The breeding systems of selected *Thysanotus* species and the influence of floral display size and interspecific pollen transfer on their reproductive success

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I declare that this thesis is my own work and that it has not previously been submitted at any tertiary educational institution. Where collaborative research is included, contributors have been acknowledged.

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Abstract

An understanding of the reproductive biology of a plant species is fundamental to understanding its viability, interactions and function within an ecosystem. This study explored the influence of pollination type, floral display size, and interspecific pollen transfer on the production of fruit and seeds in three *Thysanotus* species (*T. manglesianus*, *T. multiflorus* and *T. triandrus*). *Thysanotus* is a native, buzz-pollinated genus, and currently there is a dearth of knowledge regarding its reproductive biology. The present study aimed to fill this gap in the research by presenting a general overview of these species. The findings may then provide a basis for future research of other native, buzz pollinated species.

This study used *Thysanotus* populations at a nature reserve in Langford, Western Australia to determine breeding systems and the influence of inflorescence size and application of heterospecific pollen on their fruit and seed sets. Breeding systems for each species were determined by hand pollinating flowers with self or outcross pollen, and recording the resulting fruit set. The influence of floral display size (of *T. multiflorus* and *T. triandrus*) was determined by looking at differences in the number of fruit and seeds produced by plants with different sized floral displays. To examine the effect of heterospecific pollen on reproduction, *T. multiflorus* pollen was applied to the stigmas of *T. triandrus* flowers and, over one hour later, either outcross or self-pollen was applied and the resulting fruit and seed set was recorded.

All of the study species have a mixed mating system (i.e. produce seed from self or outcross pollen). Increased floral display size did not significantly increase fruit and seed set, or geitonogamous reproduction in *T. multiflorus*. In *T. triandrus*, a greater proportion of flowers set fruit from small floral displays than large, and large size did appear to increase geitonogamy. Interspecific pollen transfer had no effect on the fruit and seed set of *T. triandrus*, and pollinators did not distinguish between the flowers of the two species, so there was no evidence that pollinators could be instrumental in reproductive isolation.

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Introduction

The production of fruit and seeds is a highly influential process in the successful maintenance of a plant population. The number of fruit and seeds produced is influenced by a combination of factors, including the plant's floral display, floral phenology, breeding system, pollinators, pollen yield, resource availability, and their interactions (e.g. Wyatt 1982; Sargent *et al.* 2007; Khanduri 2011). An understanding of these characteristics may help to account for negative consequences such as pollen limitation or inbreeding depression, both of which may limit a species' reproductive success. Despite the abundance of *Thysanotus* species *(T. manglesianus, T. multiflorus* and *T. triandrus*), there are no detailed publications about their reproductive biology. This thesis aims to provide a general overview of the selected species, the findings of which may also be applicable to other buzz pollinated native species.

Floral display

Floral display is the number, type, and arrangement of open flowers on an individual plant at a given time (Harder and Barrett 1995). It is integral to plant fitness and reproductive success, as it can affect the number of pollinators a plant attracts and the number of flowers visited, thereby influencing pollen transfer (Wyatt 1982; Snow *et al.* 1996). In turn, this can influence the frequency of pollination and fertilisation, and the fruit and seed set which results (Wyatt 1982; Sargent *et al.* 2007). On a smaller scale, the inflorescence is the shoot system which serves for the formation of flowers (Weberling 1989). This arrangement is important for the identification of plants and their phylogenetic relationships (Judd *et al.* 2007). The number of open flowers which make up an inflorescence (i.e. inflorescence size) is a major feature of floral display, and has been the most widely researched (Harder and Barrett 1995; Snow *et al.* 1996; Harder *et al.* 2004; Valdivia and Niemeyer 2006).

Plants with different display sizes are likely to have differing success rates as pollen donors and recipients (Willson and Price 1977). Large floral displays typically attract more pollinators than small displays and are therefore considered to be advantageous (Willson and Price 1977; Gerber 1985; Schmid-Hempel and Speiser 1988; Brody and Mitchell 1997; Harder *et al.* 2004; Valdivia and Niemeyer 2006). Large floral displays are also thought to cause more pollen to be removed (Schmid-Hempel and Speiser

1988), enhance mating opportunities (Harder *et al.* 2004), and increase both male and female fitness (Stanton *et al.* 1986; Young and Stanton 1990; Kudoh and Whigham 1998; Harder and Johnson 2005). Brody and Mitchell (1997) found that larger inflorescences were also more likely to be visited first in any given foraging bout, and that plants with more open flowers also received a greater number of visits in total.

Although large floral displays are considered to be more advantageous than small displays, they are also more costly in terms of production and maintenance (Galen *et al.* 1999; Galen 2000). Furthermore, in contrast to the idea that large floral displays increase outcrossing, there is also evidence to suggest that they increase the rate of geitonogamous pollination and pollen discounting. Pollen discounting is where self-pollinations reduce the number of pollen grains available for transfer to other plants. Pollen discounting may be increased by large floral displays, as pollinators can be encouraged stay on one plant for longer, transferring more pollen between the flowers of that individual (Gerber 1985; Harder and Barrett 1995; Snow *et al.* 1996; Harder *et al.* 2004). Large floral displays may also be disadvantageous as they can attract herbivorous predators, leading to higher rates of seed predation (Brody and Mitchell 1997). A smaller number of flowers may therefore reduce seed predation (Brody and Mitchell 1997), pollen discounting (Harder and Wilson 1998), and may also decrease the number of ineffectual self-pollinations (Wyatt 1982).

Floral phenology

Floral phenology refers to the study of temporal events in the life history of a plant, including shoot growth, flowering, fruiting, and seed dispersal, all of which take place in due season (Fenner 1998). The study of these events involves observations of their timing and the selective forces which influence them (Fenner 1998). The timing of such events can usually be quantified according to frequency, time (i.e. date of start, end and peak of flowering), duration, magnitude (i.e. mean and variability) and the degree of synchrony within and between species (Newstrom *et al.* 1994). Seasonality is a well-known mechanism of phenological variation in flower production, as many plants flower at a certain time of year (Gentry 1974). The phenological patterns observed are likely to be a result of compromises between selective pressures such as seasonal climatic changes, resource availability, and the presence of pollinators (Fenner 1998). Some interdependence does occur between events, for instance, fruiting cannot occur before flowering, and seed dispersal cannot precede fruiting (Fenner 1998). Different phenologies also exist, for example, a plant species may produce a large number of flowers over several weeks, or it might produce a small number of flowers per day, but over a longer period of time (Gentry 1974). An understanding of the phenology of plants is crucial to the understanding of community function and diversity (Fenner 1998).

Competition and facilitation for pollinators

In communities where plants are co-flowering, species often share pollinators, and have interactions via these pollinators (Gentry 1974; Ashman and Arceo-Gomez 2013; Ye *et al.* 2014). At the pre-pollination stage, interactions can be positive (facilitation), negative (competition) or neutral (Gentry 1974; Ashman and Arceo-Gomez 2013). Negative interactions are also known as reproductive interference and adversely affect the fitness of at least one of the species involved (Hochkirch *et al.* 2007; Nishida *et al.* 2013), these can occur pre or post pollination (Nishida *et al.* 2013). Competition for pollinator visits may occur when plants co-flower (Waser 1978; Hochkirch *et al.* 2012), but the extent of competition between species largely depends on the patterns of pollinator foraging within the community (Brown and Mitchell 2001). Assemblages of plant species often co-exist in ways that minimize reproductive interference (Coyne and Orr 2004), which favours positive interactions.

