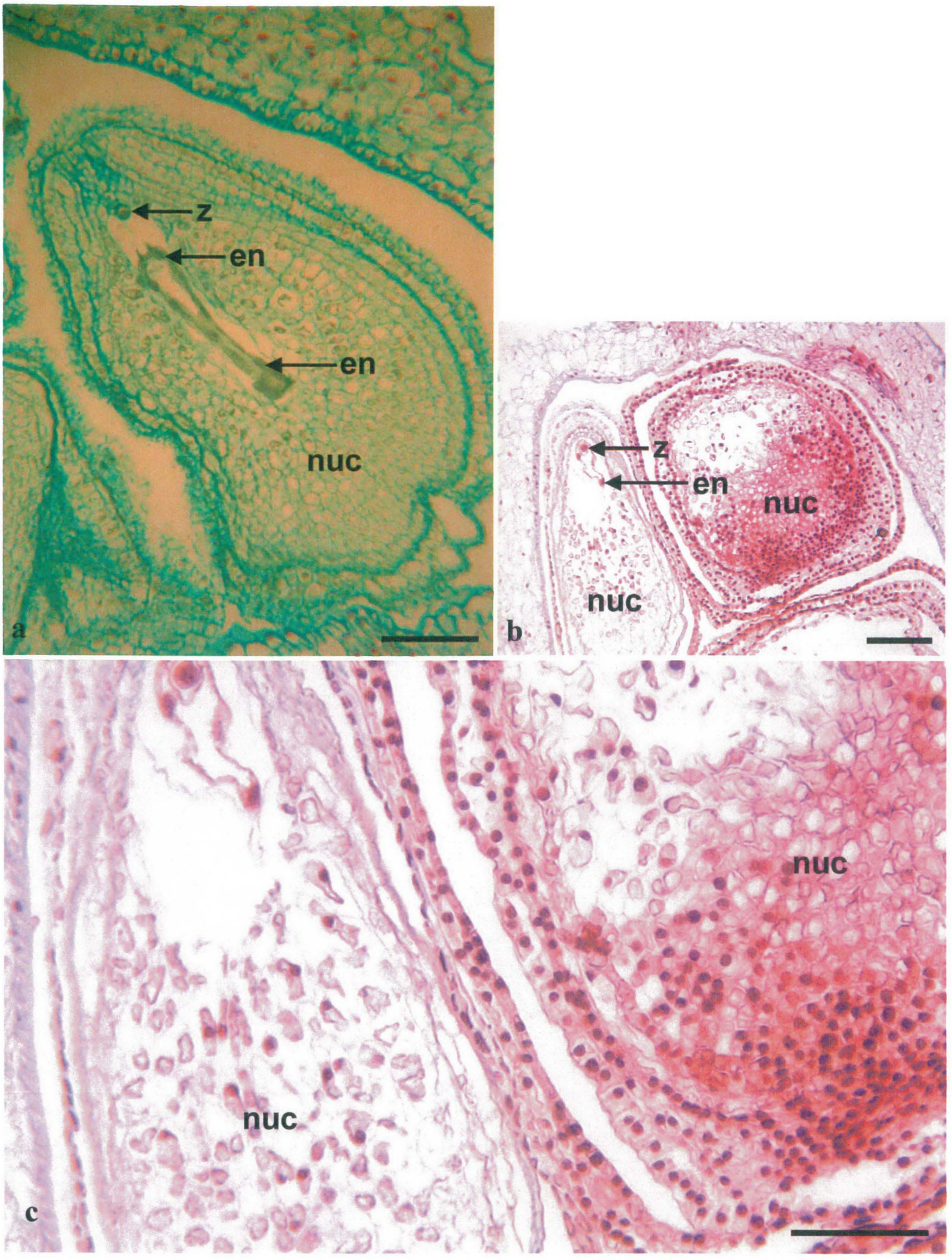

Figure 2.5. Aborted ovules. Longitudinal sections (12µm) stained with Safranin and Fast Green. (a) Self-fertilised ovule 5-days PP with zygote and free nuclear endosperm, representative of initial development, but ovule is shriveled and poorly stained, indicating abortion. (b) Self-pollinated flower at 7-days PP with an aborted ovule (left) adjacent to a viable ovule (right). (c) Nucellar tissue degenerating as ovule aborts (left) in contrast to nucellus re-abortion in developing ovule (right). Abbreviations: en, endosperm nuclei; nuc, nucellus; z, zygote. Scale Bar = 100µm.

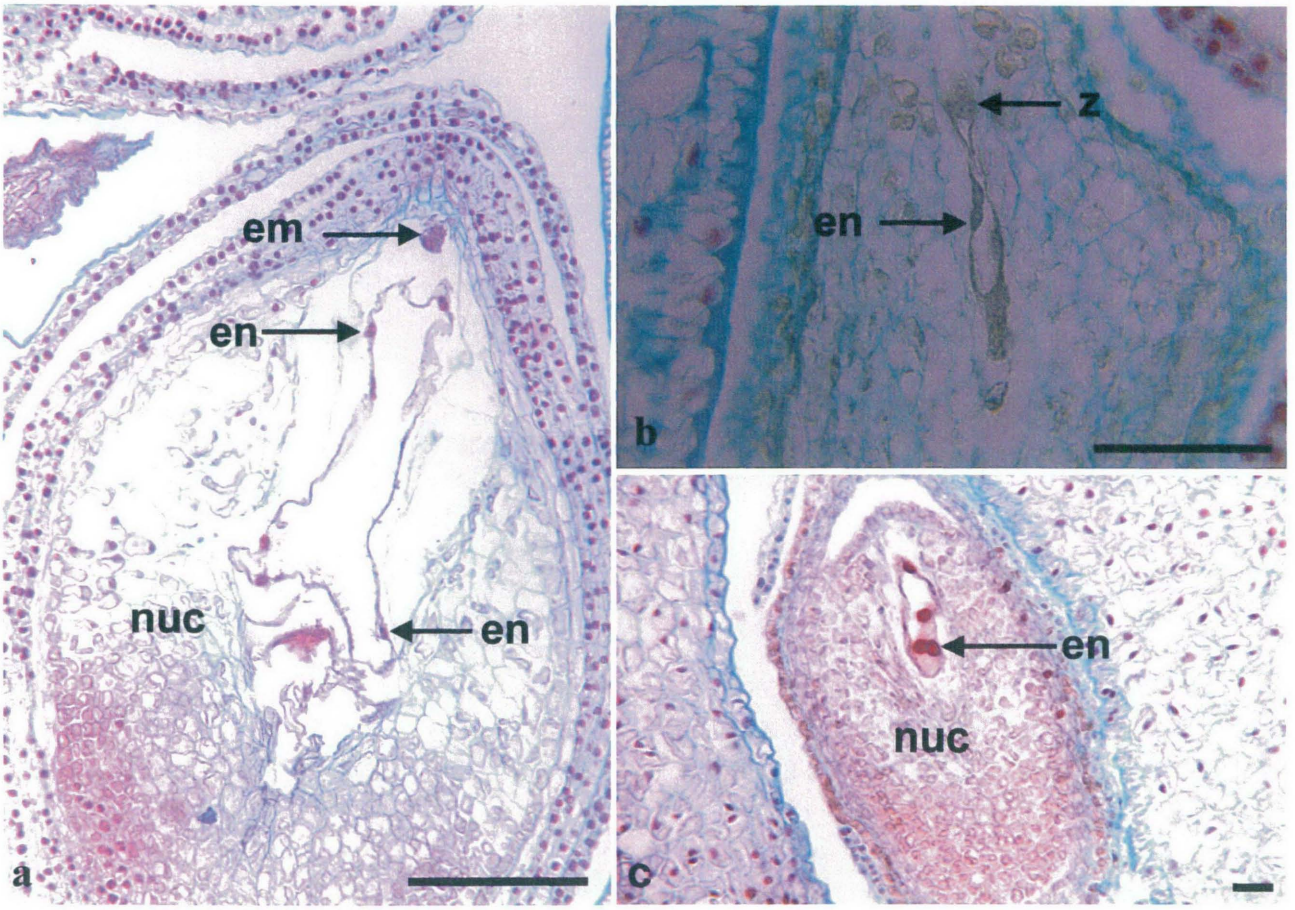

Figure 2.6. Aborted ovules. Longitudinal sections (12μm) stained with Safranin and Fast Green (a) Self-pollinated ovule at 7-days PP. (b) Self-pollinated ovule at 7-days PP. (c) Self-pollinated ovule at 5-days PP. Note that abortion has occurred at different stages in development, ovule in image (a) has a relative large endosperm and a 2-celled embryo. Ovules in (b) and (c) have a much less developed endosperm and have probably aborted at an earlier stage. Additionally, note that in (a) and (c) the nucellar tissue appears to be degenerating yet the zygote and endosperm nuclei are well stained, indicating the nucellar tissue may be first to abort and the zygote and endosperm were probably still developing at the time of harvest. Abbreviations: em, embryo; en, endosperm nuclei; nuc, nucellus; z, zygote. Scale Bar = 100μm.

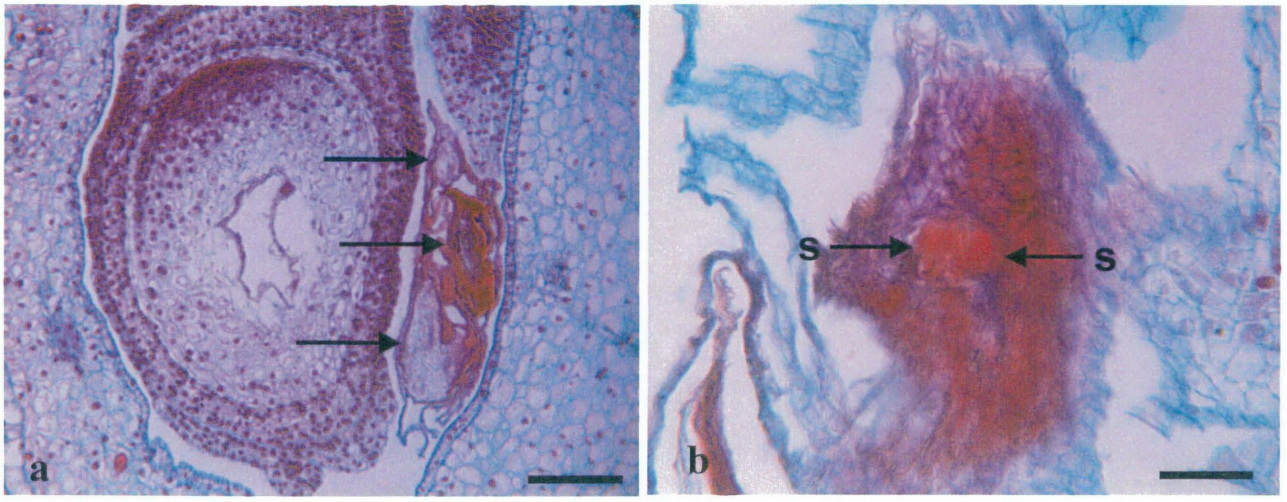

Figure 2.7. Self- and cross-pollinated flowers at 7-days PP. Longitudinal sections (12μm) stained with Safranin and Fast Green. (a) Arrows pointing to three undetermined structures within a cross-pollinated flower. (b) Ovule from a self-pollinated flower classified as unfertilised because the two synergids remain intact. Abbreviations: s, synergids. Scale Bar = 100μm.

Fertilisation frequencies

The number of fertilised ovules per ovary did not differ significantly following self- and cross-pollination (Table 2.1). On average about 11 ovules per ovary (≈ 70%) were fertilised at 2-days PP. Variation among plants was significant at 2-days PP, however there was no significant difference detected at 5- and 7-days PP (2-days, $F_{12, 60} = 2.09$, $P = 0.031$; 5- & 7-days PP, $F_{8.1, 4.2}$[1] $= 1.97$, $P = 0.180$). Levels of fertilisation did not increase by 5- and 7-days PP, indicating that fertilisation takes place within 2 days of pollination. Overall, these results demonstrate that self-pollination caused fertilisation as frequently as cross-pollination, indicating that there was no pre-zygotic barrier to self seed set development.

Table 2.1. Mean (±SE) number of ovules fertilised per ovary after self- and cross-pollination at 2-days PP, and 5- and 7-days PP. F ratios and P values are presented.

Stage of development	Pollination treatment		Statistical results		
	Self	Cross	df	F	P
2-days PP	11.81 ± 0.53	11.00 ± 0.37	1, 60	1.60	0.21
5- & 7-days PP	10.87 ± 0.52	10.95 ± 0.54	7.19, 16.82 [1]	0.00	0.96

[1] Not exact F-test (Minitab 13).

Ovule development at 5- and 7-days PP

Pollination treatment had a highly significant effect on ovule development and abortion (Fig. 2.8, Table 2.2a & b). Days PP significantly affected ovule abortion but not ovule development (Fig. 2.8, Table 2.2a & b). More importantly, for both ovule development and ovule abortion, there was a significant interaction between pollination treatment and days PP, indicating that the number of developing ovules and the number of aborted ovules in each treatment was dependant upon the days PP (Table 2.2a & b). After self-pollination, the number of developing ovules was reduced by ≈ 50% from 5-days PP to 7-days PP. For cross-pollination, the number of developing ovules was similar at 5-days PP and 7-days PP, (Fig. 2.8). Conversely, abortion increased from 5-to 7-days PP after self-pollination, but remained low at both time points for cross-pollination. There was no significant difference between plants for ovule abortion and development (Table 2.2a & b).

Undetermined ovules

The number of ovules that were unable to be determined as either fertilised or unfertilised did not differ significantly between treatments or days PP (2.86 ± 0.63 ovules per ovary), suggesting that these structures were as likely to be found after self- and cross-pollination at either 5- or 7-days PP (Table 2.2c).

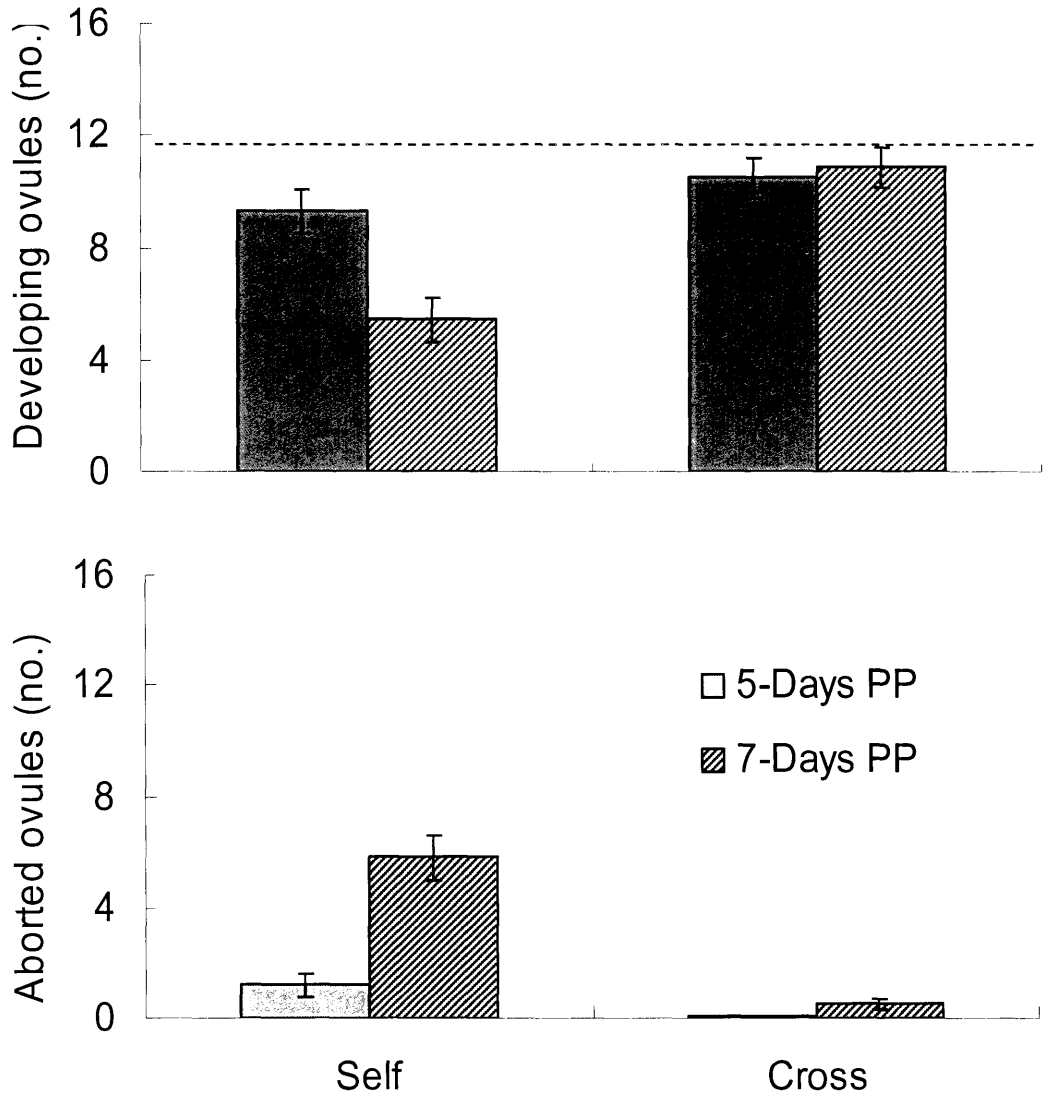

Figure 2.8. Number of developing and aborted ovules after self- and cross-pollination harvested at 5- and 7-days PP. Dotted line represents mean fertilisation. Graph relates to ANOVA results in Table 2.2

Table 2.2. Results of the Factorial random-block ANOVA examining the effects of plant (block), pollination treatment (self or cross) and days PP (5 or 7) on developing ovules. (a) ovule abortion (b) and the number of undetermined ovules (c). Plants were considered a random factor, treatment and days PP were fixed factors. These analyses correspond to Fig. 2.8.