Positive interactions may occur when one co-flowering plant species facilitates the pollination of another (Johnson *et al.* 2003; Moeller 2004; Ghazoul 2006; Liao *et al.* 2011). Facilitation is the result of a co-flowering plant species attracting pollinators that it does not use, or a result of mechanical isolation (where the pollen of different plant species is transferred to a different part of a pollinators body) (Grant 1994; Pauw 2006). Facilitation enhances pollinator visits to a focal species enough to offset reproductive costs of interspecific pollen transfer (the transfer of pollen from one plant species to the stigma of another, Morales and Traveset 2008). Through facilitation, pollen limitation can decrease because pollinator visitation increases (Moeller 2004).

Pollinators may be attracted (or deterred) by floral traits such as colour, orientation or scent (Briscoe Runquist and Moeller 2014). The link between these floral traits and the traits of pollinators is the basis for pollination syndromes (Judd *et al.* 2007). If the link is strong enough, plants may eventually become adapted to pollinators (Judd *et al.*

2007). This is considered to be one of the main mechanisms which drives evolution in plants (Coyne and Orr 2004; Johnson 2006). Flower colour is an important floral trait which can affect pollinator preference (Bradshaw 2003). Plant species which possess flowers with similar colours may have a positive or negative effect on each other. For example, de Jager *et al.* (2011) suggest that assemblages of different coloured flowers may be selected for when pollinators are unable to distinguish between sympatric co-flowering species, as this reduces reproductive success (due to interspecific pollen transfer).

For some co-flowering species, interspecific pollen transfer is a negative interaction which occurs at the post-pollination stage (Waser 1978; Mitchell *et al.* 2009; Ashman and Arceo-Gomez 2013). Interspecific pollen transfer can reduce seed production through conspecific pollen loss and heterospecific pollen deposition (Morales and Traveset 2008; Mitchell *et al.* 2009). This is undesirable as it reduces the amount of pollen available for deposit onto conspecific stigmas, therefore reducing the opportunity for outcrossing (Waser 1983). Additionally, there may be stigma blockage (Richards 1997; Brown and Mitchell 2001), allelopathic inhibition of conspecific pollen, or ovule usurpation which may reduce seed set, or result in the production of unviable seeds and/or unfit offspring (Brown and Mitchell 2001; Morales and Traveset 2008). This form of pollen transfer is likely when shared pollinators move frequently between co-flowering species during a single foraging bout (Waser 1978).

Breeding system

The terms ‘breeding system’ and ‘mating system’ are used inconsistently in the literature (Neal and Anderson 2005), however for the purpose of this study, both refer to the means of pollination and fertilisation by which a plant species can successfully reproduce. There are multiple methods of self and outcross pollen donation available to a plant (Figure 1.1). Many preferentially outcrossing species exhibit a mixed mating system, where reproduction can occur using both self and outcross pollen (Duncan *et al.* 2004).

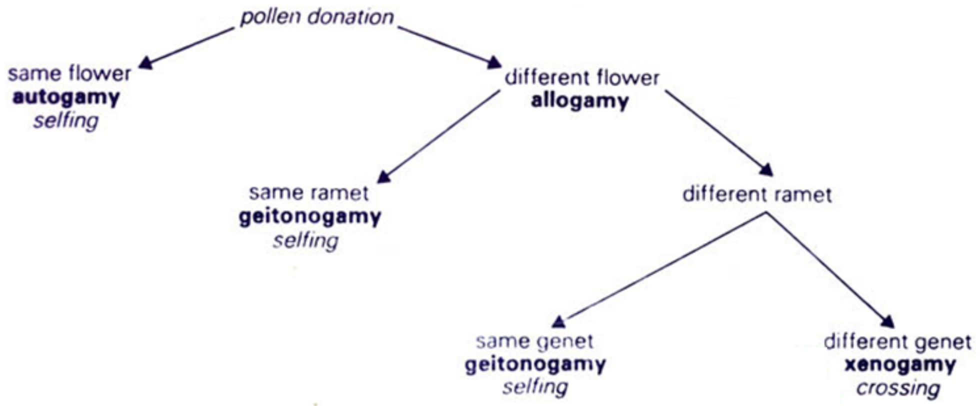

Figure 1.1 Possible types of pollen donation (Richards 1997).

Darwin (1859) proposed that inbreeding reduces fertility, and that it will therefore only evolve when it is necessary to ensure reproduction. Later, Darwin (1876) also suggested that by avoiding self-pollination, plants avoid the harmful consequences of what is now called inbreeding depression (the reduced fitness of individuals resulting from self-fertilisation in comparison to outcrossed offspring). The avoidance of self-fertilisation is made possible by a number of mechanisms, one of which is self-incompatibility, where plants are unable to reproduce successfully using their own pollen (Judd *et al.* 2007). Even when a mixed mating system is available, the successful transfer of pollen is not in itself a successful pollination (Faegri and Van der Pijl 1979; Richards 1997). For example, in *Dianella revoluta* (a buzz pollinated species native to Western Australia), Duncan *et al.* (2004) found that pollinators facilitate self-pollen transfer. However, despite the large quantities of self-pollen which reach the stigma, the species is only partially self-compatible.

Reproductive assurance refers to the beneficial nature of self-fertilisation in cases where reproductive failure (due to mate or pollinator scarcity) is a likely alternative (Richards 1997; Fausto *et al.* 2001). Self-fertilisation may evolve as a result of the interactions between taxa which exist in overlapping geographical ranges, and share pollinators (Briscoe Runquist and Moeller 2014). It can be evolutionarily advantageous, especially when it is delayed and therefore does not directly compete with outcross pollination. Self-fertilisation also guarantees the availability of pollen if the supply of outcross pollen is unreliable (Lloyd 1979). For example, Fishman and Wyatt (1999) found that *Arenaria uniflora* only has selfing populations where it overlaps with *Arenaria glabra*, and their experimental work showed that heterospecific pollen transfer was likely to be the mechanism which drove this transition in mating system. Experimental field studies have supported the reproductive assurance hypothesis by showing that self-compatibility is selected under strong pollen limitation (Kalisz *et al.* 2004; Moeller and Gerber 2005). Even within a species, different mating systems may arise (for spatially separated populations) based on the reliability of plant–pollinator interactions and the reproductive assurance value of selfing (Fausto *et al.* 2001; Moeller 2006; Brys *et al.* 2013).

Despite their advantages, self-pollination and fertilisation are generally thought to be less beneficial than outcross pollination, due to the reduced fitness associated with inbreeding (Darwin 1876; Lloyd 1992). Additionally, self-pollination does not allow for adaptation to change, and can increase the chance of developing non-beneficial mutations (Proctor *et al.* 1996). Although the genetic consequences are the same for different types of self-fertilisation, their effect on successful pollen export and reproductive success may differ (Lloyd 1979; Harder and Thomson 1989; Harder 2000). In general, delayed autogamous self-pollination is thought to be advantageous in comparison to geitonogamous self-pollination. This is because geitonogamous pollination decreases the amount of pollen available for transport to other flowers, thereby reducing outcrossing (Harder and Barrett 1995; Harder *et al.* 2004).

Pollen limitation is where a plant receives less pollen than the amount necessary for full reproductive success. This is demonstrated when supplemental pollination of flowers increases their female fertility in comparison to open-pollinated controls (Larson and Barrett 2000). Pollen limitation is often considered to be a driving force in the evolution of mating systems in plants (Lloyd 1992; Schoen *et al.* 1996; Ashman *et al.* 2004). It occurs when pollinators or plants are scarce, and therefore have a reduced probability of interaction and successful reproduction (Moeller 2004). This can result from both insufficient pollen quantity and quality (Lloyd and Schoen 1992), and is thereby a significant determinant of seed production. A possible outcome of strong pollen limitation is the evolution of traits which promote self-pollination, as this can provide reproductive assurance (Morgan and Wilson 2005; Eckert *et al.* 2006). Pollen limitation has been identified as a problem for seed set in some buzz-pollinated species, for example *Rhexia virginica* (Larson and Barrett 1999a) and *Vaccinium stamineum* (Cane *et al.* 1985). Snow (1982) also found that in self-incompatible *Passiflora vitifolia*, natural pollinations (where hummingbirds usually transferred self-pollen) set less fruit than flowers which were hand pollinated with outcross pollen.