Source of variation	d.f.	Adj MS	F	P
(a) Developing ovules				
Plant	7	45.025	2.13	0.170
Pollination	1	236.511	25.69	0.000
Days PP	1	47.921	2.29	0.173*
Plant × Days PP	7	21.143	2.30	0.036
Pollination × Days PP	1	90.502	9.83	0.002
Error	72	9.205		
(b) Ovules abortion				
Plant	7	14.491	1.24	0.405*
Pollination	1	219.803	21.79	0.002*
Plant × Pollination	7	10.237	2.24	0.155
Days PP	1	122.028	20.57	0.003*
Plant × Days PP	7	6.002	1.31	0.364
Pollination × Days PP	1	81.211	17.92	0.004*
Plant × pollination × Days PP	7	4.571	1.80	0.104
Error	58	2.537		
(c) Undetermined ovules				
Plant	7	41.815	4.09	0.041*
Pollination	1	18.534	3.11	0.082
Days PP	1	7.399	0.73	0.420*
Plant × Days PP	7	10.219	1.72	0.119
Error	73	5.959		

NB * Not an exact F-test (Minitab™ 13.1). The plant × pollination (P = 0.272) and plant × pollination × days PP (P = 0.631) interactions were non-significant and were omitted from the final analyses of developing ovules (a). The plant × pollination (P = 0.539), pollination × days PP (P = 0.658) and plant × pollination × days PP (P = 0.756) interactions were non-significant and were omitted from the final analyses of undetermined ovules (c).

Discussion

In this chapter, I described the mature ovule of *B. bulbosa* and characterised the sequence of events in the first week of fertilised ovule development. There has been little embryological research within the Asphodelaceae and only one paper examining a *Bulbine* species (*Bulbine annua*; Stenar, 1928; *Asphodelus tenuifolius*; Maheshwari & Singh, 1930; *Gasteria verrucosa*; Sears, 1937; Aloaceae; Steyn & Smith, 1998). My observations are consistent with these earlier works. *Bulbine bulbosa* has typical Asphodelaceae characteristics, such as hemitropous, arillate, bitegmic and crassinucellate ovules; polygonum-type embryo sac development, fused polar nuclei prior to fertilisation, and helobial endosperm formation after syngamy (Maheshwari & Singh, 1930; Cave, 1953; Watson & Dallwitz, 1992).

The frequency of fertilisation did not differ following self- and cross-pollination and was about 70% at 2-days PP. I developed an unambiguous classification for fertilisation, and the most common indicator at 2-days PP was the clear presence of a zygote and endosperm initiation with two free endosperm nuclei in the micropylar and chalazal chambers. This stage was consistently observed in both self- and cross-fertilised ovules. Similar fertilisation frequencies after self- compared to cross-pollination have been found in other species, although few studies determine fertilisation by direct observation of ovules. Fertilisation was inferred by pollen tube penetration of ovules in *Hymenaea stigonocarpa* and was calculated using aborted ovules and self seed set (Gibbs *et al.*, 1999; Mahy & Jacquemart,

1999). Fertilisation frequencies in *Epilobium obcordatum*, a species originally hypothesised to have a LASI mechanism, were also found to be similar after both self- and cross-pollination with direct observation of serial sections of ovaries up to 10-days PP (Seavey & Carter, 1996). Similarly, microscopic investigation of sectioned post-pollination ovules showed that the largely self-sterile *Dalbergia miscolobium* also had no significant difference in fertilisation frequencies between self- and cross-pollination (Gibbs & Sassaki, 1998). Conversely, also in a microscopic investigation of cleared developing ovules, a significantly lower fertilisation frequency after self- compared to cross-pollination was detected in *Clintonia borealis* (Dorken & Husband, 1999).

The mechanism providing self-infertility in *B. bulbosa* is clearly post-zygotic, eliminating the well known forms of physiological self-incompatibility. It has been suggested that a post-zygotic self-sterility system is unlikely to be common as it essentially wastes ovules (de Nettancourt, 1977; Lewis, 1979; Seavey & Bawa, 1986; Lipow & Wyatt, 1998). Such wastage of ovules may explain why plants have evolved to produce more ovules than seeds (Charlesworth, 1989). *Bulbine bulbosa* has a relatively high ratio of fertilised ovules:total ovules compared to other species (Krebs & Hancock, 1990; Manasse & Pinney, 1991), which suggests that there is an opportunity for selection among developing ovules.

In this study, I found that there were very few ovule abortions after cross-pollination. The number of developing ovules at 7-days PP remained at fertilisation

levels. In contrast, developing self-fertilised ovules were reduced by abortion by 5-days PP and even further by 7-days PP. The focus on developmental differences following self- vs. cross-pollination was aimed at quantifying the number of developing ovules or conversely the number of abortions, at 5- and 7-days PP. At these stages, ovules were characterised specifically to determine if they were developing or had aborted in order to ascertain if ovule abortion was an on going phenomenon or occurred at a single developmental stage.

The results of this study indicate that ovule abortion after selfing was caused by inbreeding depression, the expression of several or many genes, arresting development at different stages. Evidence provided here shows that self ovule abortion occurred in at least two time frames, between fertilisation and 5-days PP (a reduction of 9%) and 5- and 7-days PP (a reduction of 23%). Further reduction of ≈ 21% is also likely to occur between 7-days PP and maturity providing a third time frame for self ovule abortion (Owen *et al.*, 2007). In a LASI mechanism, abortions would have been evident at a similar stage in development. I did not observe abortions at any one single stage up to 7-days PP. A LASI system has been implicated in *Pseudowintera axillaris*, where a uniform failure of developing selfed ovules occurred at 15-days PP before the division of the zygote (Sage & Sampson, 2003). Similarly, in *Crocus vernus,* embryo formation is arrested just before the early globular stage (Chichiricco, 1993). Based on this study, I cannot completely rule out LASI acting during embryogenesis, in conjunction with EAID, without a histological examination of self ovule development between 7-days PP and completed embryo

formation. However, I did observe numerous globular embryos after selfing in two different plants sampled at 10-days PP (K. Owen, unpublished data). Further more, although I did not quantify the developmental stages of abortions, I observed aborted ovules that appeared to arrest at different developmental stages. Therefore, it is most likely that EAID rather than LASI is reducing self seed set comparative to cross seed set in *B. bulbosa*.

The permanent slides prepared for this chapter could be used to characterise the process of abortion in selfed ovules, as was done in a detailed study of the fate of ovules by Seavey and Carter (1996). Additionally, the comparative size and developmental stage of selfed and crossed ovules at 5- and 7-days PP could be calculated to assess the degree of inbreeding depression for these life-cycle stages. Examining ovules, with an increased sample size, at 10-days PP, with particular attention to embryo development, may rule out the possibility of LASI acting in conjunction with EAID. However, this thesis investigates the detrimental effects of inbreeding on pollination, seed set and later life cycle stages facilitated through pure selfing, mating between related individuals and mating between related individuals in close proximity.

Chapter 3

Uniparental and biparental inbreeding depression in *Bulbine bulbosa* (Asphodelaceae)

Introduction

Inbreeding is an important part of the mating system of many flowering plants (Thornhill, 1993). A common consequence of inbreeding is a relative decrease in fitness of self compared with outcrossed progeny. Inbreeding depression (δ) is expressed as a value between 0 and 1, and is estimated as: $1 - (w_s / w_c)$, where w_s is the mean fitness following selfing and wc the mean fitness following crossing. Inbreeding depression can be calculated for each life-cycle stage or trait, or a multiplicative function can be utilised to calculate overall inbreeding depression. Two hypotheses have been proposed to explain inbreeding depression in plant populations, the 'over dominance' and the 'partial dominance' theories (Charlesworth & Charlesworth, 1987; Roff, 2002). According to the over dominance theory, heterozygous genotypes have superior fitness over homozygotes (Charlesworth & Charlesworth, 1987). However, the partial dominance hypothesis is generally considered more important and proposes that inbreeding depression is caused by the expression of recessive deleterious mutations (Charlesworth & Charlesworth, 1987; Husband & Schemske, 1996; Roff, 2002).

Besides the immediate significance for the ecology and viability of inbred populations, inbreeding depression has substantial evolutionary consequences (Barrett & Kohn, 1991; Dole & Ritland, 1993; Husband & Schemske, 1995; Husband & Schemske, 1996). Inbreeding depression is the major selective force opposing the automatic gene transmission advantage of selfing (Charlesworth & Charlesworth, 1979; Holsinger, 1992; Husband & Schemske, 1996). A selfing plant can pass on genes to the next generation, as an ovule and pollen parent to its own progeny, and through pollen dispersal, giving a 50% advantage over an outcrossing individual (Fisher, 1941). However, if inbreeding depression is over 0.5, the selfing advantage is negated. Inbreeding depression is, therefore, commonly used to explain the evolution and maintenance of breeding systems that facilitate outcrossing and discourage selfing (Charlesworth & Charlesworth, 1979; Lloyd, 1979; Lande & Schemske, 1985).

Inbreeding depression has been studied widely but estimates of the magnitude of inbreeding depression are often calculated by comparing pure self and unrelated outcrossed progeny (Charlesworth & Charlesworth, 1987; Holsinger, 1992; Nason & Ellstrand, 1995). This provides an estimate of uniparental inbreeding depression and may be a restricted view of inbreeding (Nason & Ellstrand, 1995). Restricted pollen and seed dispersal contributes to genetic structure in populations, creating a situation where genetically related individuals are located in close proximity – so called genetic neighbourhoods (Levin, 1984; Heywood, 1991; Waser, 1993a; Griffin & Eckert, 2003; Herlihy & Eckert, 2004). Even though an outcrossing breeding system may be adopted, crossing between plants in close proximity can lead to inbreeding

under natural conditions (Uyenoyama, 1986; Waller, 1986). If inbreeding depression is calculated by comparing self and related outcross parents, the estimate will be reduced by the extent to which the mates are genetically related (Waller, 1993; Waser, 1993a). Mating between genetically related individuals is referred to as biparental inbreeding, and biparental inbreeding depression is the reduction in fitness of progeny resulting from matings between related individuals compared with mating between unrelated individuals (Ritland, 1984; Uyenoyama, 1986; Waller, 1993; Nason & Ellstrand, 1995).

Biparental inbreeding depression is often assessed indirectly, by comparing the fitness of progeny resulting from varying interparental distances (Price & Waser, 1979; Levin, 1984; Waser & Price, 1989; Dudash, 1990; Fenster, 1991; McCall *et al.*, 1994; Trame *et al.*, 1995; Fischer & Matthies, 1997; Byers, 1998; Hardner *et al.*, 1998; Stacy, 2001). Direct estimates of biparental inbreeding depression, assessing comparative fitness of progeny resulting from mating between individuals of known genetic relationships, have also been obtained for some species (Heywood, 1993; Nason & Ellstrand, 1995; Thompson & Tarayre, 2000; Delph, 2004).

While uniparental inbreeding depression is proposed as the major force opposing the evolution of selfing, evolutionary predictions incorporating estimates of biparental inbreeding depression are more difficult (Dole & Ritland, 1993; Waller, 1993). Uniparental inbreeding depression decreases the relative performance of selfed progeny and prevents the spread of alleles associated with selfing, whereas,

biparental inbreeding depression reduces offspring fitness while promoting genes that are associated with outcrossing (Uyenoyama, 1986; Waller, 1986). Simple models have suggested that populations will evolve unconditionally toward selfing or outcrossing if uniparental inbreeding depression is below or above the threshold of 0.5, respectively (Lloyd, 1979; Schoen & Lloyd, 1984; Lande & Schemske, 1985; Schemske & Lande, 1985). By contrast, more complex models incorporating the effects of biparental inbreeding predict stable but mixed mating systems (Uyenoyama, 1986; Uyenoyama *et al.*, 1993; Waller, 1993).

Models incorporating biparental inbreeding have two opposing genetic forces to consider (Uyenoyama, 1986; Waller, 1993). Biparental inbreeding reduces the genetic cost of outcrossing whilst simultaneously reducing the advantage of alleles that promote selfing (Waller, 1986). The degree to which the cost of outcrossing is reduced below 0.5 depends upon the relationship of the mating individuals (Uyenoyama, 1986; Griffin & Eckert, 2003). The greater the coefficient of inbreeding, the more the cost of outcrossing declines and the selfing advantage is reduced. As a result, the probability of an outcrossing mating system is increased (Waller, 1986). However, biparental inbreeding increases homozygosity, eliminating deleterious recessives alleles and favouring selfing (Waller, 1986; Kelly & Willis, 2002). It is difficult to predict which of these opposing forces may carry more weight (Uyenoyama, 1986). Biparental inbreeding, therefore, may favour a mixed mating system with the pendulum swinging towards selfing or outcrossing as contributing factors such as, the amount of biparental inbreeding and the relatedness of mating individuals vary (Uyenoyama, 1986; Waller, 1986, 1993).

Severe uniparental inbreeding depression was detected in *B. bulbosa* in a previous study (Owen *et al.*, 2007). Also, early-acting depression reduces the number of developing selfed ovules (Chapter 2). *Bulbine bulbosa* pollinators are non-specialist small insects and as a result of energy constraints pollen-dispersal distances may only be a few metres (Heinrich & Raven 1972). Therefore, pollen-dispersal in *B. bulbosa* is likely to follow a leptokurtic distribution with predominately near neighbour pollination (Kevan & Baker 1983). Additionally, *B. bulbosa* has no obvious seed dispersal structures, the seeds seemingly are simply dispersed by gravity (K. Owen, personal observation). These factors may contribute to natural populations of *B. bulbosa* developing a spatial pattern where genetically related individuals are found in close proximity. Consistent with this view, matings between individuals of *B.bulbosa* in close spatial proximity result in fewer seeds than matings between individuals that are distant from each other (Chapter 4). In natural populations of *B. bulbosa*, therefore, biparental inbreeding depression could impact upon the ecology and evolution of the mating system.

Aim

In this chapter I examine the effect of mating genetically related individuals on progeny fitness at several life-cycle stages. I hand-pollinated *B. bulbosa* flowers with self pollen, related-cross pollen, and unrelated cross pollen. I then examined seed set and progeny fitness at later life-cycle stages. Specifically, I ask the following question:

Do biparentally inbred progeny exhibit inbreeding depression during seed

development and at later life-cycle stages (i.e., seed mass and germination, seedling survival and growth, flowering, and pollen and ovule production)?

Methods

Experimental design – hand-pollinations

I assessed the effect of mating between related individuals by conducting hand-pollinations on ten family groups. To generate family groups, I cross-pollinated at least 5 flowers on each of 10 randomly chosen maternal plants from the glasshouse collection (Chapter 2). Multiple pollen donors were used for cross-pollinations. Seeds from each of the 10 maternal plants were used to generate family groups of about 15 plants. Plants were kept in the glasshouse and maintained as described in Chapter 2. Plants within each family ranged from half to full sibs, mimicking family groups in a natural population.

I compared three pollination treatments, self, biparentally inbred and outcrossed, using three focal plants from each family group (Fig. 3.1). For outcross-pollinations, flowers were emasculated and pollen was obtained from two to four donors from the same population, excluding plants in the family groups (Fig. 3.1). For the biparentally inbred-pollinations, flowers were emasculated and pollen was obtained from two to four donors within the family, excluding the three focal plants (Fig. 3.1). For self-pollinations, flowers were emasculated and pollen from the same flower was collected and used. Hand-pollinations were undertaken as described in

Chapter 2. Three replicates of each pollination treatment were randomly conducted on each focal plant, totalling nine pollinations within the first 20 flowers of an inflorescence.

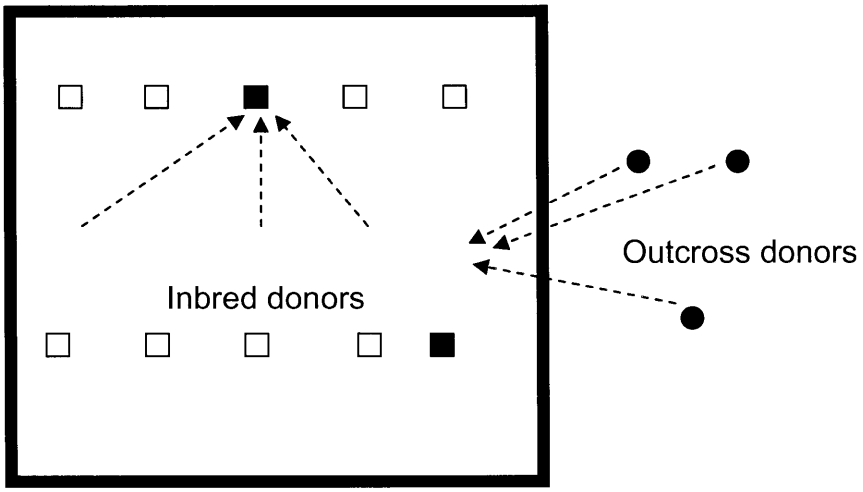

Figure 3.1. Model illustrating how pollen donors were used for biparentally inbred- and outcross-pollinations in the glasshouse. Symbols inside box (□ and ■) represent one family of full to half sibs, where ■ are pollen recipients and □ are inbred donors. Symbols outside the box (●) represent unrelated plants from the same population, which were used as outcross-pollen donors. Inbred-pollen donors were from within the family, excluding pollen recipients.

Ovule fertilisation, seed abortion and seed set

Mature fruits were harvested when the capsules began to split open. I scored the number of unfertilised ovules, aborted seeds and seeds in all matured fruits. Ovules less than 0.5mm were scored as unfertilised. Shrivelled structures of varying sizes and ≥0.5mm were scored as aborted seeds. Mature seeds were counted if they were dark brown or black and firm. Aborted seeds may have been underestimated as very early abortions may have gone undetected (Chapter 2). Seed set was calculated as a proportion of the total number of ovules (seeds + abortions + unfertilised ovules). The sum of seed abortions and the number of mature seeds equalled ovule fertilisation. Seed abortion was calculated as a proportion of the number of fertilised ovules.

Later life-cycle stages

To determine the effect of pollination treatment on later life-cycle stages, seeds from each treatment, from each plant, were placed in small envelopes and stored for approximately one month. Because not all plants produced selfed seeds, selfed seeds were pooled across plants within families to assess the effect of selfing on later life-cycle stages.

Seed mass

To determine the effect of pollination treatment on seed mass, ten seeds from each plant were weighed individually to the nearest 0.01mg (N = 246 and 256 for

biparentally inbred and outcross seeds, respectively). All available selfed seeds were also weighed ($N = 130$).

Seed germination

I assessed seed germination to determine if inbred seeds were less viable than outcross seeds. I randomly selected 20 biparentally inbred and 20 outcross seeds from each plant (N = 3) within each family (N = 10). Seeds were then sterilised in a bleach solution (3 parts H_2O: 1 part 5% bleach). For biparentally inbred and outcross treatments, seeds from each plant were placed on moist seed germination paper in separate Petri dishes. For the self treatment, seed families were placed in individual Petri dishes. Dishes were maintained at alternating temperatures of 20°C for 12 h light and 10°C for 12 h dark in a germination cabinet for the duration of the experiment. Germination, scored when radicle appeared, was recorded daily for eight weeks. Germinated seeds were removed and placed in separate Petri dishes labelled by germination date. Percent germination and the number of days to 50% germination were calculated for each Petri dish (i.e., plant within each family for biparentally inbred and outcrossed progeny, and family groups for selfed progeny).

Seedling survival, leaf length and biomass

To examine seedling growth and survival, 10 biparentally inbred- and 10 outcross-germinated seeds were randomly selected from each maternal plant (N = 3) within each family (N =10). All selfed seeds that germinated were used. Seedlings were planted into small tube pots (110ml) using a standard potting mix (1:1:1 sand, loam,

peat) and placed on trays in a glasshouse. Each tray (360mm × 295mm) of 20 pots consisted of biparentally inbred and outcrossed progeny from each plant within each family. A total of 30 trays, in addition to 10 trays (10 families) of selfed plants, were randomly relocated every two weeks to minimise position effects. Although selfed plants were on different trays to families of biparentally inbred and outbred plants, randomly relocating all trays also minimized the chance that selfed plants experienced different conditions to that of biparentally inbred and outbred plants. Plants were fertilised monthly with 20 ml of half-strength liquid fertilizer (Aquasol) and watered regularly. At seven months I harvested plants and recorded survival. Roots and shoots were placed separately in brown paper bags and material was then dried in a 60° oven for 72 hours and weighed.

Flowering

The effect of pollination treatment on flower production was assessed by counting the number of days to the first open flower. The number of flowers on the first inflorescence and the number of inflorescences per plant were counted when plants were harvested.

Pollen and ovule production

To determine the effect of pollination treatment on pollen production, pollen grains were counted, measured and assessed for viability. The first flower bud, from each of five randomly chosen biparentally inbred and outcross plants within each family, was harvested at maturity (N = 150 flower buds). The anthers were placed in a small

plastic vial and the ovary was placed in a separate vial in 70% ethanol. The number of pollen grains for each flower was counted by adding 0.6mL of lactophenol stain, mixing pollen grains thoroughly to suspension using a vortex mixer and counting four replicate haemocytometer grids (Kearns & Inouye, 1993). The total number of pollen grains was recorded, and the number of viable and inviable grains noted. Grains that were more or less oval in shape and stained well were classed as viable, and misshapen, collapsed or unstained grains were classed as inviable. The mean length of ten randomly chosen pollen grains was recorded per flower to assess pollen grain size. The pollen grains were broadly-oval to oval, and the length was measured to the nearest 1.75 μm (calibration of smallest graticule width) along the longest axis, using an ocular gradicule at 20X magnification. The number of ovules per ovary was counted with a stereo microscope at 20× magnification. All ovule-like structures extending from the placental tissue were counted, even if they were relatively smaller and may have been non-functioning ovules (see Chapter 2).

Relative performance and inbreeding depression

I estimated the relative performance of selfed (RPs) and biparentally inbred (RPbp) progeny for each trait. The performances of self, outcross and biparentally inbred progeny are W_S, W_C and W_{bp}, respectively. Relative performance of selfed progeny was calculated as: $RP_S = W_S/W_C$. Relative performance of biparentally inbred progeny was calculated as: $RP_{bp} = W_{bp}/W_C$. Because higher values of seed abortion and days to 50% germination represent reduced performance, I calculated the relative

performance of these traits as the ratio of cross-to-self performance: $RP_S = W_C/W_S$ and $RP_{bp} = W_{bp}/W_C$ (Ramsey and Vaughton, 1996).

I estimated uniparental inbreeding depression (uniδ) and biparental inbreeding depression (biδ) for each trait as: uni$\delta = 1 - RP_S$ and bi$\delta = 1 - RP_{bp}$.

I estimated the relative cumulative fitness of selfed (RF_S) and biparentally inbred (RF_{bp}) progeny as a multiplicative function of the relative performances for seed set, seedling survival and biomass at 7 months, numbers of flowers per inflorescence and inflorescences per plant, numbers of ovules and pollen grains per flower, and pollen viability. These stages were chosen because they are considered components of fitness, independent of one another, and a statistical difference was detected between pollination treatments (Ramsey *et al.*, 2003).

I estimated cumulative uniparental and biparental inbreeding depression as: uniδ = 1 - RFs and biδ = 1 - RFbp.

Relative performances and inbreeding depression of selfed and biparentally inbred progeny, for each plant family, were calculated separately as described above.

Statistical analysis

The effects of pollination treatment on seed set, seed abortion, ovule fertilisation, seed mass, dry mass, days to first flower, number of flowers, number of inflorescences, number of ovules, pollen grain number, pollen viability and pollen grain size was determined using partially hierarchical ANOVAs (Model III). Family and plants were considered as random factors and treatment was a fixed factor. Percent germination, speed of germination and seedling survival were compared using two-way ANOVAs (Model III) with family and treatment as factors. For biomass, a covariate, seedling age, was included in the analysis. The treatment × seedling age and family × seedling age interactions were not significant and were removed from the final analyses (all $P > 0.2$). For percent seed set, zero values were included where no fruits matured, but, for seed abortion and ovule fertilisation, fruits that did not develop to maturity were not scored. For seed development, the three pollination treatments (self, biparental and outcross) were compared. For later life-cycle stages, only biparentally inbred and outcrossed progeny were compared; sample sizes for selfed progeny were insufficient for statistical analysis.

All data were checked for normality and homogeneity required for ANOVA. Percent ovule fertilisation, seed germination, seedling survival and pollen grain viability were arcsine transformed. A log 10 transformation was used for seed mass. The germination speed and days to first flower were square-root transformed and an inverse square-root transformation was used for pollen grain size. The remaining traits were not transformed. Untransformed means ± SE are presented. The General Linear Model (GLM) feature of Minitab™ 13.1 was used for analysis.

Results

Seed production

Percent seed set and seed abortion differed significantly between pollination treatments (Fig. 3.2, Table 3.1). Percent seed sets of biparentally inbred and outcross treatments did not differ from each other (Tukey Test, $P > 0.05$). Seed set after outcross-pollination was approximately 33% and decreased by three-fold after self-pollination (Fig. 3.2). Self-pollination resulted in the most seed abortion, outcross-pollination the least and biparentally inbred abortion was intermediate (Fig. 3.2, Table 3.1). There was no significant difference between treatments for ovule fertilisation (Fig. 3.2, Table 3.1). For all traits, there were significant differences between plants within families but no differences between families (Table 3.1).

Progeny fitness

Biparentally inbred progeny had inferior performance compared with outcrossed progeny. Differences between biparentally inbred and outcrossed progeny were significant for seedling survival, dry mass, days to first flower, numbers of flowers and inflorescences, numbers of ovules and pollen grains, and pollen grain size and viability (Tables 3.3, 3.4, 3.5, 3.6 and 3.7). Interactions between treatment and family were significant for seed mass and pollen grain size, indicating, for these traits, the effect of pollination treatment depended on family. Variation between families was marginally significant for seed mass but not other traits. Variation among plants within families was more pronounced and occurred for seed mass, dry mass, days to

first flower, numbers of flowers and inflorescences, pollen grain size and viability, and pollen grain number (Tables 3.2, 3.5, 3.7 and 3.4).

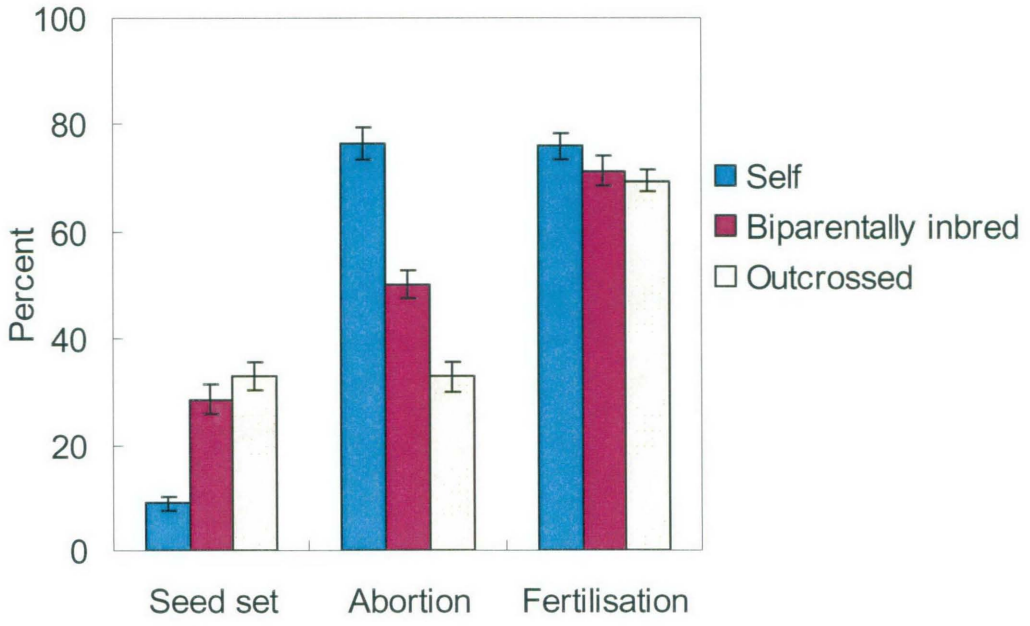

Figure 3.2. Percent (± SE) seed set, seed abortion and ovule fertilisation following self-, biparentally inbred- and outcross-pollination treatments. Percent seed sets after biparentally inbred- and outcross-pollination treatments did not differ. Fertilisation frequencies after all treatments did not differ (Tukey Tests, $P > 0.05$). Graph corresponds to ANOVA results in Table 3.1.

Table 3.1. ANOVA results for seed set, seed abortion and ovule fertilisation. Model III partially hierarchical ANOVA of the effects of family, plants, pollination treatment (self, biparentally inbred and outcrossed) and their interactions on seed set, seed abortion and ovule fertilisation. For ovule fertilisation and seed abortion the treatment × plants interaction could not be tested because some fruits did not develop to maturity. Data correspond to those presented in Fig. 3.2

Source of variation	d.f.	Adj MS	F	P
Seed set				
Family	9	0.24	1.96	0.102*
Plants (family)	20	0.08	1.90	0.041
Pollination	2	1.49	18.00	0.000
Family × pollination	18	0.08	1.89	0.047
Treatment × plants (family)	40	0.04	1.29	0.131
Error	180	0.03		
Seed abortion				
Family	9	0.10	0.87	0.567*
Plants (family)	20	0.10	2.36	0.002
Pollination	2	2.71	45.01	0.000*
Family × pollination	18	0.06	1.49	0.101
Error	139	0.04		
Ovule fertilisation				
Family	9	0.11	1.07	0.420*
Plants (family)	20	0.10	2.94	0.000
Pollination	2	0.07	1.45	0.257*
Family × pollination	18	0.05	1.44	0.124
Error	139	0.03		

NB * Not an exact F-test (Minitab™ 13.1)

Table 3.2. ANOVA results for seed mass, percent germination and days to 50% germination. Model III partially hierarchical ANOVA of the effects of family, plants, pollination treatment (biparentally inbred and outcrossed) and their interaction on seed mass. Two-way mixed model ANOVAs of the effects of family, treatment (biparentally inbred and outcrossed) and their interactions on seed germination and days to 50% germination. Means (± SE) for seed mass, percent germination and days to 50% germination after self-, biparentally inbred- and outcross-pollination treatments are provided in Table 3.8.

Source of variation	d.f.	Adj MS	F	P
Seed mass				
Family	9	0.20	2.35	0.045*
Plants (family)	20	0.08	18.02	0.000
Pollination	1	0.02	1.65	0.229*
Family × pollination	9	0.02	3.58	0.000
Error	480	0.00		
Percent germination				
Family	9	0.05	0.71	0.690
Pollination	1	0.02	0.21	0.658*
Family × pollination	9	0.07	1.07	0.409
Error	36	0.07		
Days to 50% germination				
Family	9	1.05	2.69	0.078
Pollination	1	0.06	0.15	0.705*
Family × pollination	9	0.39	0.87	0.556
Error	36	0.45		

NB * Not an exact F-test (Minitab™ 13.1)

Table 3.3. ANOVA results for seedling survival at 7 months. Model III ANOVA of the effects of family, pollination treatment (biparentally inbred and outcrossed) and their interaction on seedling survival at 7 months. Means (± SE) for seedling survival at 7 months after are provided in Table 3.8.

Source of variation	d.f.	Adj MS	F	P
Seedling survival at 7 mths				
Family	9	131.5	1.07	0.459
Pollination	1	1551.5	12.63	0.006*
Family × pollination	9	122.6	0.89	0.541
Error	36	137.4		

NB * Not an exact F-test (Minitab 13)

Table 3.4. ANOVA for dry mass at 7 months. Model III partially hierarchical ANOVA of the effects of family and pollination treatment (biparentally inbred and outcrossed) and their interactions on dry mass at 7 months. Seedling age was a covariate. The seedling age × family and seedling age × treatment interactions were not significant and were omitted from the analysis ($P > 0.2$). Means (± SE) are provided in Table 3.8.

Source of variation	d.f.	Adj MS	F	P
Dry mass at 7 mths				
Seedling age	1	3.920	3.68	0.056
Family	9	3.971	0.97	0.465*
Plants (family)	19	4.351	4.09	0.000
Pollination	1	32.982	30.98	0.000*
Family × pollination	9	0.870	0.82	0.601
Error	437	1.065		

NB * Not an exact F-test (Minitab 13)

Table 3.5. ANOVAs for days to first flower, number of flowers and inflorescences. Model III partially hierarchical ANOVA of the effects of family, plants, pollination treatment (biparentally inbred and outcrossed) and their interactions on days to the first open flower, number of flowers of the first inflorescence and number of inflorescences. Means (± SE) for days to first flower, number of flowers and number of inflorescence are provided in Table3.8.

Source of variation	d.f.	Adj MS	F	P
Days to first flower				
Family	9	7.091	1.18	0.378*
Plants (family)	19	5.553	2.69	0.000
Pollination	1	151.715	71.56	0.000*
Family × pollination	9	2.091	0.86	0.562
Error	404	2.434		
Number of flowers				
Family	9	530.2	1.30	0.313*
Plants (family)	19	392.8	2.62	0.000
Pollination	1	6233.5	35.86	0.000*
Family × pollination	9	176.1	1.18	0.309
Error	404	149.9		
Number of inflorescence				
Family	9	1.338	1.09	0.435*
Plants (family)	19	1.132	1.75	0.026
Pollination	1	10.366	13.82	0.004*
Family × pollination	9	0.760	1.18	0.307
Error	404	0.645		

NB * Not an exact F-test (Minitab 13)

Table 3.6. ANOVA for the number of ovules produced per ovary. Model III partially hierarchical ANOVA of the effects of family, plant, pollination treatment (biparentally inbred and outcrossed) and their interactions on the number of ovules. Means (± SE) are provided in Table 3.8.

Source of variation	d.f.	Adj MS	F	P
Numbers of ovules				
Family	9	13.731	1.62	0.265*
Plants (family)	19	6.995	1.06	0.398
Pollination	1	161.073	19.99	0.001*
Family × pollination	9	8.125	1.23	0.279
Error	239	6.623		

NB *Not an exact F-test (Minitab 13)

Table 3.7. ANOVAs for pollen production. Model III partially hierarchical ANOVA of the effects of family, plants, pollination treatment (biparentally inbred and outcrossed) and their interactions on pollen grain size, pollen viability and total number of pollen grains. Means (± SE) for pollen grain size, percent viability and number of pollen grains are provided in Table 3.8.