Cross fertilisation generally has a positive selective value as it allows genetic variability in a population (Faegri and Van der Pijl 1979). Cross pollination can be favoured by way of sexual segregation, dichogamy, heterostyly, structural mechanisms, post-zygotic abortions, and sterility to self-pollen (Darwin 1876; Harder and Barrett 1995). The spatial separation of the anthers and stigma (herkogamy) can also encourage cross pollination, because as herkogamy distance increases, the likelihood of autogamous self-pollination decreases (Harder and Barrett 1995). Despite being thought of as beneficial, reproduction by outcrossing still depends on the frequency and quality of the interaction between pollen vectors and individual flowers (Harder *et al.* 2004).

Pollinator behaviour

As plants are stationary, they depend on external forces to bring their gametes together (Judd *et al.* 2007). Darwin (1876) stated that insects were the most important means of pollen transfer within and between flowers. More recently it has been found that nearly 75 percent of all angiosperms rely on animal vectors to move pollen, and that floral display plays a highly significant role in attracting these vectors (Mitchell *et al.* 2009). To maximise pollinator visitation, plants can use attractants (visual or olfactory), rewards (pollen or nectar) or deceptive strategies (e.g. Orchids which resemble the look and smell of a female wasp, which attracts the male wasp by which they are pollinated) (Teixido and Valladares 2013). Not all visitors are pollinators of a given plant species as a pollinator must deposit sufficient pollen on the correct and receptive stigma, and that pollen must be conspecific and viable.

Although flowers are adaptations for pollination, insects do not visit to facilitate plant reproduction, but rather to acquire rewards such as pollen, oils, and nectar (Judd *et al.* 2007). Foraging theory predicts that animal pollinators will visit flowers in the most energy efficient way possible. Therefore, if a more profitable food source exists nearby, pollinators will visit that, and will cease to fly when available sources are not profitable (Richards 1997). As the species in this study are buzz pollinated, only this pollination mechanism will be dealt with here. Pollen collection by buzzing (sonication) is a widespread phenomenon among angiosperms (Buchmann 1983), occurring in many species with poricidal anthers. Bees collect pollen by anchoring firmly to the anthers and vibrating their thoracic flight muscles, which dislodges pollen from the anther and transfers it to the insect's body (Buchmann 1983). The floral characteristics associated with this pollination syndrome are typically blue, purple or yellow coloured flowers which open during the day (Judd *et al.* 2007), often lack nectar (Buchmann 1983), and have apically porose anthers. This is true for the buzz pollinated species of *Thysanotus* that are the focus of the current research (*T. manglesianus*, *T. multiflorus* and *T. triandrus*).

Duncan *et al.* (2004) suggest that the release of pollen by anther vibration may predispose buzz pollinated species to an increased level of facilitated self-pollen transfer. However, it is suggested that the advantages that anther morphology (in buzz pollinated species) present for pollen dispensing and transport outweigh the potential costs that might lower seed output (Duncan *et al.* 2004). In one of few detailed accounts of pollen transfer in buzz pollinated species, Larson and Barrett (1999a) suspected that facilitated selfing might occur in *Rhexia virginica*. However, they concluded that the limited natural seed set was best explained by infrequent pollinator visitation. In contrast, having quantified facilitated selfing for *Dianella revoluta*, Duncan *et al.* (2004) concluded that the most likely explanation for low natural seed set was excessive selfing.

Pollen yield

The production of seeds is dependent on the production of pollen (Khanduri 2011). Estimating pollen production per plant may therefore be useful in determining reproductive success. The number of pollen grains transferred may be influenced by anther size, stigma area and depth, and the pollen-bearing area of the pollinator (Cruden 2000), and this can vary among plants within a population (Devlin 1989). Breeding systems also influence pollen production, for example, outcrossing populations tend to produce more pollen grains per flower than selfing populations (Wyatt 1984). In general, it is thought that large flowers may contain more pollen grains and ovules than small flowers (Small 1988; López *et al.* 1999). A negative relationship between the number of pollen grains and pollen grain size is often documented (Small 1988; Vonhof and Harder 1995; Cruden 1996), and is often attributed to a size and number trade off, or selective pressures such as stigma depth and stigma area (Cruden 2000). Cruden

(2000) also suggested that the difficulty of dispensing pollen from anthers of vibratory flowers (such as *Solanum* and *Cassia*) may select for small pollen size.

Summary

Floral display, pollinators, breeding systems and interactions all influence fruit and seed sets and are therefore important to population maintenance. As there are very few published studies on the reproductive biology of native, buzz pollinated species, this contribution on *Thysanotus* will help to provide a general overview which may be applicable to other genera with the same pollination syndrome. *T. manglesianus* is a species that flowers early in the season and was examined to develop skills in hand pollination and to identify its breeding system. *T. multiflorus* and *T. triandrus* flower later and present a high level of visual similarity as both possess fringed purple flowers with three stamens. They also flower at the same time and, in this case co-exist in a sympatric population (Brittan 1981) where they are both buzz pollinated.

This study used manual pollinations to explore the breeding systems of the three study species. It is predicted that self-pollen will be less successful than the outcross pollen. Secondly, *T. multiflorus* and *T. triandrus* were analysed to determine whether floral display size influences fruit set and geitonogamy. It is predicted that large floral displays will produce more fruit, but also have more fruit with one or two seeds (i.e. increased geitonogamy), as there is more potential for pollinators to be attracted and then remain on the same plant for longer than they would on a plant with a small floral display size**.** As the role of post-pollination interactions in co-flowering communities are less well known than pre-pollination (Ashman and Arceo-Gomez 2013), this study also examined the effect of heterospecific pollen deposition on the later, conspecific pollination of *T. triandrus*. It is predicted that the presence of heterospecific pollen will lower the fruit set which results from conspecific pollination of *T. triandrus* (due to stigma clogging or possible usurpation of ovules by heterospecific pollen tubes). In addition to the central aims of this research, pollinator observations, and observations of floral phenology have also been included to provide more information on these *Thysanotus* species.

Methods

Study species

The *Thysanotus* genus (from the Greek word thysanos, meaning fringe) was established by Robert Brown (Brittan 1981). All but one species of the genus are only found in Australia, and 38 species occur in the South-West of Western Australia (Brittan 1981). The focal species for this project were *T. manglesianus*, *T. multiflorus* and *T. triandrus*. All three are herbaceous perennials, *T. multiflorus* and *T. triandrus* are made up of plantlets with a fibrous rootstock, while *T. manglesianus* twines over other plants (Brittan 1981). *T. multiflorus* and *T. triandrus* can be found on humus rich sands in the South-West of Western Australia, but are only known to occur sympatrically in five localities (Brittan 1981). The high level of visual similarity presented by their flowers raises questions about pollinator attraction and reproductive success when they co-flower at the same location, as is the case for the present study. Visual comparisons and characteristics of the three study species are presented below (Table 2.1).

Table 2.1 Summary of characteristics of the *Thysanotus* species used in this research

	T. manglesianus (Kunth)	*T. multiflorus* R. Br	*T. triandrus* (Labill.) R.Br
Photo of species			
Plant life form	Hemicryptophyte	Hemicryptophyte	Hemicryptophyte
Flower size (Brittan 1981)	Perianth segments >10mm long, outer three: 2.5-3mm wide, inner three: 6mm wide	Perianth segments 7-17mm long: outer three 2-2.5mm wide, inner three 6-8mm wide	Perianth segments 9-15 mm long: outer three 3mm wide, inner three 6-7mm wide
Inflorescence type	Solitary flower in axils of bracts	Large, single terminal umbel, may produce a second umbel below	Large, single terminal umbel
Leaves	Absent	Radical, in plantlets, glabrous	Radical, in plantlets, hirsute
Anthers	3 straight, 3 curved	3 curved, all on one side of the flower	3 curved, all on one side of the flower
Style form	Curved	Curved	Curved
Unfertilised flowers retained	No	Yes	Yes
Ovules	Trilocular ovary (2 ovules per locus)	Trilocular ovary (2 ovules per locus)	Trilocular ovary (2 ovules per locus)

Study site

Research was carried out at a reserve in Langford, Western Australia (Figure 2.1) from August to December 2013. This area is under the management of the City of Gosnells (Bush Forever site 456), and covers approximately 15 hectares. It is situated on the Bassendean Dunes, with Roe Highway to the south and Nicholson Road to the west.

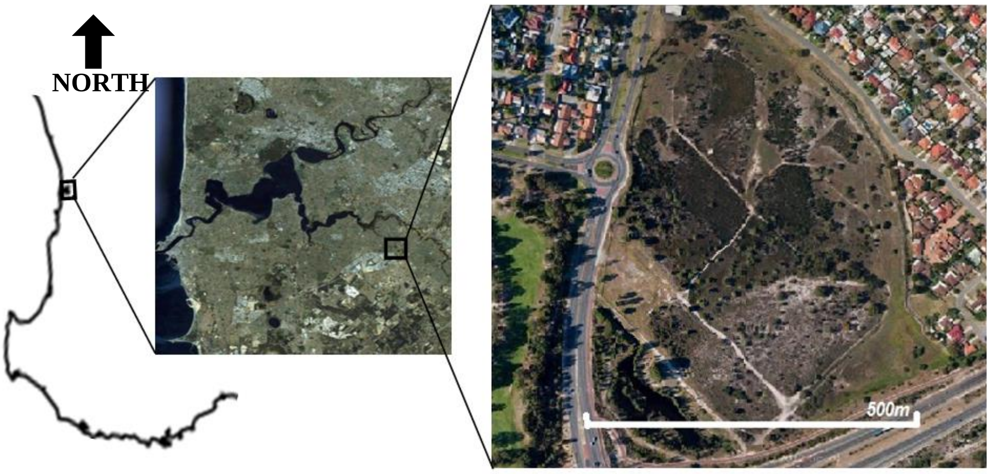

Figure 2.1 The location of the study site in Langford, near Perth, Western Australia (32°03’02.64”S, 115°55’57.19”E). Adapted from Google maps (2013).

Climatic patterns for this site are similar to those of Perth and the inner coastal region of the South-West of Western Australia. According to the Bureau of Meteorology (2014), for the City of Gosnells (1991-2013), the maximum annual mean temperature was 25.5°C, and the minimum was 13.4°C. The average annual rainfall was 822.6mm (Bureau of Meteorology 2014). The site itself contains an ephemeral swamp to the southwest, with wetland vegetation described as herb-rich shrublands, shrublands on dry clay flats, and *Melaleuca preissiana* damplands. The uplands support a *Banksia attenuata* and *Banksia menziesii* woodland (Department of Planning 2000). *Callitris pyramidalis* dominates parts of the area, with sparse *Jacksonia sternbergiana* throughout. Understorey vegetation includes sedges, *Pericalymma elliptica, Daviesia decurren*s, invasive grasses and native herbs such as perennial *Thysanotus manglesianus, T. multiflorus* and *T. triandrus*.

T. manglesianus

Fruit and seed sets were counted on four *T. manglesianus* plants in 2005, and six plants in 2006 (P. Ladd, unpublished data). Information on the breeding system of *T. manglesianus* was obtained by hand pollination experiments during September 2013, toward the east of the study site (Figure 2.2). This area covered approximately 4720m^2.

Figure 2.2 The area within the study site (32°03’02.64”S, 115°55’57.19”E) where the *T. manglesianus* investigation took place (Google maps 2013).

Six *T. manglesianus* plants were randomly selected and covered using 1.5x2mm mesh that had been stapled at the edges to form a sleeve. Sleeves were supported by bamboo stakes and placed over plants to prevent insect pollinations. Five nearby plants were also selected but left uncovered, and all plants were labelled using flagging tape. Different pollination treatments were carried out (Table 2.2). Where these involved manual pollinations, sharpened tweezers were used to cut lengthwise along the anthers. This exposed pollen which was then transferred onto the stigma of the recipient flower.

Table 2.2 The pollination treatments carried out by hand on *Thysanotus* species

Pollination treatment	Description
Covered	
Self	Self-pollen was applied to stigmas to test for self-compatibility
Outcross	Pollen from a plant at least 5m away was applied to stigma, and flower was emasculated. In *T. manglesianus,* pollinations from long and short anthers were recorded separately
Autogamous	Flowers tagged but not manipulated
Uncovered	
Open	Flowers tagged but not manipulated, in order to determine the degree of natural pollination (i.e. control)
Supplementary	Outcross pollen was applied to stigma of open (treatment) flowers

All flowers used were marked by wrapping a wire tag around the pedicle; different coloured tags represented the different pollination treatments. If a tagged flower did not form a fruit, the tag would drop off and it was collected. After allowing time for fruits to develop, the flowers that set fruit were collected, and the number of fruit and seeds were counted. Ideally, equal numbers of each treatment would have been used per plant, however, this did not always occur as the different plants had different numbers of flowers per day. On the five uncovered plants, there were 64 supplementary and 64 open pollinations altogether. On the six covered plants, there were 76, 75, 73 and 71 pollinations (for autogamous, self, outcross long and outcross short pollination respectively).

To determine the natural proportion of flowers that set fruit in the population, sections of 20 *T. manglesianus* plants were selected and tagged at peak flowering. After fruit had matured, the tagged sections were collected and observed under a dissecting microscope to record the number of fruit and flowers. The number of flowers that had not formed fruit was determined by observing the end of each peduncle, and counting the scars left after flower abscission. The flower to fruit ratio was determined by (Σ scars + fruit): number of fruit. Additional fruiting data for this species in 2005 and 2006 was provided by P. Ladd (unpublished). In order to quantify flowering intensity near peak flowering, 20m x 20m quadrats were marked with stakes and flagging tape in the *T. manglesianus* area (Figure 2.2) and the total number of *T. manglesianus* flowers per quadrat per day was counted. This was undertaken on three separate days during the flowering period to give a mean number of flowers per day.

Pollinator observations also took place and involved recording the time, date and weather conditions before watching a plant and recording whether any bees visited. These observations were recorded in 10 minute intervals, but individual plants were often watched for at least half an hour. From the 18th – 22nd of September, a video camera was also set up to record a second *T. manglesianus* plant for 20 minutes. In total, 100 minutes of footage was collected, and was watched later to determine whether any bees had visited.

T. multiflorus

Breeding systems data for *T. multiflorus* were obtained by P. Ladd in 2004-05 using the same open, self and outcross pollination treatments that were outlined for *T. manglesianus* (Table 2.2). Based on this breeding systems data, further studies were carried out from the 8th October - 8th November 2013. At this time, *T. multiflorus* was found across approx. 1800m^2, covering 400m^2 in Area A and 1400m^2 in Area B (Figure 2.3).