Source of variation	d.f.	Adj MS	F	P
Pollen grain size				
Family	9	0.003	0.37	0.932*
Plants (family)	19	0.004	2.66	0.000
Pollination	1	0.036	5.55	0.042*
Family × pollination	9	0.007	4.66	0.000
Error	199	0.002		
Percent viability				
Family	9	300.2	1.10	0.431*
Plants (family)	19	224.1	1.66	0.044
Pollination	1	6701.3	36.29	0.000*
Family × pollination	9	186.9	1.38	0.197
Error	239	149.9		
Number of pollen grains				
Family	9	2304	0.70	0.702*
Plants (family)	19	3121	2.36	0.001
Pollination	1	89549	57.86	0.000*
Family × pollination	9	1558	1.18	0.308
Error	239	1320		

NB * Not an exact F-test (Minitab 13)

Relative performance and inbreeding depression

This chapter focused on assessing biparental inbreeding; the relative performance of uniparentally inbred progeny was included as an extreme measure of inbreeding. Selfed progeny were the poorest performers for all traits, with the exception of ovule fertilisation (Table 3.8). The relative performances of selfed progeny varied from 0.27 for seed set to 1.10 for ovule fertilisation; cumulative fitness was 0.001 (Table 3.8).

Outcrossed progeny were the fittest overall, and biparentally inbred progeny were intermediate in all stages except ovule fertilisation (Table3.8). The relative fitness of biparentally inbred progeny compared with outcrossed progeny varied from 0.63 for seed abortion to 1.05 for ovule fertilisation (Table 3.8). Overall, biparentally inbred progeny were only 18% as fit as outcrossed progeny.

Inbreeding depression

The severity of inbreeding depression varied between traits, but, for all traits, biparental inbreeding depression was less than uniparental inbreeding depression. The greatest reduction in inbreeding depression was at the seed set stage, where biparental inbreeding reduced inbreeding depression six-fold compared with uniparental inbreeding, indicating biparental inbreeding masks the effect of deleterious recessive alleles (Fig. 3.3). For the remaining traits, with the exception of pollen grain size, mating between related individuals resulted in considerable reductions to inbreeding depression (Fig. 3.3). Uniparental inbreeding depression

ranged from $\delta = 0.05$ for pollen grain size to $\delta = 0.73$ for seed set, whereas biparental inbreeding depression values ranged between $\delta = 0.05$ for pollen grain size and $\delta = 0.37$ for seed abortion.

Cumulative uniparental inbreeding depression was $\delta = 0.99$ compared with $\delta = 0.74$ for biparental inbreeding depression. Although biparental inbreeding masks the effects of deleterious recessive alleles for various traits, the cumulative effects of inbreeding, either uniparentally or biparentally, indicate inbred progeny are unlikely to survive to reproductive maturity. On the other hand, cumulative biparental inbreeding depression varied between plants families, ranging from $\delta = 0.52$ and $\delta = 0.91$ (Fig. 3.4). This indicates that, for at least one plant family, biparentally inbred progeny were approximately half as fit as outcrossed progeny and may have had an increased chance of survival in comparison to other biparentally inbred families.

Table 3.8. Summary of performance following self-, biparentally inbred- and outcross-pollinations across a range of life-cycle stages in *Bulbine bulbosa*. Data are means (± SE) pooled over 10 families. Relative self performance (RPs) and relative biparentally inbred performance (RPbp) were calculated as the ratio of self:outcross performance and biparentally inbred:outcross performance, respectively, with the exception of days to 50% germination and days to first flower, which were the inverse of these ratios. Traits considered as components of fitness and where there was a statistical difference between biparental and outcross treatments were used for the calculation of cumulative fitness and are indicated by *.

Life-cycle stage	**Pollination**				
	Self	**Biparentally inbred**	**Cross**	**RPs**	**RPbp**
Seed Development (%)					
Ovule fertilisation	74.32 ± 2.92	71.23 ± 2.17	67.81 ± 3.08	1.10	1.05
Seed abortion	75.84 ± 3.02	50.30 ± 2.79	31.77 ± 2.83	0.42	0.63
Seed set*	8.83 ± 1.23	28.64 ± 2.48	32.98 ± 2.96	0.27	0.87
Seeds					
Mass (mg)	2.55 ± 0.07	3.18 ± 0.04	3.23 ± 0.05	0.79	0.98
Germination (%)	90.10 ± 5.50	95.61 ± 1.46	96.18 ± 1.96	0.94	0.99
Days to 50% germ.	24.5 ± 3.1	23.10 ± 1.6	22.30 ± 1.10	0.94	0.96
Seedling survival (%)					
7 months*	69.50 ± 5.83	89.96 ± 2.45	97.14 ± 1.42	0.71	0.93
Plant growth (mg)					
Biomass 7 mths *	1.95 ± 0.13	2.98 ± 1.12	3.58 ± 1.13	0.55	0.83
Flowering (number)					
Days to 1st flower	65.08 ± 2.41	54.32 ± 1.59	37.92 ± 1.32	0.58	0.70
Flowers*	27.77 ± 2.55	31.00 ± 0.89	41.00 ± 0.86	0.68	0.76
Inflorescences*	0.87 ± 0.11	1.54 ± 0.05	1.84 ± 0.05	0.47	0.84
Pollen and ovule production					
Ovules number*	13.9 ± 0.5	15.12 ± 0.3	16.6 ± 0.2	0.83	0.91
Pollen grain size(μm)	42.93 ± 0.71	43.04 ± 0.42	45.10 ± 0.27	0.95	0.95
Pollen grains (number)*	4984 ± 374	8079 ± 232	10485 ± 202	0.48	0.77
Pollen viability (%)*	54.84 ± 4.01	81.18 ± 1.74	93.08 ± 0.83	0.59	0.87
Cumulative fitness				**0.01**	**0.26**

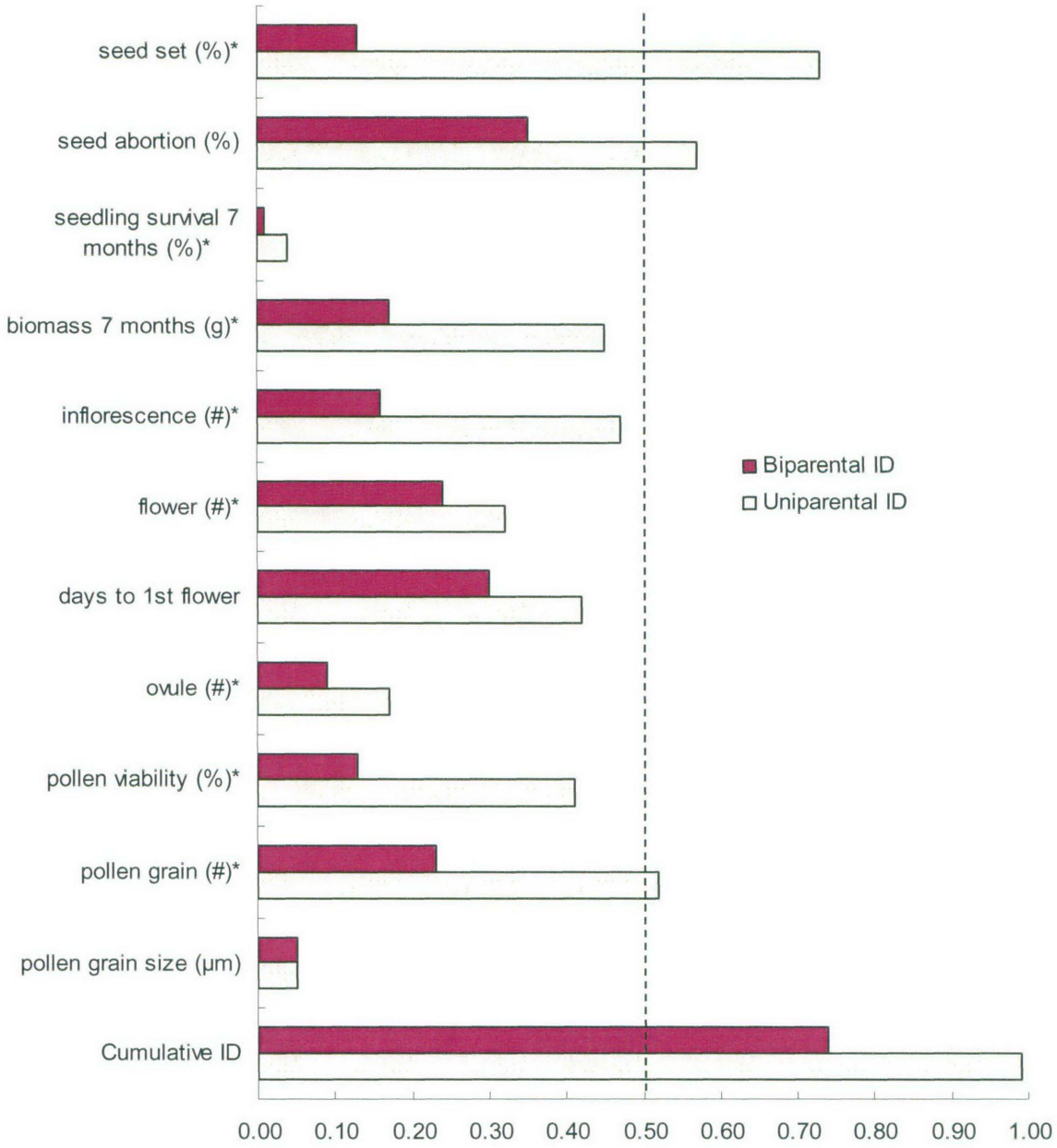

Figure 3.3. Inbreeding depression estimates for a wide range of life-cycle traits. Cumulative δ was estimated from the relative fitness of selfed and biparentally inbred plants for seed set, seedling survival and biomass at 7 months, number of flowers and inflorescences, number of ovules and pollen grains, and pollen viability; traits marked with *. The broken line represents the threshold level of δ below which selfing should increase in frequency. This threshold will shift lower for biparental inbreeding to the extent to which the mating individuals are related.

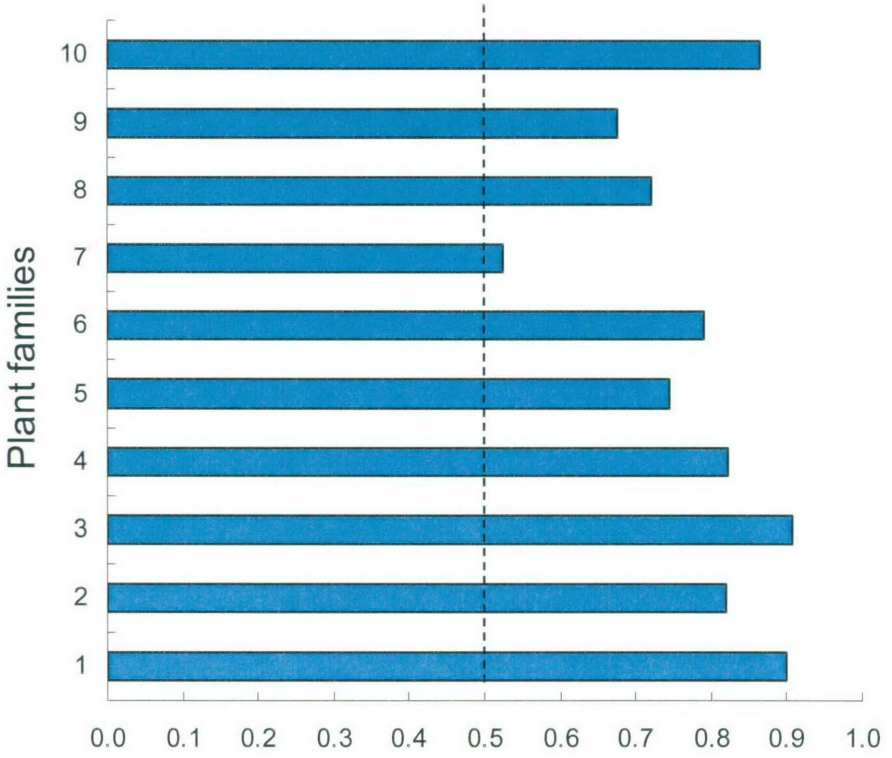

Figure 3.4. Cumulative biparental inbreeding depression for 10 plant families. Cumulative biparental inbreeding depression for each family was estimated from the relative fitness of biparentally inbred progeny for seed set, seedling survival and biomass at 7 months, days to first flower, number of flowers and inflorescences, number of ovules and pollen grains, and pollen viability. The broken line represents the threshold level of δ below which selfing should increase in frequency. This threshold will shift lower for biparental inbreeding to the extent to which the mating individuals are related. Uniparental inbreeding depression was greater than $\delta = 0.89$ for all families.

Discussion

Inbreeding had an important effect on a broad range of traits in the life-cycle of *B. bulbosa*, with uniparental and biparental inbreeding reducing overall fitness, by 99% and 74% respectively, compared with outcrossing. Uniparental inbreeding depression, for a limited range of traits, was previously detected in *B. bulbosa* (Owen *et al.*, 2007). This study provides evidence that mating between related individuals is also detrimental and both uniparental and biparental inbreeding continue to have detrimental effects at a wide range of later life-cycle stages.

The timing and magnitude of the expression of inbreeding depression is often linked to the breeding system (Husband & Schemske, 1996). *Bulbine bulbosa* exhibits very high levels of uniparental inbreeding depression in the early stages of development (eg., seed set $\delta = 0.73$), consistent with an outcrossing breeding system (Barrett & Harder, 1996; Husband & Schemske, 1996). Biparental inbreeding has a lesser effect on the earlier stages of development, as mating between related individuals appears to mask the effects of lethal recessive alleles. At later life-cycle stages mildly deleterious alleles are expressed through uniparental inbreeding and biparental inbreeding.

Estimates of inbreeding depression have been calculated for many species; it is an important component for assessing breeding system evolution. Many studies, however, restrict the estimate by using only traits of female function. For *B. bulbosa*,

strong uniparental inbreeding depression, and to a lesser extent biparental inbreeding depression, were detected in the male function traits of pollen viability and pollen grain number. The harmful effects of inbreeding depression on male function have been detected in other species (Krebs & Hancock, 1990; Willis, 1993; Carr & Dudash, 1995, 1996, 1997; Stephenson *et al.*, 2001; Good-Avila *et al.*, 2003). The development of the male gametophyte via the microsporangium and the microspores is dependant on maternal tissue (e.g., Tapetum, Maheshwari, 1950), so it follows that the quality and/or successful development of pollen depends upon the vigour of the maternal plant. Good-Avila (2003) suggested that differences in size and *in vitro* performance of pollen were due to differences between inbred and outbred maternal plants. It appears that, for *B. bulbosa*, inbred plants also have a reduced capacity to produce the same quality and quantity of pollen as outcross plants.

The effects of biparental inbreeding in *B. bulbosa* were intermediate to that of pure selfing and outcrossing. Other studies have detected similar results after biparental inbreeding (Nason & Ellstrand, 1995; Richards, 2000; Delph, 2004). Delph (2004) found the cumulative fitness of inbred progeny, in *Silene acaulis,* was intermediate to self and outcrossed progeny and seed germination declined with inbreeding in *Silene alba* (Richards, 2000). An investigation of five levels of inbreeding, in *Raphanus sativus*, showed a declining and linear relationship between cumulative fitness and inbreeding (Nason & Ellstrand, 1995). In the present study, the fitness of biparentally inbred progeny was assessed using a combination of two levels of inbreeding; half and full sibs, so the fitness estimate was an average of these two levels of relationship. Further experimentation assessing the effect of mating

between full and half sibs, in *B. bulbosa*, separately may also reveal a negative, linear relationship between relatedness and fitness.

Cumulative biparental inbreeding depression for *B. bulbosa* was $\delta = 0.74$. This is probably an underestimate of the strength of inbreeding depression in the wild, given that inbreeding depression was calculated in the glasshouse, rather than in the field where inbreeding depression is likely to be higher (Schemske, 1983; Dudash, 1990; Ramsey & Vaughton, 1998). This suggests that very few inbred progeny are likely to survive to reproductive maturity under natural conditions. Fine scale genetic structure is likely to exist within populations of *B. bulbosa* as the seeds lack an obvious dispersal mechanism and genetic neighbourhoods may consist of maternal plants and their offspring. *Bulbine bulbosa* is pollinated by small generalist insects so pollen transfer is likely to be limited, facilitating biparental inbreeding within genetic neighbourhoods. It is unlikely, however, that the genetic structure is perpetuated by biparental inbreeding because the likelihood of biparentally inbred progeny reaching reproductive maturity is greatly reduced by inbreeding depression. In *B. bulbosa*, mating between individuals close together results in lower seed set compared to mating between individuals further apart. This indicates related individuals, perhaps maternal plants and their offspring, are found in close proximity (Chapter 4). Overall, recruitment to the next generation of *B. bulbosa* may be severely affected if the level of biparental inbreeding is a major component of the mating system of natural populations. Further study of biparental inbreeding, in *B. bulbosa* under natural conditions, could include emasculating focal and surrounding flowers and comparing

seed set with unmanipulated, open-pollinated flowers to assess the degree to which selfing, geitonogamy and/or biparental inbreeding contributes to natural seed set.

Assessing the impact of biparental inbreeding in a population may be more important than assessing inbreeding depression through pure selfing, because in some species biparental inbreeding may be more likely to occur. Many hermaphroditic species have strategies, such as dichogamy and herkogamy, which reduce selfing. Natural selection, probably through inbreeding avoidance, has facilitated the evolution of such traits in *B. bulbosa*. *Bulbine bulbosa* is herkogamous, reducing within-flower pollen transfer, and a reduced floral display per plant assists in reducing geitonogamy. While these traits may serve to reduce selfing by promoting outcrossing they are simultaneously promoting crossing between related individuals.

Uniparental inbreeding via pure selfing may be prevented by a physiological incompatibility mechanism, although this mechanism may not completely prevent mating between related individuals (e.g., *Gaillardia pulchella*, Heywood, 1993; *Raphanus sativus*, Nason & Ellstrand, 1995). Histological investigation of early seed development indicates that *B. bulbosa* does not have a physiological barrier to selfing, rather inbreeding depression lowers self-fertility (Chapter 2). In the present study, biparental inbreeding only had a minimal effect on seed set compared with pure selfing (RP_{bp} =0.87 vs RP_s = 0.27), a result that is also *inconsistent* with a physiological self-incompatibility mechanism (Vander Kloet & Lyrene, 1987).

Siblings would share *S*-alleles causing partial or full cross-incompatibility (Krebs & Hancock, 1991). Furthermore, a self-incompatible species should show *S*-allele segregation within families, forming separate cross-compatible and cross-incompatible groups (Seavey & Bawa, 1986). For *B. bulbosa*, there is no variation between families for seed set or seed abortion, rather variation among plants within families – a result inconsistent with segregation of *S*-alleles and consistent with the expression of lethal recessive alleles.

Biparental inbreeding depression alters the potential consequences for breeding system evolution. Similar to uniparental inbreeding depression, it can counteract the advantage of alleles promoting selfing and reduce the genetic cost of outcrossing, although any reduction in the cost of outcrossing will be proportional to the degree of relatedness between mates (Waller, 1993; Herlihy & Eckert, 2004). Alternatively, biparental inbreeding can promote the evolution of selfing, via the gene transmission advantage, while simultaneously passing on characteristics promoting outcrossing (Fisher, 1941; Charlesworth & Charlesworth, 1979; Uyenoyama, 1986). Through these two opposing forces, biparental inbreeding may favour a mixed mating system (Uyenoyama, 1986; Yahara, 1992). Any reduction in the cost of outcrossing or the advantage of selfing, however, may be outweighed by strong inbreeding depression (Uyenoyama, 1986; Griffin & Eckert, 2003). The population estimate of biparental inbreeding depression in *B. bulbosa* is so severe ($\delta = 0.74$), and much higher than the maximum threshold that counteracts the selfing advantage, that it is unlikely to positively influence the evolution of selfing or maintain a mixed mating system.

On the other hand, an increase in the frequency of selfing or the establishment of a mixed mating system may be facilitated by a variation for self-fertility in *B. bulbosa.* Evolutionary predictions are often based on a population estimate of inbreeding depression. These may not be appropriate for populations with variation in self-fertility among plants (Holsinger, 1988, 1991). *Bulbine bulbosa* exhibits a variation for self-fertility (ranging between 0.16 and 0.51 at seed set; K. Owen, unpublished data). The present study found variation between 10 plant families for relative fitness of biparental inbred progeny, ranging from $\delta = 0.09$ to $\delta = 0.48$ (Fig. 3.4). Family groups exhibiting lower levels of inbreeding depression may be indicative of a history of inbreeding and purging of deleterious recessive alleles (Holsinger, 1988; Uyenoyama & Waller, 1991). With an increased sampling of plant families in *B. bulbosa*, one or more genotypic lines may exhibit relative fitness levels of biparentally inbred progeny that exceed the genetic cost of outcrossing, providing the opportunity for an increase in the frequency of selfing or the stabilisation of a mixed mating system in some family lines.

Chapter 4

The effect of distance between mates on seed production in *Bulbine bulbosa* (Asphodelaceae).

Introduction

Genetic structure within plant populations is the non-random distribution of genotypes determined by the interaction of mutation, migration, natural selection, and genetic drift (Loveless & Hamrick, 1984). Breeding system traits, such as limited pollen and seed dispersal causing restricted gene flow and non-random mating, also have an important impact on genetic structure. Additionally, clonal reproduction might contribute (Trame *et al.*, 1995; Nuortila *et al.*, 2002; Souto *et al.*, 2002). Two individuals selected randomly from a population, which has genetic structure, have a greater probability of genetic similarity compared to those chosen from an unstructured population (Silvertown & Lovett Doust, 1993). A decreased genetic similarity between individuals is associated with an increasing distance in structured populations (Trame *et al.*, 1995; Glaettli *et al.*, 2006). Many plant populations are characterised by having a genetic structure (e.g., *Delphinium nelsonii* Waser, 1987; *Eucalyptus globulus ssp. globulus* Hardner *et al.*, 1998; *Silene acaulis* Gehring & Delph, 1999; *Alstroemeria aurea* Souto *et al.*, 2002).

If genetic structure exists, matings between near and far neighbouring plants can reduce fecundity and/or progeny fitness (Price & Waser, 1979). For near neighbour matings, pollen tube attrition can reduce fertilisation. Pollen tube attrition can be mediated by a either a pollen-pollen or pollen-style interaction (Cruzan, 1990). For example, the style can act to retard the growth of pollen tubes from related individuals and promote the chances of cross-pollen tube growth and fertilisation (Waser & Price, 1991b; Souto *et al.*, 2002; Glaettli *et al.*, 2006). Post-zygotically, inbreeding depression can inhibit successful seed maturation or reduce progeny fitness at later life-cycle stages (Levin, 1984; Uyenoyama, 1986; Charlesworth & Charlesworth, 1987; Waser & Price, 1989, 1991b, 1993; Trame *et al.*, 1995; Fischer & Matthies, 1997; Byers, 1998; Stacy, 2001; Nuortila *et al.*, 2002; Souto *et al.*, 2002; Glaettli *et al.*, 2006). For far neighbour matings, outbreeding depression can reduce fitness. Outbreeding depression is caused by either the disturbance of co-adapted gene sequences or the loss of genes that are adapted to the local environment (Fenster & Dudash, 1994; Dudash & Fenster, 2000; Fenster & Galloway, 2000). Outbreeding depression has been detected both within and between populations (Price & Waser, 1979; Sobrevila, 1988; Waser & Price, 1989; Waser, 1993b; Waser & Price, 1993; Fischer & Matthies, 1997; Waser *et al.*, 2000).

Studies examining both inbreeding and outbreeding depression test an extensive range of potential mating distances to determine an optimal pollination distance (McCall *et al.*, 1991; Waser & Price, 1991b, 1994). The spatial threshold, below and above which inbreeding and outbreeding depression is expressed, is of importance for the conservation of threatened plant species (Quilichini *et al.*, 2001).

For outcrossing plants with reduced population sizes, inbreeding depression may increase the risk of extinction (Barrett & Kohn, 1991; Lande, 1994; Kirkpatrick & Jarne, 2000). For small fragmented, selfing or mixed mating populations, which are also threatened, mixing genes from other differentiated populations may cause outbreeding depression (Quilichini *et al.*, 2001). Optimal pollination distances have been found for some species (e.g. Price & Waser, 1979; Trame *et al.*, 1995; Fischer & Matthies, 1997; Fenster & Galloway, 2000; Paschke *et al.*, 2002).

Bulbine bulbosa is partially self-fertile with self seed set less than cross seed set (Owen *et al.*, 2007). Ovule fertilisation after self- and cross-pollination is similar, with inbreeding depression the most likely cause of reduced self seed set (Chapter 2). In addition *B. bulbosa* progeny are strongly affected by inbreeding depression after mating between related individuals (i.e., biparental inbreeding depression, Chapter 3). Historically, the natural distribution of *B. bulbosa* was probably represented as a relatively continuous carpet in open woodlands and grasslands. It is likely that this distribution has been severely reduced and fragmented by European activities, as most populations are now found in nature reserves and on road sides (K. Owen, personal observation). The reduction of breeding individuals into smaller patches could have resulted in a loss of within population genetic diversity and an increase in genetic differentiation between populations (Schoen & Brown, 1991), suggesting that *B. bulbosa* might be vulnerable to inbreeding and/or outbreeding depression.

Aim

In this chapter I examine the effect of mating on seed production between individuals at varying distances. Specifically I ask the following questions:

1. Does mating between individuals in close proximity lower seed set due to inbreeding depression?

2. Does mating between populations (5km apart) also adversely affect seed set due to outbreeding depression?

3. Is there an optimal mating distance for *B. bulbosa*?

Methods

Experimental plants

This study was conducted in the glasshouse in spring 2002 using plants harvested from "Yallaroo" the previous year. Yallaroo is a privately owned, ungrazed property situated 25km west of Armidale NSW Australia (30°30' 00"S, 151°40' 00"E). *Bulbine bulbosa* plants were found in loose to dense patches in open eucalypt woodland. To test the effect of pollination distance on seed set, plants were harvested from pre-determined positions, so that glasshouse pollinations could imitate natural pollinations of varying distances (10cm, 1m, 10m and 5km). At the field site, four patches approximately 10m apart, consisting of 30 or more plants each, were randomly chosen and marked A, B, C, and D. Within each patch, four clumps approximately 1m apart, with more than 10 plants each, were chosen and marked 1, 2, 3, and 4. Within each clump, four plants, approximately 10cm apart, were

harvested. Each plant ($N = 64$) received a unique label to reflect a position within the clump and patch, thereby identifying the distance of each plant from all other plants. For example, the four plants harvested from patch A, clump 1 were marked A1a, A1b, A1c and A1d respectively. For the 5km treatment, plants from the Tea Tree Gully population 5 km away were used (i.e., glasshouse collection; Chapter 2). Plants were potted in potting mix of equal parts loam, sand and peat and kept in glasshouse conditions. Pollinating insects were excluded and pots were randomly moved fortnightly to reduce any position effects caused by glasshouse conditions. Pots were fertilised at fortnightly intervals with half strength soluble fertilizer (Aquasol ^{TM} 0.8g/L) and watered regularly.

Experimental design – hand pollinations

I assessed the effect of mating between individuals at varying proximities by conducting hand pollinations on 16 focal plants. One plant was randomly chosen from each clump. Three focal plants died during the experiment. I compared five pollination treatments corresponding to mating at four distances (10cm, 1m, 10m and 5km) and a self treatment. For each distance treatment, 3 pollen donors were used corresponding to the appropriate distances apart. For the 10cm treatment (within clump) the three other plants in the clump were used as donors. For the 1m treatment (between clumps within a patch) one donor was randomly chosen from each of the other clumps and for the 10m treatment (between patches) one donor was randomly selected from each of the other patches. Donors for the 5km treatment were randomly selected from the 'glasshouse collection'. For self-pollinations, pollen from the focal plant was used. Hand pollinations were undertaken as described in Chapter

2. Three flowers were pollinated for each treatment on each plant. Treatments were administered, in a random order, within the first 20 flowers of an inflorescence. Pollinations were undertaken between 10.00 and 11.00 am eastern Australia daylight savings time (EADST).

Ovule fertilisation, seed abortion and seed set

Mature fruits were harvested when capsules began to split open. I scored the number of unfertilised ovules, aborted seeds and seeds in all matured fruits, as described in Chapter 3.

Statistical analysis

All data were checked for normality and homogeneity required for ANOVA. Percent seed set was arcsine transformed. Percent seed abortion and fertilisation were not transformed. Untransformed means ± SE are presented. The effect of pollination treatment on seed set was determined using a random block design. Pollination treatment was considered a fixed factor and plant as a random factor. Percent seed abortion and ovule fertilisation were tested using one-way ANOVAs (Model 1). For percent seed set, zero values were included where no fruits matured. For seed abortion and ovule fertilisation, fruits that did not develop to maturity were not scored and if more than one fruit matured for each treatment on each plant the mean per plant was used.

Results

Seed set

Pollination treatment and plant strongly affected seed set (Fig. 4.1, Table 4.1). Self-pollination resulted in less seed than any of the distance treatments (Fig. 4.1). Overall, pollination from donors at further distances (10m and 5km) resulted in higher seed set than pollination from closer donors (0.1m and 1m; Tukey tests, Fig. 4.1). However, there was no difference within the two closer treatments or the two further treatments (Tukey tests, Fig. 4.1). In addition, there was a significant interaction effect, indicating that maternal plants reacted differently to pollen originating from different distances
(Table 4.1).

Seed abortion

Pollination treatment strongly affected seed abortion (Fig. 4.1, Table 4.1). Although abortion after self-pollination was significantly higher, Pairwise comparisons showed no difference between the distance treatments, indicating no effect of pollination distance on seed abortion (Tukey tests, Fig. 4.1, Table 4.1).

Ovule fertilisation

There was no significant difference between treatments for percent ovule fertilisation (Fig. 4.1, Table 4.1).

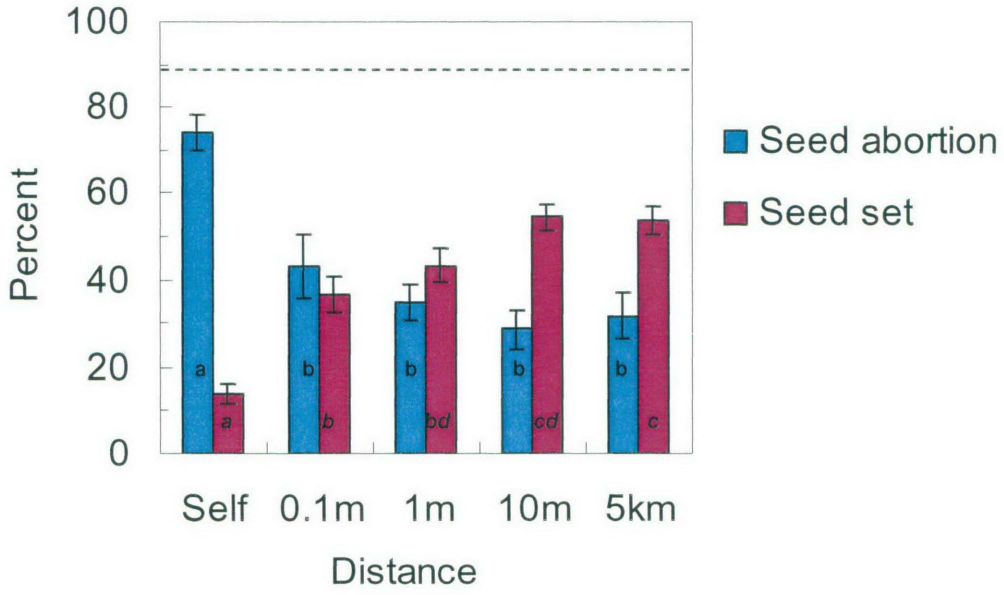

Figure 4.1. Percent (± SE) seed set and seed abortion following self, 0.1m, 1m, 10m, and 5 km distance pollination treatments. Broken line represents mean ovule fertilisation. Means with different letters differed significantly (Tukey tests, $P < 0.05$). Graph corresponds to ANOVA results in Table 4.1.

Table 4.1. ANOVA results for seed set, seed abortion and ovule fertilisation. Random-block ANOVA of the effects of plants and pollination treatment (self, 0.1m, 1m, 10m, and 10km) and their interaction on seed set. Model I one-way ANOVAs of the effects of pollination treatment (self, 0.1m, 1m, 10m, 10km) on seed abortion and ovule fertilisation in *B. Bulbosa*. Data correspond to those presented in Fig. 4.1

Source of variation	d.f.	MS	F	P
Percent seed set				
Plant	12	2102.6	4.73	0.000
Pollination	4	8000.4	18.01	0.000
Plant × pollination	48	444.3	2.08	0.000
Error	195	213.1		
Percent seed abortion				
Pollination	4	4273	11.81	0.000
Error	58	362		
Percent ovule fertilisation				
Pollination	4	111	0.80	0.527
Error	58	138		

Discussion

In this chapter I used plants from a natural population, to determine if distance between mates affects seed set. Overall, seed set was affected by distance between pollen donor and recipient. On average, close matings (0.1m and 1m) reduced seed set compared to the more distant matings (10m and 5km) by a mean of 14%. The absence of a distance effect in the analysis of seed abortion can only be explained by the reduced sample size owing to the exclusion of fruits that did not mature. Seed abortion should be the inverse of seed set. For the close matings 21% of fruit were unable to be scored, and for the more distant matings only 5% were lost. Aborted fruits would have contained many aborted seeds.

Biparental inbreeding depression is the likely cause of reduced seed production after near neighbour mating compared to far neighbour mating. *Bulbine bulbosa* was affected by biparental inbreeding depression, with mating between sibs compared to outcrossing decreasing seed set and increasing seed abortion (Chapter 3, Table 3.8). Biparental inbreeding depression after mating between close neighbours has been found in other species with limited seed dispersal (e.g., *Gentianella germanica* Fischer, 1997; *Amianthium muscaetoxicum* Redmond, 1989; *Impatiens capensis* McCall *et al.*, 1991).

The reduction in seed set after mating between plants in close proximity, provides evidence that the near neighbours are related and that this population of *B. bulbosa* is genetically structured. The detection of inbreeding depression between close neighbours has been used by other researchers as an indication of genetic structure (Price & Waser, 1979; McCall *et al.*, 1991; Fischer & Matthies, 1997; Hardner *et al.*, 1998). The correlation between distance and relatedness is often made with distance used as a surrogate for relationship in experimental studies (e.g. Price & Waser, 1979; Fischer & Matthies, 1997; Stacy, 2001). Collecting genetic and ecological data simultaneously, however, can provide a more accurate assessment of the existence of genetic structure. For *Alstroemeria aurea, Agave schottii*, and *Silene vulgaris*, genetic data confirmed the study populations were structured, after reductions in fitness were detected between matings of individuals in close proximity (Trame *et al.*, 1995; Souto *et al.*, 2002; Glaettli *et al.*, 2006).

The extent of inbreeding depression, facilitated through near neighbour mating during later life-cycle stages, was not investigated in this chapter. However, biparental inbreeding depression, over a wide range of later life-cycle traits, was examined in Chapter 3. Cumulative biparental inbreeding depression was very high $\delta = 0.82$ for *B. bulbosa* (Table 3.8, Chapter 3). In addition, plants with an outcrossing breeding system are likely to express inbreeding depression throughout their entire life-cycle (Barrett & Harder, 1996; Husband & Schemske, 1996). Consequently, it is highly likely that the reduction in progeny fitness of near neighbour mating compared to far neighbour mating for *B. bulbosa* would continue to occur at later life-cycle stages. Inbreeding depression, in later life-cycle traits, after mating

between close neighbours, has been detected in other self-incompatible species across a range of plant families (Byers, 1998; Stacy, 2001; Paschke *et al.*, 2002).