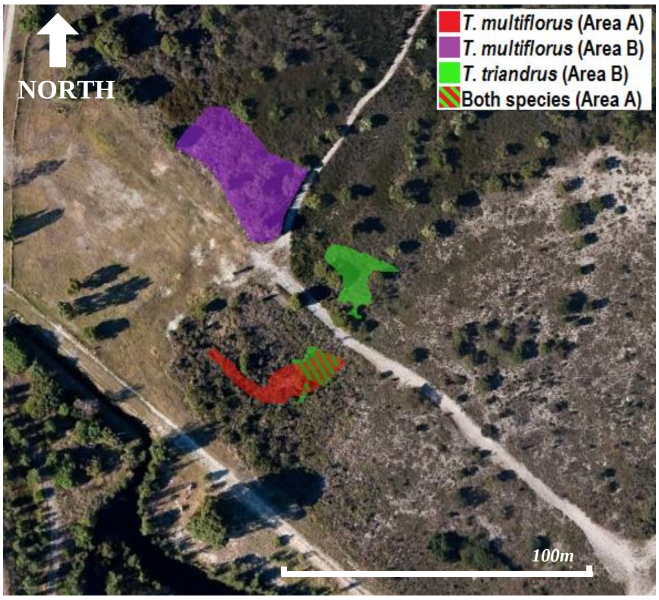

Figure 2.3 The different study areas within the site (32°03'02.64"S, 115°55'57.19"E) showing the location of *T. multiflorus* and *T. triandrus*, and where the two species overlap (Google maps 2013).

To determine whether floral display size influenced fruit set and geitonogamous self-fertilisation, ten 'large' (>5 flowers) and ten 'small' (≤5 flowers) *T. multiflorus* plants were chosen in Area B, and five inflorescences were tagged on each plant. At 08:00am each morning, the number of flowers on these plants was counted and recorded. If the number of flowers on 'small' floral displays exceeded five, excess flowers were removed. To keep flowers (and resulting fruit) on the tagged inflorescences, flowers were always removed from the untagged inflorescences first. Starting at 12:00pm daily, the plants were checked for pollinator visitation (determined by the presence of pollen on the stigma and/or petals) using a 12x hand lens. The numbers of unvisited and visited flowers per plant were counted and recorded. After fruiting, the tagged inflorescences were collected, and the numbers of flowers, fruits and seeds for each were recorded. Pollinator observations for *T. multiflorus* were also carried out, in the same manner as those conducted for *T. manglesianus*.

Overall, flower to fruit conversion for the area where *T. multiflorus* occurred alone (Area B) was determined from the collection of three inflorescences from 10 randomly selected plants that had not been used for the inflorescence study. This was also done in the area where *T. multiflorus* was coincident with *T. triandrus*. The total number of *T.*

multiflorus flowers were recorded for both areas. The first recording was made on the 24th of September (the start of flowering), and the final recording was made on the 16th of December, thus the flowering period was around 83 days. Fruit and seed sets were also collected in these areas in 2011 and 2012 by P. Ladd (on 30 plants per area).

T. triandrus

Breeding systems data for *T. triandrus* were obtained in 2011 by P. Ladd, using self, outcross, open, and supplementary pollinations. Like *T. multiflorus*, fruit and seed sets were also collected on 30 plants in *T. triandrus* in each area in 2011 and 2012 (P. Ladd, unpublished data). In 2013, *T. triandrus* display size and interspecific pollen transfer were investigated in both Area A (200m^2) and Area B (399m^2), Figure 2.3. Conditions outlined by Morales and Traveset (2008) were used to determine whether interspecific pollen transfer was possible between *T. multiflorus* and *T. triandrus*. As some conditions were already met (flowers occurred in the same area, flowering times overlapped, and they shared a pollinator species), pollinators were observed to see whether they moved between both species during single foraging bouts. These observations were recorded in the same manner as for *T. manglesianus* and *T. multiflorus,* however, if multiple *Thysanotus* species were visited, all were recorded.

In 2013, *T. triandrus* observations began on the 5th November, and ended on the 15th of December (when flowering finished). To investigate the influence of floral display size, 30 *T. triandrus* plants were randomly selected, and five inflorescences on each plant were tagged. A ring of chicken wire was placed around the plants in open areas to prevent rabbit herbivory. The number of flowers on each of the 30 plants was counted each day. After fruits had matured sufficiently to count the seeds, the tagged *T. triandrus* inflorescences were collected and the numbers of flowers, fruits and seeds were counted for each. The overall flower to fruit conversion (for the area where both species occurred together) was determined from collection of five inflorescences from 10 plants that had not been selected for the display size investigation. The total number of flowers in Area A and Area B were also counted for *T. triandrus* in the 2013 flowering season, starting on the 5th of November and ending on the 15th of December (40 days).

To determine the influence of the visual similarity of *T. multiflorus* and *T. triandrus,* the colour of the flowers of each species were compared using an ASD FieldSpec 4

Spectroradiometer (standard resolution). Secondly, to test whether heterospecific pollen inhibited the production of fruit, six *T. triandrus* plants with six or more inflorescences were selected (in *T. triandrus* Area B) and covered with mesh bags to prevent pollinator interaction. Six inflorescences were labelled per plant, using pink tape for self-pollination (3), and orange for outcross pollination (3). At 07:30am each day (from the 10th– 23rd November [as this was when flowering ended on the selected plants]), every flower on the labelled inflorescences was hand pollinated with *T. multiflorus* pollen. At least an hour later, the flowers were pollinated with either self or outcross *T. triandrus* pollen, according to their tag. Ideally, the effect of heterospecific pollen deposition on the growth of pollen tubes would have also been observed, however the pollen tubes of these *Thysanotus* species do not fluoresce under UV light when stained with decolourised aniline blue.

Pollen Counting

To determine the pollen production of flowers, mature buds of each species were collected and stored in Formalin-Acetic Acid-Alcohol (FAA) solutions. Individual anthers were placed into plastic test tubes (10mL capacity, with cap) with 5mL of acetic anhydride and 1mL of sulphuric acid (acetolysis mixture). The dimorphic anthers of *T. manglesianus* were recorded separately from one another. To create a spore suspension, one *Lycopodium* spore tablet (containing 18,583 spores) was disaggregated in 10mL of water in a volumetric flask. Once completely suspended, 2mL of spore suspension was added to each test tube, and after acetolysis had finished, tubes were topped up to 10mL with DI water. Samples were centrifuged (at 2,500 revolutions for 5 minutes), decanted, and then topped up to 10mL again, the process was repeated until the solution was clear (usually three times). Once the final decant had taken place (only leaving the pollen, spores, and a very small amount of water), a glass pipette was used to add 8 drops of glycerol into each sample. A glass rod was used to stir a solution and transfer one drop onto a slide, where it was examined under a Leitz diaplan microscope at 400x magnification. The ratio of pollen to spores was counted for each slide. Six slides were counted for each test tube of solution, and 10 flowers for each species were used.

Data analysis

To evaluate study objectives, fruit set and seed production data were analysed using generalised linear mixed models (GLMMs) in R 3.0.2 (R Core Team 2013), using the lme4 package (Bates *et al.* 2012). Mean proportions and 95% confidence intervals were calculated and comparisons between pollination treatments, years, areas, and display sizes were made graphically. Probabilities were assessed against an alpha level of .05 unless otherwise stated.

GLMMs describe a relationship between a response variable and covariates which were observed with the response (Bates 2010). They are a combination of two widely used statistical frameworks in ecology (Bolker *et al.* 2009), incorporating both linear mixed models (analysing both fixed and random effects using a linear predictor, Bates 2010), and generalised linear models, which allow response variables from different distributions (i.e. handle non-normal data by using link functions and an exponential family such as normal, Poisson or binomial distributions).