For the study population of *B. bulbosa*, it is likely that reduced seed dispersal is the most important factor in maintaining genetic structure. *Bulbine bulbosa* has no obvious seed dispersal mechanisms. Inflorescence stalks are often between 0.5m and 1.0m high and seeds are small and light (K. Owen, personal observation). Hardner *et al.* (1998) used a formula of 'twice the height of the tree' to estimate the likely dispersal distance of Eucalypt seeds (Potts & Wiltshire, 1997). This supported their detection of related individuals within 50m, but not 250m, of 25m high trees. Following Hardner's method of estimation of seed dispersal distance, genetic neighbourhoods of *B. bulbosa* may exist within 1m of parental plants as offspring of mating between individuals at this distance were less fit compared to individuals 10m apart. For *B. bulbosa*, severe inbreeding depression after mating between sibs is likely to result in very few or no offspring surviving and contributing to the genetic structure (Chapter 3). It is highly likely, therefore, that the genetic neighbourhoods would consist of maternal plants and their immediate offspring but not their inbred grandoffspring.

Outbreeding depression was not detected in this study at the seed production stage. The maximum pollination distance was 5km, whereas some studies have conducted between population pollinations of up to 2000km (Fenster & Galloway, 2000). Others have experimented with pollination distances of around 30km (Byers,

1998; Stacy, 2001; Paschke *et al.*, 2002). Also, outbreeding depression may not have been detected because traits beyond seed set were not examined. Waser and Price (1994) stress that the same logic applies for studies of outbreeding depression as for inbreeding depression and it is important to examine later life cycle stages. Biparental inbreeding depression was relative low at seed set for *B. bulbosa* but stronger at later life-cycle stages (Chapter 3, Fig.3.3). For *Cochlearia bavarica* and *Anchusa crispa*, outbreeding depression was not detected at seed set but was apparent in later life-cycle traits (Quilichini *et al.*, 2001; Paschke *et al.*, 2002). Therefore, the existence of an optimal crossing distance, by detecting both inbreeding and outbreeding depression, cannot be determined without further experimentation examining crosses greater than 5km apart and later life-cycle stages including reproduction and subsequent progeny fitness.

The data collected in this chapter suggest some possible consequences for *B. bulbosa* populations, both immediate and longer term. Biparental inbreeding depression, through mating between parents in close proximity, will strongly affect fecundity, although this could purge deleterious alleles from the population and reduce inbreeding depression (Lande & Schemske, 1985). Reduced fecundity, however, will severely affect recruitment to the population and increase the risk of local population extinction within an already fragmented distribution (Lande, 1994). As reduced seed dispersal maintains the genetic structure that facilitates biparental inbreeding depression, selection could favour the evolution of a seed dispersal mechanism. But, if pollination from further distances occurs, new genotypes would

be recruited to the neighbourhood and selection should additionally favour increased pollen dispersal (Watson & Dallwitz, 1992).

Chapter 5

Poor quality pollen interference and pollen limitation in *Bulbine bulbosa* (Asphodelaceae).

Introduction

Most hermaphroditic plant species produce fewer fruit than flowers and fewer seeds than ovules (Andersson, 1993; Burd, 1998; Anderson & Hill, 2002). Many ecological and genetic factors may interact to thwart the ability of a plant to maximise seed set. Pollen availability is one way that seed set can be limited (i.e., pollen limitation). Natural populations of plants, across a broad taxonomic range of species and families, are thought to be frequently pollen limited (Burd, 1994; Larson & Barrett, 2000; Ashman *et al.*, 2004; Knight *et al.*, 2005).

Natural seed set can be pollen limited through inadequate pollen quantity or quality. To detect pollen limitation, many researchers compare the reproductive success of open-pollination to pollen supplementation. If supplementation increases seed set, open plants are pollen limited (Larson & Barrett, 2000; Knight *et al.*, 2005). If natural seed set is limited due to insufficient pollen deposition, it can be because of stochastic pollinator behaviour or the general failure of either abiotic or biotic vectors to transport pollen (Burd, 1995; Ashman *et al.*, 2004). Small populations may experience Allee effects, when reduced numbers of plants have a reduced floral display that fails to attract pollinators to ensure sufficient pollination (Groom, 1998;

Knight, 2003). Pollen limitation may, therefore, be a particular hazard to rare species and plants affected by anthropogenic influences, such as habitat fragmentation (Jennersten, 1988; Kearns *et al.*, 1998; Colling *et al.*, 2004).

Pollen limitation studies are well documented in the literature, however, most focus on reduced fecundity through limited pollen quantity and many fail to distinguish between pollen quantity and quality (Manasse & Pinney, 1991; Burd, 1995; Colling *et al.*, 2004 but see Ramsey, 1995b). Less well studied, is pollen limitation caused by the quality of pollen deposited (Byers, 1995; Ramsey, 1995b; Ramsey & Vaughton, 2000; Anderson & Hill, 2002; Duncan *et al.*, 2004). If the standard pollen supplementation experiment is conducted and seed set does not increase with added pollen, seed set may still be pollen limited by poor pollen quality (i.e. mate limitation: Campbell, 2001). An audit of pollen deposition, assessing ovule fertilisation, and a comparison with cross-pollination will help to clarify pollen limitation by quantity or quality (Ramsey, 1995a; Ramsey & Vaughton, 2000). If pollen deposition is high and fertilisation rates after open- and cross-pollination are similar, open-pollination may be limited by pollen quality.

Natural seed set can be pollen limited simply because the pollen deposited is predominately poor quality. Alternatively, poor quality pollen may interfere with appropriate pollen (i.e., unrelated cross pollen). For plants with a physiological self-incompatibility system, incompatible pollen may clog the stigmatic surface or stylar tract, preventing compatible pollen from germinating or blocking cross pollen tube

growth (Waser & Price, 1991a; Broyles & Wyatt, 1993). However, some opportunity may remain for outcrossing. Histological examinations or pollen chase experiments can be undertaken to investigate the interference of self pollen in self-incompatible plants (e.g., Manasse & Pinney, 1991; Griffin & Barrett, 2002; Bittencourt Jr. & Semir, 2004). For plants that have a late-acting self-infertility system, either a late-acting self-incompatibility (LASI) or exhibit early-acting inbreeding depression (EAID), poor quality pollen interference may be more problematic. For plants with LASI, self-pollen tubes can arrest in the ovary and prevent cross pollen from entering the micropyle (e.g., Kenrick *et al.*, 1986; Chichiricco, 1993). If ovules are self-fertilised, EAID can cause the arrest of ovule/seed development (e.g., Seavey & Bawa, 1986; Krebs & Hancock, 1990; Manasse & Pinney, 1991; Husband & Schemske, 1996). Late-acting systems can completely pre-empt ovules. If self-fertility is controlled by LASI or EAID ovules are wasted and unavailable for fertilisation by cross pollen resulting in pollen discounting (Waser & Price, 1991a; Sage *et al.*, 1999; Herlihy & Eckert, 2002; Cesaro *et al.*, 2004).

Pollen from related individuals may also cause pollen interference in the same ways as self pollen (Waser & Price, 1983; Ramsey & Vaughton, 2000). Crosses between related individuals, can be prevented by a physiological self-incompatibility system, if the individuals have the same genotype at the *S*-locus (Nason & Ellstrand, 1995). Additionally, crosses between related individuals can increase homozygosity and so increase the chance that deleterious recessive alleles will be expressed during seed development (i.e., Biparental inbreeding: Chapter 3; Uyenoyama, 1986; Charlesworth & Charlesworth, 1987; Kelly & Willis, 2002).

Bulbine bulbosa is a partially self-fertile perennial, flowering in spring in grasslands and open Eucalypt woodland. The cost of selfing in *Bulbine bulbosa* was investigated previously, and pollen limitation, through high levels of selfing, was determined as a restraint to fecundity under natural conditions (Owen *et al.*, 2007). *Bulbine bulbosa* is an excellent system for further study of pollen limitation. In the current project I have found that biparental inbreeding as well as uniparental inbreeding reduces progeny fitness through inbreeding depression (Chapter 3). In addition, plants mating with individuals in close proximity produce less seed than plants mating with individuals further apart, probably because of biparental inbreeding depression (Chapter 4). Additionally, the plant population size and genetic structure may also influence pollen deposition. *Bulbine bulbosa* is a geophyte with winter dormancy. It is possible that, for some genotypes, the dormancy may last over several years if favorable conditions do not trigger spring re-growth. This would result in a seasonal variation in population genetic structure, influencing both the number of flowering plants and their genetic relationship. It is likely that in *B. bulbosa* populations, the deposition of and interference by self pollen as well as pollen from related plants increases pollen limitation.

Aim

In this Chapter, I assessed pollen limitation, over three flowering seasons, by conducting a field experiment in a natural population of *B. bulbosa.* A study over a number of seasons is valuable because it is likely to capture variation caused by the highly stochastic patterns of insect pollinator behaviour and the seasonal population genetic makeup. A cross-pollinated treatment and open-pollination and pollen

supplementation were included to distinguish between pollen quality and quantity limitation. I assessed self-pollen interference, as a mechanism for increasing pollen limitation through the deposition of poor quality pollen prior to cross pollen deposition. Specifically, I examined the extent of pollen limitation under natural conditions and assessed self pollen interference by asking the following questions:

1. Is seed set limited by the quantity of pollen deposited?
2. Is seed set limited by the quality of pollen deposited?
3. Does self pollen interfere with cross pollen?

Methods

Study site

This study was carried out over four years, from 2001 to 2004, at Yallaroo (See Chapter 3). Flowering densities of *B. bulbosa* are dependant on autumn to early spring rainfall (K. Owen, personal observation) and in each year of this study the flowering density in the experimental population varied. In 2001 and 2004 the plants were distributed in loose to dense patches in moist and sheltered microhabitats. In 2003, after good early spring rainfall, flowering density was very high and the plants formed a continuous mat in the population area. The populations were not counted but in 2001 and 2004 appeared to be several thousand plants and in 2003 the number of plants was probably two- or three-fold more. In contrast, very few *B. bulbosa* populations were evident in the local Armidale area in spring 2002 after a period of below average rainfall. The establishment and growth of *B. bulbosa* has also been

linked to time since fire and appropriate establishment gaps in grasslands (Lunt, 1994; Hitchmough *et al.*, 1996).

Pollen deposition

To further examine pollen limitation by quantity, I assessed pollen deposition relative to ovule production under natural field conditions in 2001, 2003 and 2004. In each year, for 10 randomly chosen days during peak flowering, five flowers were harvested at approximately midday, the end of floral life ($N = 50$). Pollen deposition was assessed only on fine days when pollinators were active. Stigmas were placed onto cubes of basic fuchsin stained glycerin jelly and squashed under a coverslip (Kearns & Inouye, 1993). Ovaries were preserved in small vials of 70% alcohol. I counted the total number of pollen grains deposited on the stigmatic surface at 40× magnification and assessed the number of ovules per flower at 20× magnification. For pollen deposition, I did not discriminate between germinated and ungerminated grains. I compared pollen deposition in each year by a nested ANOVA, with year and day considered as random factors. Raw data satisfied assumptions of normality and homogeneity. I compared pollen deposition to ovule production in each year using a paired t test.

Field Experiment

I conducted a field experiment, comparing self-, cross-, open- and supplemented-pollination treatments, to determine whether natural-seed set was pollen limited either by the number of pollen grains deposited (pollen quantity) or by the type of

pollen deposited (pollen quality). In each year I randomly assigned focal plants for controlled pollinations (2001, $N = 11$; 2003, $N = 16$; 2004, $N = 14$). Plants were at least 3m apart. A wooden stake was driven into the soil adjacent to the plant and a long rectangular mesh bag, approximately 20 cm wide, was used to cover the inflorescence to exclude pollinators when appropriate (Fig. 5.1). Pollen was applied as described in Chapter 2. I randomly administered three to four replicates of four pollination treatments within the first 20 flowers of the inflorescence of each plant. Pollinations were conducted daily over a period of about 1 month till all treatments were completed on each plant. Pollination treatments were as follows: *Self* – Plants were covered with mesh bags the day before pollination. Flowers were emasculated and the anthers used to pollinate the same flower; *Cross* – Plants were covered with mesh bags the day before pollination. Flowers were emasculated and donor pollen obtained from two to four flowers from at least 10m away; *Open* – Flowers were left open to natural pollination; *Pollen supplementation* – Flowers were left open to natural pollination for several hours. Cross pollen from two to four donors, from at least 10m away was applied to the stigma between 11am and midday. Although flowers begin to close between 12 midday and 1pm stigmas are still receptive and pollen viable at this time (Owen *et al.*, 2007). Fruits were harvested 25 days after pollination. I scored the number of unfertilised ovules, aborted seeds and seeds, as described in Chapter 3.

Figure 5.1. Field site 2003. *Bulbine bulbosa* plants bagged and open. Wooden stakes approximately 1m in height.

The effect of pollination treatment and year on ovule fertilisation, seed abortion and seed set, was first determined with a two-way model III ANOVA to test the interaction between treatment and years. For all traits the interaction was non-significant (all $P > 0.2$) and was removed from the final analysis. Treatment (self-, cross-, open- pollination and pollen supplementation) was a fixed factor and year (2001, 2003 and 2004) was a random factor. For seed set, zero values were included where no fruits matured. For ovule fertilisation and seed abortion, only fruits that developed to maturity were scored. Plant was used as a replicate, so fruits were considered as a subsample and the mean seed abortion and ovule fertilisation per plant was calculated. All data were checked to satisfied assumptions of normality and homogeneity required for analysis of variance (ANOVA). Seed set and seed abortion data were arcsine transformed and ovule fertilisation data were not.

Natural selfing rates

The field pollination treatments also provided a means to estimate the zygote selfing rate in a natural population of *B. bulbosa*. I used the non-genetic method developed by Charlesworth (1988). *Bulbine bulbosa* is polyploid (Watson, 1986) and has proven difficult to electrophorese because banding patterns are not able to be scored (M. Ramsey, personal communication, 2000). Moreover, selection against selfing in *B. bulbosa* occurs predominately at the seed production stage and it is more appropriate to use a method that estimates the selfing rate at pollination and/or fertilisation (Charlesworth, 1988; Ritland, 1990). I estimated the selfing rate of naturally pollinated plants as $S = (p_c - p_o)/(p_c - p_s)$, where p_c is the mean percentage seed set after cross-pollination, p_s is the mean seed set after self-pollination and p_o is

the mean percentage of seed set after open-pollination (Charlesworth, 1988). All three treatments were conducted on each plant. The variance (V) of the S estimate was obtained by the 'delta method' (Charlesworth, 1988).

The mean value for seed set was obtained by using all fruits. Zero values were used where fruits did not mature, therefore taking into account fruit set. Charlesworth's method assumes that the seed set of open-pollinated plants is not limited by the quantity of pollen deposited on stigmas. This assumption holds for the present study (see Pollen deposition results this chapter). In addition, a more accurate estimate of S is obtained if open-pollinated seeds are obtained by either selfing or out-crossing as opposed to biparental inbreeding (Charlesworth, 1988). If open-pollination includes mating between related individuals and inbreeding depression reduces open seed set, values of S would be overestimated (Charlesworth, 1988). For *B. bulbosa*, biparental inbreeding and biparental inbreeding depression are likely to occur (Chapters 3 and 4). Although Charlesworth (1988) suggests that the inaccuracy of the estimate due to biparental inbreeding is unlikely to be great, for *B. bulbosa* it may be indicative of the degree of natural biparental inbreeding and therefore the estimate should be considered as an upper-bound limit for pure selfing.

Inbreeding depression

The pollination treatments, over a period of three field seasons (2001, 2003, 2004), were used to estimate the relative performance of selfed progeny and inbreeding depression at seed set under natural conditions. Relative performance (RP) was

calculated as: $RP = w_s/w_c$, where w_s and w_c are the performances of selfed and crossed progeny, respectively. I estimated inbreeding depression as: $\delta = 1 - (RP)$.

Pollen interference

To determine if self-pollen tubes interfere with cross-pollen tubes and pre-empt ovules, I conducted a glasshouse pollen-chase experiment. I divided pollen-chase treatments into three components. Broadly, these were;

(a) *Pollen application (spatial)* - two application methods that roughly reflect spatially how successive pollen deposits, by insects, might occur under natural conditions;

(1) - Self, then cross on successive sides of the stigma (Fig. 5.2).

(2) - An 'all over' application of self-pollen followed by an 'all over' application of cross pollen.

(b) *Pollen interference (temporal)* – three pollen-chase treatments replicating how self and/or cross pollen might be deposited over time;

(3) - Cross pollen, then self pollen.

(4) - Cross, then cross pollen.

(5) - Self, then self pollen.

(c) *Cross-pollen type (genetic)* – a comparison of different cross donors. The cross pollen used was taken from four different plants numbered 43, 44, 53, and 66;

(6) - Self, then cross pollen 43.

(7) - Self, then cross pollen 44.

(8) - Self, then cross pollen 53.

(9) - Self, then cross pollen 66.

(4) - Self, then cross pollen.[1]

The experiment was conducted in the glasshouse in 2004 using 10 randomly chosen plants from the glasshouse collection of plants (see Chapter 2). There was no difference in seed set after examining *pollen application type* (see results), so, for treatments examining *self-pollen interference* and *cross-pollen type*, pollen was first deposited onto one side of the stigmatic surface at approximately 7am and the side (left or right) was noted on a small jewellers tag attached to the pedicel. The second application was deposited onto the other side of the stigmatic surface about four hours later. The stigma of *B. bulbosa* is not lobed, but flowers are zygomorphic, so the stigmatic surface was visually divided in half along the longitudinal plane of symmetry (Fig. 5.2). Approximately equal amounts of pollen were deposited, by using the end a fine metal probe, resulting in a pollen to ovule ratio on each side of about 6:1, a sufficient number to fertilise all ovules (K. Owen, unpublished data).

All pollinations were undertaken using a dissecting microscope at 10× magnification; the potted plant was laid on its side and the flower positioned on the

[1] With the exception of the individual cross-chase treatments 6, 7, 8 and 9 all other cross-pollinations were an even mixture of pollen from plants 43, 44, 53, and 66 (see below).

microscope stage. Prior to pollinations, each target flower was emasculated and the pollen stored in individually-labelled plastic vials. For self-pollinations, pollen from the same flower was used. For cross-pollinations, other than single donor treatments, four donors were used from the same population but not from experimental plants. The same number of anthers from each donor was placed into a small plastic vial and mixed for 60 seconds with a vortex mixer. All pollen was applied using a fine metal probe. Three replicates of nine treatments were administered randomly to each plant ($N = 27$). Flower position has no significant effect on seed set for cross-pollinated plants in the glasshouse or open-pollinated plants in the field (K. Owen, unpublished data). Mature fruits were harvested as capsules began to split open. I scored the number of seeds as a percentage of the total number of ovules, as described in Chapter 3.

The results of the self-pollen interference experiment were analysed in three separate two-way Model III ANOVAs. I compared: (1) the effect of the method of application of self then cross pollen; (2) the four treatments exploring the effect on seed set of the delay between first and second pollen deposits and (3) the relative success of the four different cross donors and a mixture of all donors after self-pollination. In all ANOVAs, plant was considered a random factor and pollination treatment was fixed. For pollen deposition type the interaction was non-significant and was omitted from the final analysis ($P = 0.192$). All data were arcsine transformed to satisfy assumptions of normality and homogeneity required for analysis of variance (ANOVA).

Figure 5.2. *Bulbine bulbosa* flowers. Flowers are zygomorphic which allowed the stigmatic surface to be visually divided into left and right sides along the longitudinal plane of symmetry.

Results

Pollen deposition

The number of pollen grains deposited on the stigmatic surface was significantly greater than the number of ovules produced per flower in all years (2001 – 116.5 ± 5.3 vs. 15.66 ± 0.3, $t_{49} = 18.88$, P = 0.000; 2003 – 114 ± 6.1 vs. 15.58 ± 0.3, $t_{49} = 15.93$, P = 0.000; 2004 – 93.9 ± 7.8 vs. 16.00 ± 0.2, $t_{49} = 10.04$, P = 0.000). The number of deposited pollen grains exceeded the number of ovules by greater than 5-fold in all years. Pollen deposition did not vary between years or among days within years ($F_{2, 27} = 2.92$, $P = 0.071$, $F_{27, 120} = 1.6$, $P = 0.135$ respectively). This result indicates that the quantity of pollen deposited did not limit seed set in this *B. bulbosa* population in 2001, 2003 or 2004.

Field experiment

Overall, natural-seed set was pollen limited by the quality of pollen deposited. For seed set, seed abortion and ovule fertilisation, the preliminary analysis indicated that the year × pollination interaction was not significant (all P> 0.20), and this interaction was omitted from the final analysis to test the significance of the main effects (Table 5.1). Years were also pooled for presentation (Fig. 5.3). Pollination treatment had a highly significant effect on seed set and seed abortion, but not on ovule fertilisation (Fig. 5.3, Table 5.1). Seed set was significantly lower after self-pollination than after all other treatments which did not differ (Fig. 5.3, Tukey test P < 0.05). However, seed set after cross-pollination increased by ≈8%, compared to

open- and supplemented-pollination, although it was marginally non-significant ($P = 0.08$, Tukey test). There was significantly greater seed abortion after self-pollination and conversely significantly less after cross-pollination than after any other treatment (Fig. 5.3, Tukey test $P < 0.05$). There was no difference between open- and supplemented-pollination treatments for seed abortion (Fig. 5.3, Tukey test $P < 0.05$).

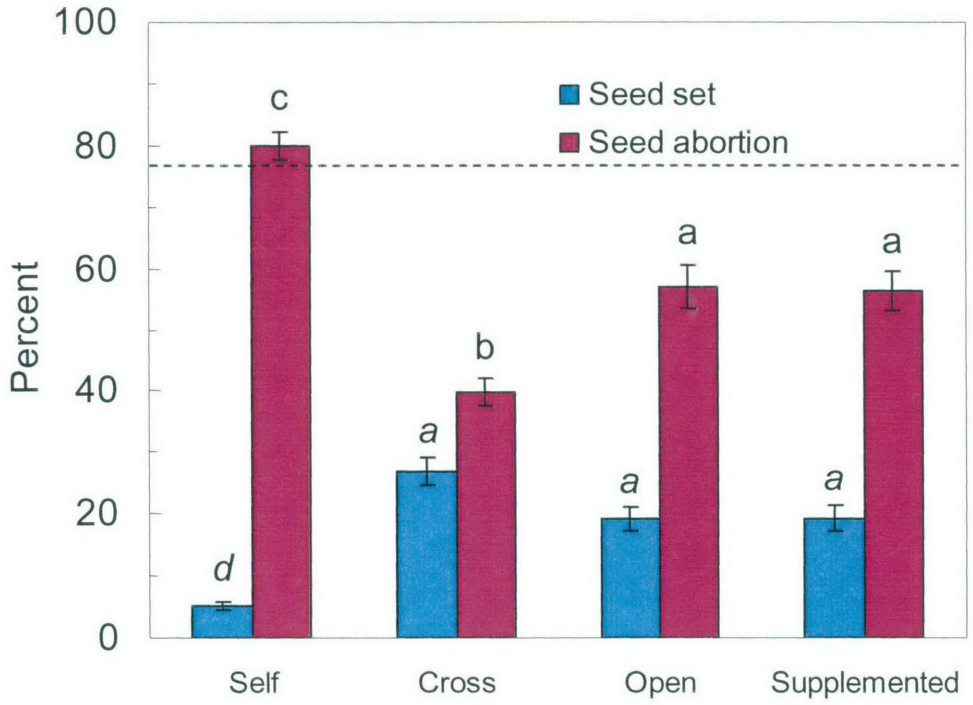

Figure 5.3. Mean percent seed set and seed abortion (± SE) following self-, cross-, open- and supplemented-pollination treatments in 2001, 2003 and 2004. Broken line represents mean fertilisation. Bars with the same letters were not significantly different (Fig. 5.2, Tukey test $P > 0.05$). Graph corresponds to ANOVA results in Table 5.1.

Table 5.1. ANOVA results for seed set, seed abortion and ovule fertilisation. Model III two-way ANOVA of the effects of year (2001, 2003 and 2004), and pollination treatment (self-, cross-, open-pollination and pollen supplemented) on seed set, seed abortion and ovule fertilisation. For all analysis the year × pollination interaction was non-significant (all $P > 0.2$) and were omitted from the final analysis. Data correspond to those presented in Fig. 5.3

Source of variation	d.f.	Adj MS	F	P
Seed set				
Year	2	701.9	7.84	0.001
Pollination	3	2380.8	26.58	0.000
Error	158	89.6		
Seed abortion				
Year	2	501.5	3.47	0.034
Pollination	3	6212.0	42.95	0.000
Error	146	144.6		
Ovule fertilisation				
Year	2	0.01160	0.66	0.520
Pollination	3	0.01434	0.81	0.489
Error	146	0.01765		

Natural selfing rates

By comparing open, self and cross seed set in the field, I estimated natural selfing rates. Selfing was intermediate in all years but slightly higher in 2003 (2001, $S = 0.29 \pm 0.18$; 2003, $S = 0.51 \pm 0.10$; 2004, $S = 0.29 \pm 0.23$). The higher estimate for 2003 probably reflects the higher flowering density compared to 2001 and 2004. This may have facilitated a higher level of biparental inbreeding, with more related individuals in close proximity, inflating the value of S (Charlesworth, 1988).

Inbreeding depression

In all three years of the field experiment, progeny from self-pollinated plants were less fit than from cross-pollinated plants. Inbreeding depression at the seed production stage was: 2001, $\delta = 0.86$; 2003, $\delta = 0.76$; 2004, $\delta = 0.84$, substantially higher than 0.5, the threshold below which selfing will increase in frequency (Fisher, 1941).

Pollen interference

Pollen application – There was no difference between two pollen-chase treatments comparing method of application (8.53 ± 2.30 vs. 6.22 ± 2.21, Table 5.2a). There was no variation among plants for percent seed set (Table 5.2a).

Self-pollen interference – There was no significant difference between treatments where cross pollen was applied first (Fig. 5.4; Tukey test, $P > 0.05$). Similarly, there was no difference between treatments where self-pollen was applied first (Fig. 5.4; Tukey test, $P > 0.05$). However, there was significantly more seed set (≈25%) when

cross pollen, compared to self, was applied first, regardless of the second type of pollen application (Fig. 5.4, Tukey test $P < 0.05$). The plant × pollination interaction was significant, indicating plants reacted differently to the pollen-chase treatments (Table 5.2b). Seed set also varied among plants (Table 5.2b).

Cross-pollen type – There was no significant difference between treatments for seed set comparing different cross-pollen types following self-pollination (Table 5.2c). Mean percent seed set ranged from 7.00 ± 2.27 for self + cross 43 to 18.67 ± 3.00 for self + cross 44. Seed set after self- + cross-pollination with all cross donors was intermediate at 11.24 ± 2.52.

Collectively, these results indicate that self-pollen pre-empts ovules, regardless of the way in which the pollen is applied or the genotype of cross pollen that follows the initial self-pollination. After selfing, even if delayed cross-pollination occurs, ovules are likely to be already self-fertilised and subsequent abortion reduces seed set.

Table 5.2. ANOVA results for seed set after pollen interference experiments. (a) Pollen application type – Model III two-way ANOVA of the effects of plants, pollination (all over self + all over cross and self one side + cross other side). The pollination × plant interaction was $P = 0.192$ and was omitted from the final analysis. (b) Self-pollen interference – Model III two-way ANOVA of the effects of plant, pollination treatment (self + cross, self + self, cross + self, cross + cross) and the pollination × plant interaction on seed set. (c) Cross-pollen type – Model III two-way ANOVA of the effects of plant, pollination (self + all cross donors, self + cross 53, self + cross 44, self + cross 66 and self + cross 43) and the pollination × plant interaction on seed set. Data from (b) correspond to those presented in Fig. 5.4

Source of variation	d.f.	Adj MS	F	P
(a) Pollen deposition type				
Plant	9	382.1	2.67	0.013
Pollination	1	149.9	1.05	0.311
Error	49	142.9		
(b) Self-pollen interference				
Plant	9	731.1	2.38	0.039
Pollination	3	7476.1	24.36	0.000
Plant × pollination	27	306.8	1.69	0.038
Error	80	181.5		
(c) Cross-pollen type				
Plant	9	1235.8	4.83	0.000
Pollination	4	575.0	2.25	0.083
Plant × pollination	36	255.7	2.46	0.000
Error	100	104.0		

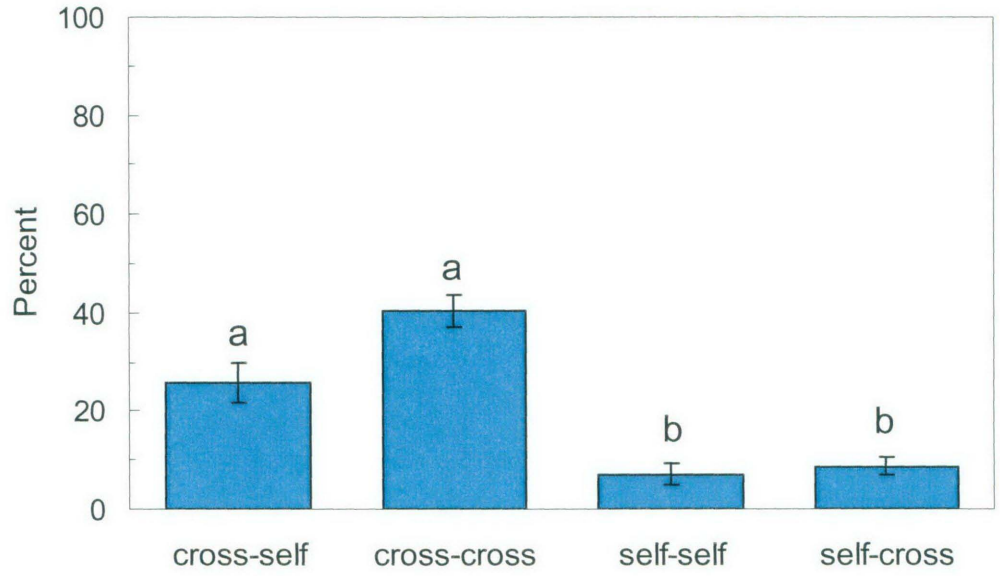

Figure 5.4. Mean percent seed set (± SE) following cross- + self-, cross- + cross-, self- + self-, and self- + cross-pollination treatments. Bars with the same letters were not significantly different (Tukey Tests). Graph corresponds to Table 5.2b.

Discussion

Natural seed set in the study population of *B. bulbosa* was pollen limited in three flowering seasons. On average, seed set after open-pollination was 11% less than after cross-pollination. Pollen limitation is common among flowering plants and to vary within and among flowering seasons (Copland & Whelan, 1989; Burd, 1994; Dudash & Fenster, 1997; Larson & Barrett, 2000; Ramsey & Vaughton, 2000; Totland, 2001; Ashman *et al.*, 2004). In this study, the effect of pollination treatment on seed set was independent of year, indicating pollen limitation may be a constant feature of this population of *B. bulbosa*.

To distinguish between pollen quantity and quality limiting seed set, fertilisation rates should be compared between open- and cross-pollination treatments. Additional data on natural pollen deposition can also assist with interpretations. For *B. bulbosa*, a similar number of ovules were fertilised in all pollination treatments and the mean number of pollen grains naturally deposited was at least six times the amount required for full fertilisation. Open seed set was not limited by the amount of pollen deposited. Severe uniparental inbreeding depression was detected in all years of this study. For the open treatment, more seeds aborted due to inbreeding depression, indicating that pollen quality was responsible for lowering seed set. Pollen quality limiting seed set has been found in *B. bulbosa* previously (Owen *et al.*, 2007).

Facilitated autogamy was found to be a major cause of pollen limitation in *B. bulbosa* (Owen *et al.*, 2007). Owen *et al.* (2007) found seed set after experimental self- and open-pollination to be similar (16% vs. 20%). However, in the present study, seed set after self-pollination was significantly less than open seed set (5% vs. 19%). The disparity in self seed set levels between this and the previous study probably arises because in this study experimental self-pollinations were undertaken in the field, and in Owen *et al.* (2007) selfing was undertaken in the glasshouse. Glasshouse conditions probably masked inbreeding depression, inflating self seed set.

In the present study, open seed set was intermediate to self and cross, indicating that poor quality pollen deposition consisted of either a mixture of self and cross pollen (Owen *et al.*, 2007), pollen from related near neighbours or a mixture of related and self pollen. Biparental inbreeding increases the survival chances of inbred progeny relative to uniparental inbreeding. Biparental inbreeding reduces seed set, but not as severely as uniparental inbreeding (Chapter 3), providing a possible explanation for intermediate open seed set relative to self- and cross-pollination. Separating biparental seed set from a mixture of self and cross seed set is impossible without genetic analysis. However, open-pollination for *B. bulbosa,* serviced by small generalist insects, is likely to be restricted to within a few metres. Pollination from within a radius of ≈1m reduced seed set by ≈ 14% compared to pollination from further distances, and was attributed to biparental inbreeding depression (Chapter 4), so it is possible open seed set was uniparentally as well as biparentally inbred. Self

and related pollen amounts to 'inappropriate' pollen, which can affect seed abortion and consequently seed set.

Calculated selfing rates for each year were also high (2001, $S = 0.29$; 2003, $S = 0.51$; 2004, $S = 0.29$) comparative to very low self seed set, suggesting that estimates probably include biparental inbreeding (Charlesworth, 1988). Jacquemart and Thompson (1996) outline four contributing factors to the selfing rate: (1) autonomous selfing, (2) facilitated selfing, (3) geitonogamy, and (4) biparental inbreeding. With the exception of autonomous selfing, all of these modes of selfing are probably important in *B. bulbosa* (Owen *et al.*, 2007 Chapter 3, Chapter 4). Further studies, including genetic analysis of open-pollinated progeny, are required to tease out the relative importance of each component of selfing.

For many pollen limitation investigations, comparisons are made between open and pollen-supplemented treatments (Larson & Barrett, 2000). In these studies, the presence of pollen limitation (quantity) is indicated by increased seed set after supplementing open-pollination with cross pollen. This approach, however, will miss pollen limitation by quality as pollen supplementation will not increase seed set if ovules have already been fertilised (Waser & Price, 1991a; Ramsey, 1995a). For *B. bulbosa*, pollen supplementation did not increase seed set, compared to open pollination, and both were significantly less than seed set after cross pollination despite similar fertilisation in all three treatments. For open-pollination and pollen supplementation ovules were already fertilised by poor quality pollen and seed set

was reduced due ovule abortion due inbreeding depression. This indicates that interference by poor quality pollen either deposited before or simultaneously, can exacerbate pollen limitation.