The advantages of implementing GLMMs for analyses in the present study were twofold. Firstly, the response variables in the present study, fruit set and seed set, followed binomial and Poisson distributions (respectively), so the ability to choose among various distributions and link functions afforded by GLMMs was desirable. Secondly, the present study investigated both fixed and random effects. Fixed effects are those for which all levels or conditions of interest to the study within each variable have been sampled (Field *et al.* 2012), and for the present study those were display size, pollination type, year, and area. Random effects are those for which only a small, random sample of the population of interest has been sampled (e.g. plant number in the present study) (Field *et al.* 2012). By treating plant number as a random effect, GLMMs enabled inferences to be made about plants in the wider *Thysanotus* population beyond those that were sampled (Bates 2010; Field *et al.* 2012).

Analysis proceeded following the suggestions of Bolker *et al.* (2009) for analysing non-normally distributed data that also include random effects. GLMMs were fit by maximum likelihood using a binomial distribution with a logit link for fruit set data (presence/absence). For seed set data (counts between 1 and 6), GLMMs were implemented using a Poisson distribution with a log link. Parameter estimates were determined by adaptive Gauss-Hermite Quadrature (GHQ), as recommended by Bolker et al. (2009) for models with binomial and Poisson distributions that include less than three random effects. GLMMs were also tested for overdispersion using overdisp_fun( ), and no overdispersion was detected in any of the models.

Breeding systems. GLMMs were implemented for the proportion of flowers setting fruit, and the mean number of seeds per fruit. For these GLMMs, pollen treatment was the predictor, and plant number was the random effect. Open pollination was the baseline (control) that each pollination treatment was compared to. At the time of collection, breeding systems data were pooled for *T. multiflorus*. Therefore, a lack of observations attributed to individual plants precluded this species from being analysed statistically or having confidence intervals estimated.

Multiple year comparison. Fruit and seed set were compared in 3 different years for each species (2005, 2006 and 2013 for *T. manglesianus* and 2011-13 for *T. multiflorus* and *T. triandrus*. For the latter two, two different areas (referred to as Area A and Area B) were also compared between years. GLMMs were again carried out using the proportion of flowers setting fruit, and mean number of seeds per fruit as the dependent variable. Area and year were the treatments (or year only, in the case of *T. manglesianus*), and plant number was again the random effect.

Floral display size. GLMMs were applied for fruit and seed set, with display size as the treatment variable, and plant number as the random effect. To compare the frequencies of fruit with each number of seeds (1-6), a chi test for goodness of fit was carried out. For *T. triandrus,* the 30 sample plants were separated into different size categories (≤30, 31-60, ≥61) based on their maximum number of flowers.

Interspecific pollen transfer. To determine whether interspecific pollen transfer reduced reproductive success, the fruit and seed set data were graphed and compared to the results obtained in the breeding systems experiment. The proportion of fruit with each possible seed number (1-6) was also graphed for self and outcross pollination, again comparing interspecific pollen transfer to the breeding systems experiment. Chi tests were used to compare the frequencies for outcross and self-pollinations between the breeding system and interspecific pollen transfer experiments. This occurred for the proportion setting fruit, mean number of seeds per fruit, and the proportion of fruit with each seed number. Analysis of variance procedures were used to test for significant differences between the mean number of pollen grains counted for each species.

Results

T. manglesianus, *T. multiflorus* and *T. triandrus* all have mixed mating systems. Across the three study species, the greatest mean proportion of flowers setting fruit, and greatest mean number of seeds per fruit were observed from outcross or supplementary pollination treatments. In the floral display size investigation, *T. multiflorus* samples with small floral displays had a greater proportion of visited flowers than those with large displays. The mean proportion of flowers setting fruit and the mean number of seeds per fruit were both greater for large *T. multiflorus* displays, however neither effect was statistically significant. The hypothesis that self-pollination would increase for samples with large floral displays was not supported for *T. multiflorus*. The opposite was true for *T. triandrus*, as plants with smaller floral displays had a greater proportion of flowers setting fruit, and large displays showed more geitonogamy. Overall, there were only 18 bees seen in a total of 13 hours and 40 minutes of plant observations. Contrary to expectations, the presence of heterospecific pollen on *T. triandrus* did not inhibit the success of later conspecific pollen, as there was no difference between the breeding system and experimental fruit and seed sets.

T. manglesianus breeding system

T. manglesianus has a mixed mating system, as flowers set fruit from both self and outcross pollinations (Figure 3.1). Across the whole site, the mean proportion of *T. manglesianus* flowers setting fruit (via open pollination on 20 non-manipulated plant samples) was 0.29 (95% CI = ±0.05), which was somewhat lower than (although within the confidence interval for) the proportion of open pollinated flowers setting fruit in the hand pollination study.

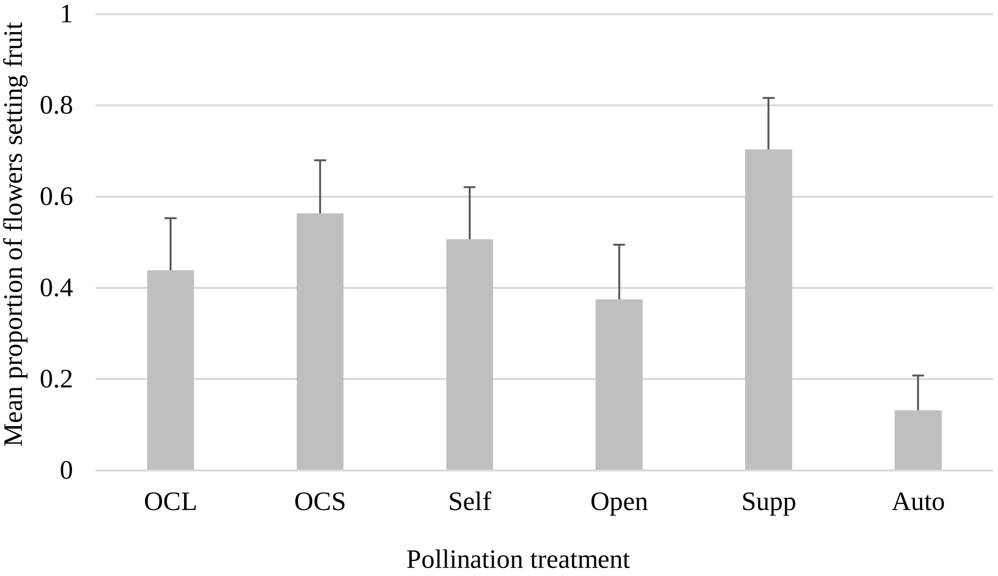

Figure 3.1 Mean proportion (+95% CI) of *T. manglesianus* flowers setting fruit for each pollination treatment. Where OCL and OCS are outcross pollen from long and short anthers, Supp is supplementary pollination and Auto is autogamous pollination.

The supplementary hand pollinations carried out for *T. manglesianus* resulted in a significantly greater proportion of flowers setting fruit than open pollinated flowers. The proportion of flowers to set fruit for autogamous pollination was significantly less than open pollination (Table 3.1).

Table 3.1 GLMM for the proportion of *T. manglesianus* flowers which set fruit for each pollination treatment (compared to open pollination)

Pollination treatment	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.52	0.43	-1.20	0.23
Autogamous	-1.81	0.63	-2.87	0.00**
Outcross (long)	0.08	0.57	0.13	0.89
Outcross (short)	0.66	0.57	1.15	0.25
Self	0.45	0.57	0.79	0.43
Supplementary	1.46	0.39	3.78	0.00***

Note. Open and supplementary: *n*=64, auto: *n*=76, outcross long: *n*=73, outcross short: *n*=71, and self: *n*=75.
** result is significant at p<.01.