The pollen-chase experiment indicated that, if self pollen is deposited first, seed set is low, and conversely seed set is high if cross pollen is deposited first; the genotype of the second pollen donor makes little difference. This indicates both self and cross-pollen tubes probably grow at similar rates (Ramsey, 1995a; Mahy & Jacquemart, 1999; Gibbs *et al.*, 2004). Pollination after a 50:50 mixture of self:cross pollen resulted in intermediate seed set, compared to pure self- and pure cross-pollination, also suggesting that self and cross-pollen tubes grow at similar rates (Owen *et al.*, 2007). Cross pollen is unable to out-compete self pollen, and subsequent abortion of already selfed ovules, due to inbreeding depression, lowers seed set. Open-pollination in *B. bulbosa* probably consists of inappropriate pollen that pre-empts ovules, making them unavailable for later arriving cross-pollen tubes. Similar results were found for *Blandfordia grandiflora* and *Burchardia umbellata,* where pollen supplementation to open plants did not increase seed set; however, a comparison with cross-pollination indicated that seed set was pollen limited by quality (Ramsey, 1995b; Ramsey & Vaughton, 2000).

Self-incompatible species are more commonly pollen limited than self-compatible species (Burd, 1994; Larson & Barrett, 2000). Furthermore, the type of self-incompatibility mechanism may influence the level of pollen limitation. Pollen

limitation studies indicated that *Eupatorium resinosum*, *Linanthus parviflorus* and *Scorzonera humilis* are pollen limited by quality, yet they all responded positively with higher seed set after pollen supplementation (Byers, 1995; Goodwillie, 2001; Colling *et al.*, 2004). These species all have a sporophytic self-incompatibility (SSI) system, which prevents incompatible pollen from germinating on the stigma. Plants with an earlier acting self-incompatibility mechanism should have an increased chance of later deposited cross pollen successful fertilising ovules, unless self pollen clogs stigmas and or styles. *Blandfordia grandiflora*, *Burchardia umbellata and Bulbine bulbosa* have late-acting systems, where ovules are wasted by self-fertilisation and subsequent abortion (Waser & Price, 1991a; Ramsey, 1995b; Ramsey & Vaughton, 2000; Chapter 2).

Natural pollination by insects is likely to consist of a sequence of visits that may or may not affect the entire surface of the stigma (Ashman *et al.*, 2004). A species with a SSI inhibiting self-fertilisation on a portion of the stigma, might have a later chance cross-pollination (Duncan *et al.*, 2004). For species with early-acting self-incompatibility mechanisms, the vagaries of natural pollination might work in their favour. The pollen-chase experiment for *B. bulbosa* indicated there was no advantage to a delivery of self then cross pollen on alternate sides of the stigma compared to an all over delivery of self then cross pollen.

Natural pollination is likely to consist of a number of pollen donors, and pollen competition may result in higher numbers of better quality seeds compared to

pollination resulting from one donor (Winsor *et al.*, 2000; Ashman *et al.*, 2004). For *B. bulbosa*, the relative competitive value of four different donors and all four together did not differ. In all treatments, cross-pollination after prior selfing did not differentially increase seed set.

Chapter 6

General Discussion and Conclusions

The overall aim of this thesis was to investigate the causes of self-sterility and consequences of inbreeding on seed set and progeny fitness in a perennial geophyte *B. bulbosa*. The facilitation and costs of selfing in *B. bulbosa* have been investigated by Owen *et al.*, (2007). In this thesis I built upon the previous study broadening our understanding of inbreeding in *B. bulbosa* and further contributing to the knowledge of mating systems within the Asphodelaceae.

In this final chapter, I provide a summary and discussion of my research findings based around three conclusions:

(1) The mechanism of self-infertility in *B. bulbosa* is inbreeding depression;

(2) Self pollen and pollen from related individuals interferes with cross pollen causing ovule wastage and ovule discounting;

(3) Inbreeding depression effects a wide range of life-cycle stages and cumulative inbreeding depression is severe.

Here I draw evidence from each data chapter to support, elaborate and discuss these conclusions.

Early-acting inbreeding depression controls self-fertility

Two possible mechanisms can limit self-fertility in hermaphroditic flowering plants: physiological self-incompatibility and inbreeding depression. Determining which of

these mechanisms is responsible for reduced seed set is important to the understanding of plant mating systems because inbreeding depression may be a selective force that can led to the evolution of self-incompatibility (Charlesworth & Charlesworth, 1987). To determine the self-infertility mechanism operating in *B. bulbosa*, I made direct observations of ovules after self- and cross-pollination at 2-, 5- and 7-days PP. Rarely, are observations made at very early post pollination stages, rather the distinction between early-acting inbreeding depression and late-acting self-incompatibility is made on theoretical grounds only (Seavey & Bawa, 1986).

For the observation of 2-days PP ovules, I used a relatively quick and easy method. I cleared whole ovules in a clove oil based medium (Herr, 1971). This allowed the observation of large sample sizes of selfed and crossed ovules at a very early stage of seed development. There was no distinction between fertilisation frequencies in self- and cross-pollinated ovaries at this stage of development (both around 70%) indicating the absence of a genetically controlled sporophytic or gametophytic self-incompatibility mechanism. A similar developmental stage, with a zygote and endosperm initiation, was observed at 2-days PP in both self- and cross-fertilised ovules (Fig. 2.2, Chapter 2). My results indicated the arrest in development of selfed ovules occurred post-zygotically.

The post-zygotic nature of self-infertility in *B. bulbosa* has profound implications for the mating system of *B. bulbosa.* Selfed ovules that arrest in

development, even immediately after syngamy, are wasted and unavailable for crossing (ovule discounting). Post-zygotic self-sterility mechanisms are in contrast to mechanisms that operate pre-zygotically, where despite self-pollination, opportunities may still exist for ovules to be fertilised by later arriving cross-pollen tubes. Aborted ovules from a post-zygotic self-sterility system are wasted and the process has been described as maladaptive (Barrett, 2002a). However, this could explain why many plants have evolved to produce more ovules than seeds because the maternal plant aborts ovules fertilised with genetically inferior pollen exerting a selective force (Charnov, 1979; Charlesworth, 1989; Marshall & Folsom, 1991).

I observed the development of self- and cross-pollinated ovules at 5- and 7-days PP. A large sample size was observed by preparing permanent slides after sectioning and staining whole initiated fruits. My results indicated that the mechanism controlling self-fertility in *B. bulbosa* is consistent with early-acting inbreeding depression rather than late-acting self-incompatibility. Firstly, I did not observe a uniform arrest of selfed ovules at any time before 7-days PP, which would be indicative of a single gene controlled self-incompatibility system (Seavey & Bawa, 1986; Lipow & Wyatt, 2000). Secondly, in developing selfed fruits, ovules appeared to abort in at least three different stages (Chapter 2). Developing selfed ovules were reduced, on average, from 12 to 9 between fertilisation and 5-days PP, from 9 to 4 between 5- and 7-days PP and would probably be reduced to ≈ 2 seeds per fruit at maturity (Chapter 2; Owen *et al.*, 2007). Furthermore, I observed the apparent arrest of development at different stages within the 5- and 7-days PP samples (Chapter 2). This indicates the expression of several to many deleterious

recessive alleles, causing selfed ovules to abort and reduce the fitness of selfed progeny compared to crossed progeny. Inbreeding depression can be expressed at any time during a plant life-cycle, including strong effects in very early seed development, where a large number of essential genes are first expressed (Seavey & Carter, 1994; Husband & Schemske, 1996).

Pollen interference causes ovule wastage and ovule discounting

Natural seed set can be limited by the deposition of an insufficient number of pollen grains or by the deposition of poor quality pollen (i.e., self pollen or pollen from related individuals). Pollen deposition is dependant on a number of factors, such as the level of pollinator activity and foraging behavior. Low pollinator activity could decrease the amount of pollen deposition. Foraging within small areas could also decrease the deposition of good quality pollen. Such limited foraging behavior might increase rates of facilitated selfing, geitonogamy or the transfer of pollen between related plants in close proximity. Additionally, the plant population size may also influence pollen deposition (i.e., Allee affect: Groom, 1998; Knight, 2003). Both pollinator and plant population characteristics can vary within and between seasons so I examined pollen deposition and natural seed set over a number of seasons.

I audited pollen deposition and compared the number of ovules to the number of deposited pollen grains. I compared open-pollinated seed set to cross-pollinated seed set. On average, pollen deposition did not vary from year to year and exceeded ovule numbers 5-fold, indicating the quantity of pollen deposition did not limit seed

set. However, open seed set was less than cross seed set in all years of this study despite similar fertilisation frequencies, indicating that natural seed set was not limited by pollen quantity but by pollen quality (Chapter 5; Fig. 5.3).

Open seed set was intermediate to cross and self seed set in all years of this study (Chapter 5; Fig. 5.3). Similarly, biparental inbreeding increased seed set compared to uniparental inbreeding but was still less than outcrossing (Chapter 3). Inferior seed set was obtained from mating between individuals at close distances (i.e., due to biparental inbreeding depression) compared to further distances (Chapter 4). Collectively, these results indicated that open-pollination probably consisted of the deposition of pollen from related individual situated nearby. Inferior quality pollen pre-empted ovules that subsequently aborted due to inbreeding depression resulting in ovule wastage.

Ovules that are wasted due to inbreeding depression are unavailable for outcrossing resulting in ovule discounting (Bertin & Sullivan, 1988; Sage *et al.*, 1999). The number of ovules discounted after self- and open-pollination can be estimated by multiplying the percentage of abortion attributable to inbreeding depression (i.e., self- and open-abortion minus cross-abortion $\approx 40\%$, $\approx 15\%$, respectively) by the average number of ovules ($N = 16$; Chapter 5). This resulted in self- and open-pollination reducing the number of ovules available for outcrossing by six and two respectively (i.e., ovule discounting).

Supplementing open-pollinated flowers with cross pollen, towards the end of the floral life, did not increase seed set. Open- and supplemented-pollination both resulted in about 20% seed set. Percent ovule fertilisation and abortion were similar after open-pollination and pollen supplementation (Chapter 5; Fig 5.3). Supplementing open-pollination with good quality pollen did not increase ovule fertilisation because ovules were probably already fertilised by poor quality pollen. Open pollination for *B. bulbosa* could simply consist of only poor quality pollen but if good quality pollen was available, it appears that prior or simultaneous deposition of poor quality pollen could interfere with cross pollen by pre-empting ovules. This would result in natural seed set being limited further by the interference of poor quality pollen.

I tested whether self pollen interferes with cross pollen in a glasshouse pollen chase experiment. The results from this experiment support the indication from the field experiments that self pollen interference and subsequent inbreeding depression reduces natural seed set (Chapter 5). Delayed cross- after self-pollination resulted in seed set similar to that of two successive depositions of self pollen (Fig. 5.4). This indicated that if cross pollen is deposited later it did not have the ability to out-compete self pollen. Overall, the first pollen deposited fertilises ovules and determines seed set.

Self pollen interference is suggested to be one of the most important selective forces behind the evolution of floral diversity (Lloyd & Webb, 1986). For *B.*

bulbosa, costs incurred by within flower selfing and biparental inbreeding between near by individuals, appear to be high. However, biparental inbreeding may be an unavoidable consequence of floral adaptations that promote outcrossing (Barrett, 2003). As restricted pollen and seed dispersal distances may be largely responsible for the genetic structured populations facilitating biparental inbreeding, selection may favour increased pollen dispersal distances and the evolution of a seed dispersal mechanism. It is proposed that the spatial and temporal separation of stigmas and anthers (herkogamy and dichogamy), have evolved to reduced interference between pollen dispersal and deposition (Lloyd & Webb, 1986; Webb & Lloyd, 1986).There is no temporal separation of male and female functions in *B. bulbosa* but plants are weakly herkogamous (Owen *et al.*, 2007). If variation for herkogamy exists within *B. bulbosa* population, the degree to which herkogamy reduces self pollen interference could be tested by emasculation experiments (e.g., Snow, 1982). Selection for increased herkogamy may benefit reduced facilitated selfing but could be detrimental for outcrossing if small pollinating insects fail to contact stigmas.

Inbreeding depression

A complete understanding of the ecology and evolution of mating systems, in plant populations requires a full evaluation of the severity of inbreeding depression. The current study adds to that of Owen *et al.*, (2007) by establishing that biparental inbreeding depression in addition to uniparental inbreeding depression is important for *B. bulbosa*. Further more, it is important to examine a wide range of life-cycle stages.

For *B. bulbosa*, uniparental and biparental inbreeding resulted in inbreeding depression at a wide range of life-cycle stages reducing overall fitness, by 99% and 74% respectively, compared to outcrossing. The effect of biparental inbreeding was intermediate to pure selfing and outcrossing indicating that after mating between sibs fewer loci containing deleterious alleles are expressed as recessive homozygotes compared to pure selfing. Uniparental and biparental inbreeding depression was detected during seed development, seedling growth, number of flowers and inflorescences, speed of flowering, the number of ovules and pollen grains, and pollen grain viability. Biparental inbreeding adds considerable complexity to predictions of breeding system evolution. Whilst biparental inbreeding reduces the genetic cost of outcrossing, biparental inbreeding depression can negate the genetic transmission advantage of selfing. Simultaneously, biparental inbreeding can also increase the frequency of selfing via the selfing advantage and pass on characteristics promoting outcrossing (Chapter 3; Fisher, 1941; Charlesworth & Charlesworth, 1979; Uyenoyama, 1986; Waller, 1993; Herlihy & Eckert, 2004). These opposing forces, may favour a mixed mating system in populations practicing biparental inbreeding (Uyenoyama, 1986; Yahara, 1992). Any reduction in the cost of outcrossing or the advantage of selfing, however, may be outweighed by strong inbreeding depression (Uyenoyama, 1986; Griffin & Eckert, 2003).

Although it is unlikely that any biparentally inbred progeny survive to reproductive maturity, *B. bulbosa* populations may still maintain a genetic structure through limited pollen and seed dispersal distances. Therefore, I hypothesised that mating between close neighbours could also represent biparental inbreeding.

Additionally, outbreeding depression may result from mating between distances much further apart. I tested this by conducting a glasshouse experiment on plants harvested from set distances apart in the field (Fig. 4.1, Chapter 4). Mating between individuals in close proximity produced less seed set than mating between individuals at further distances apart. These results supported the existence of a genetic structure within populations resulting in biparental inbreeding depression between individuals in close proximity. However, there was no evidence of reproductive isolation between the populations indicated by outbreeding depression. Additionally, an optimal distance was not determined but further experimentation including mating at even further distances apart may uncover outbreeding depression.

Inbreeding depression as defined by $\delta = 1\text{-}RF$ (see Chapter 3) was detected in all experiments comparing inbred and outbred progeny conducted in this project. I have summarised these estimates in Table 6.1, including inbreeding depression calculated at 7-days PP and the estimation from the previous study (Chapter 2; *Owen et al.*, 2007). Inbreeding depression for seed set was severe in all calculations, except for mating within sibships and mating between individuals in close proximity, where the affect of deleterious mutations appears to have been masked. However, the cumulative affect of both uniparental and biparental inbreeding is severe (all $\delta > 0.74$; Fig 6.1). Additionally, if mating between individuals in close proximity represents biparental inbreeding then cumulative inbreeding depression after close proximity mating is also likely to be severe.

Table 6.1. Summary of inbreeding depression. Inbreeding depression estimates are divided into field and glasshouse calculations. Field calculations are for seed set over 3 flowering seasons (Chapter 5). Glasshouse inbreeding depression estimates result from uniparental and biparental inbreeding, and close proximity matings (Chapter 3 & 4). Inbreeding depression at 7-days PP and previous study are also included (Owen *et al.*, 2007; Chapter 2).

			Traits		
			7-days PP	Seed set	Cumulative ID
Field	Uniparental ID	2001	–	0.86	–
		2003	–	0.76	–
		2004	–	0.84	–
Glasshouse			–	0.73	0.99
			0.64	–	–
		2001 (Owen *et al.*, 2007)	–	0.67	0.85
	Biparental ID	Half–full sibs	–	0.13	0.74
		Close proximity	–	0.26	–

Under natural conditions, severe inbreeding depression at the seed development stage represents a significant cost through seed discounting (Lloyd & Schoen, 1992; Morgan *et al.*, 1997; Herlihy & Eckert, 2002). Seeds sired by self or genetically related pollen might survive to fruit maturity but are unlikely to survive to reproduce because cumulative inbreeding depression is so severe (Table 6.1). Developing inbred seeds represent a sink for resources that could otherwise be used for crossed seeds. For perennial plants, resources used in one year for self seed development are effectively subtracted from the stores that might otherwise be used for development of crossed progeny at a later time (Lloyd & Schoen, 1992; Goodwillie, 1999).

Severe inbreeding depression causes other ecological and evolutionary consequences for *B. bulbosa.* The frequency with which progeny are produced through either selfing or outcrossing has a major impact on the viability and genetic structure of plant populations (Hamrick, 1982; Richards, 1997; Holsinger, 2000). Severe inbreeding depression will reduce fecundity by limiting recruitment to the next generation and risking population viability. The benefit of this is that inbred progeny are unlikely to perpetuate a genetic structure in the population (Chapter 4). If no inbred progeny survive, deleterious alleles will be maintained increasing the genetic load in the population (Barrett & Eckert, 1990; Lande *et al.*, 1994). Consequently, enforced inbreeding through reduction in population size may increase inbreeding depression to unity (Kirkpatrick & Jarne, 2000). However, survival of any inbred progeny could purge deleterious alleles from the populations (Lande & Schemske, 1985; Lande, 1994). Finally, for *B. bulbosa,* inbreeding

depression is so severe after pure selfing or mating between related individuals that it will negate the genetic transmission advantage of selfing and oppose any increase in frequency of selfing. For *B. bulbosa* inbreeding depression appears to be a major selective force maintaining a predominately outcrossing mating system.

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