*** result is significant at p<.001.

For *T. manglesianus*, the mean number of seeds produced per fruit was uniformly high and greater than open pollination in each pollination treatment except autogamy (Figure 3.2).

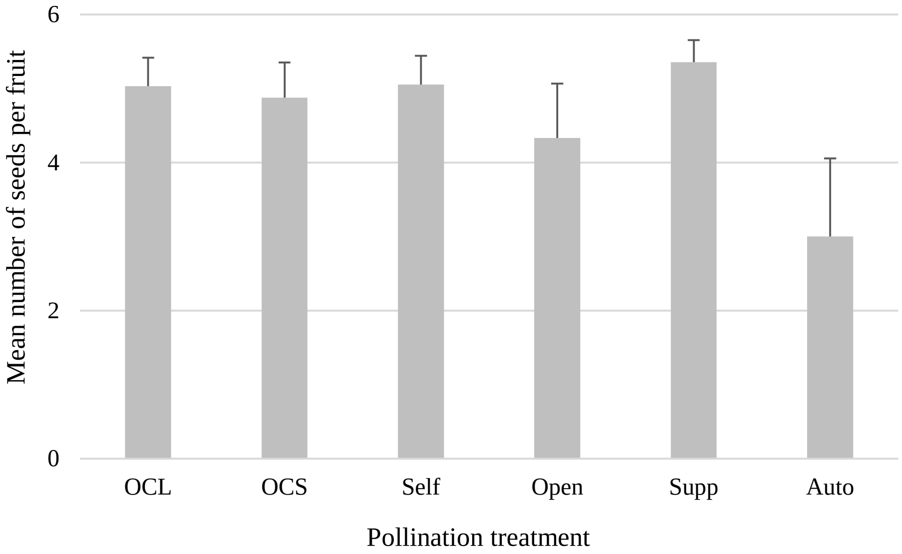

Figure 3.2 Mean (+95% CI) number of seeds per fruit in *T. manglesianus* for each pollination treatment. Where OCL and OCS are outcross pollen from long and short anthers, Supp is supplementary pollination and Auto is autogamous pollination.

Although the mean number of seeds produced by autogamous pollination was lower than open, and the mean number of seeds produced by supplementary pollinations was greater than open, these differences were not significant (Table 3.2).

Table 3.2 GLMM for the mean number of seeds per fruit for each pollination treatment in *T. manglesianus* (compared to open pollination)

Pollination treatment	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.47	0.10	14.95	<2e-16 ***
Autogamous	-0.37	0.21	-1.77	0.08
Outcross (long)	0.16	0.13	1.23	0.22
Outcross (short)	0.12	0.12	0.97	0.33
Self	0.15	0.12	1.26	0.21
Supplementary	0.21	0.12	1.81	0.07

Note. Open: *n*=24, auto: *n*=10, outcross long: *n*=31, outcross short: *n*=42, self: *n*=39 and supp: *n*=45.
*** result is significant at p<.001.

T. manglesianus flowering

The mean number of *T. manglesianus* flowers per day at the study site during 2013 peak flowering was 1496 (95% CI = ±13.38), approximately 0.32 flowers per square metre. The mean proportion of *T. manglesianus* flowers setting fruit, and the mean number of seeds per fruit were both lower in 2006 than in 2005 and 2013 (Figure 3.3).

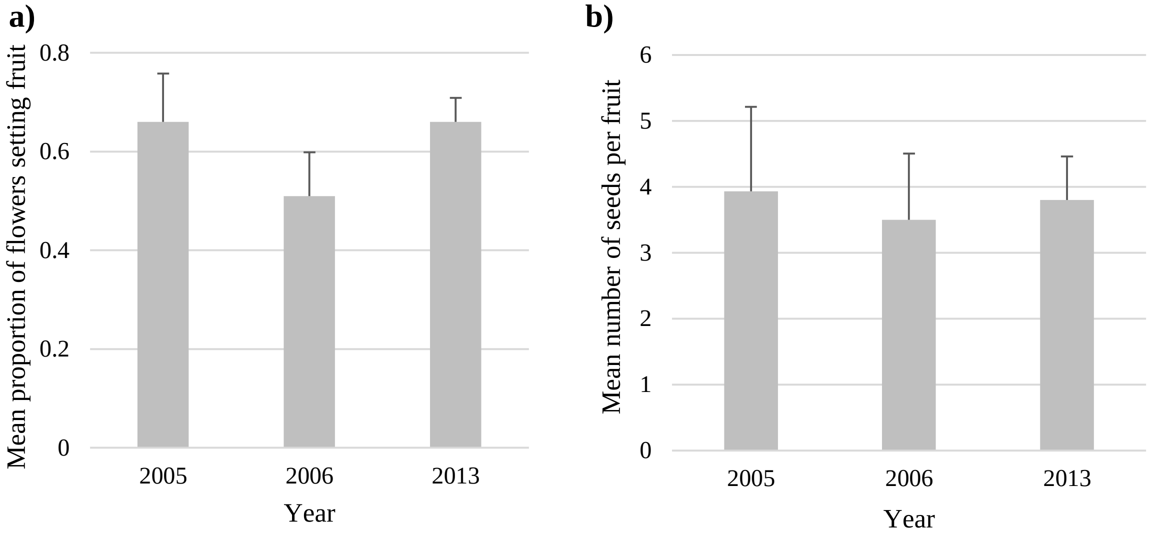

Figure 3.3 a) Mean proportion (+95% CI) of *T. manglesianus* flowers which set fruit and b) mean (+95% CI) number of seeds per fruit in 2005, 2006 and 2013.

Despite the lower means in 2006, these were not significantly different from the results obtained in 2005 (Table 3.3).

Table 3.3 GLMMs for a) the mean proportion of flowers setting fruit and b) mean number of seeds per fruit for *T. manglesianus* in 2006 and 2013, compared to 2005

		Estimate	Std. Error	z value	Pr(>|z|)
a	(Intercept)	-0.27	0.24	-1.15	0.25
	as.factor(Year)2006	-0.31	0.30	-1.03	0.30
	as.factor(Year)2013	-0.67	0.27	-1.04	0.06
b	(Intercept)	1.30	0.14	9.23	<2e-16***
	as.factor(Year)2006	0.02	0.18	0.11	0.92
	as.factor(Year)2013	-0.02	0.22	-0.11	0.91

Note. a) 05: *n*=513, 06: *n*=435, 13: *n*=223; b) 05: *n*=218, 06: *n*=582, 13: *n*=101.
*** result is significant at p<.001.

T. multiflorus and *T. triandrus* breeding systems

Breeding systems data for *T. multiflorus* (collected by P. Ladd in 2004 and 2005) shows this species has a mixed mating system (Figure 3.4). Open pollination had the lowest proportion of flowers setting fruit, and the proportion of self-pollinated flowers that set fruit was less than outcross pollination in both years. Three seeds per fruit was the most frequently observed number in fruit produced by open pollinations.

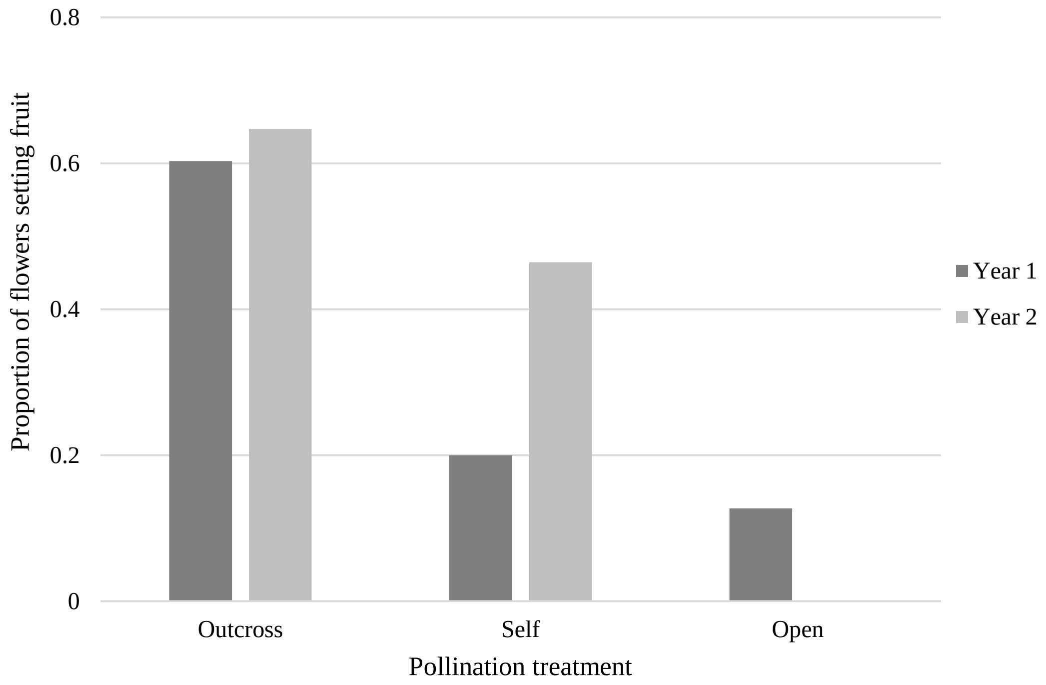

Figure 3.4 The proportion of flowers setting fruit for each pollination treatment for *T. multiflorus* over two years (data from P. Ladd). CI calculation and significance testing was not possible, as replicate data were not recorded separately.

The mean number of seeds per fruit in *T. multiflorus* was greatest in the outcross pollinated samples and lowest in self-pollinated samples for both years (Figure 3.5).

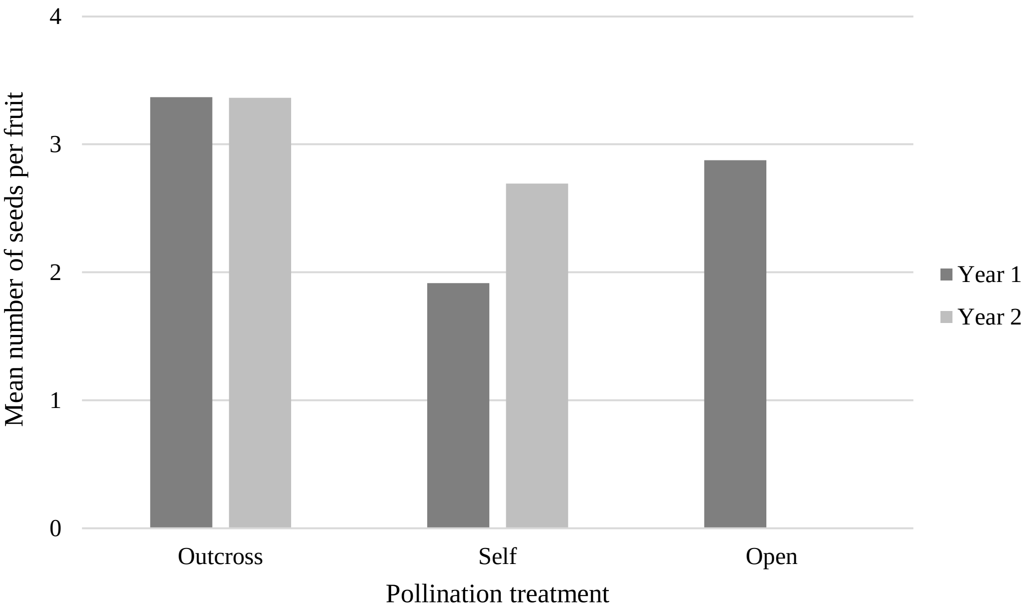

Figure 3.5 The mean number of seeds per fruit for each pollination treatment in *T. multiflorus* over two years (data from P. Ladd). CI calculation and significance testing was not possible, as replicate data were not recorded separately.

Breeding systems data were obtained for *T. triandrus* by P. Ladd in 2011. *T. triandrus* has a mixed mating system, as fruit were produced from both self and outcross pollinations (Figure 3.6). However, autogamous pollination did not set any fruit, and open pollination only set one fruit on the plants that were used for the breeding trial.

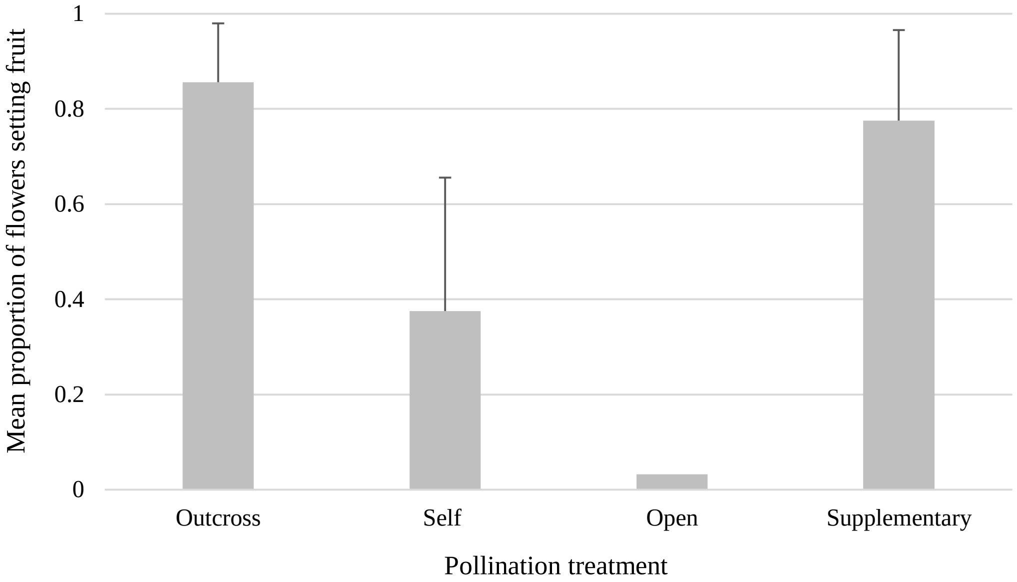

Figure 3.6 Mean proportion (+95% CI) of *T. triandrus* flowers setting fruit for each pollination treatment.

The proportion of outcross, supplementary, and self-pollinated flowers which set fruit were all significantly greater than the proportion which set fruit from open pollination (Table 3.4).

Table 3.4 GLMM for the proportion of *T. triandrus* flowers which set fruit for each pollination treatment (compared to open pollination)

Pollination treatment	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-3.66	1.01	-3.62	0.00***
Outcross	5.30	1.09	4.85	1.25e-06***
Self	3.17	1.06	2.99	0.00**
Supplementary	5.15	1.10	4.70	2.59e-06 ***

Note. Open: *n*=40, outcross: *n*=43, self: *n*=45, supplementary: *n*=38.
** result is significant at p<.01.
*** result is significant at p<.001.

The mean number of seeds per fruit for each pollination treatment (Figure 3.7) was lowest for self-pollinated flowers, and greatest for supplementary pollination (closely followed by outcross pollination), however the differences were not significant (Table 3.5).

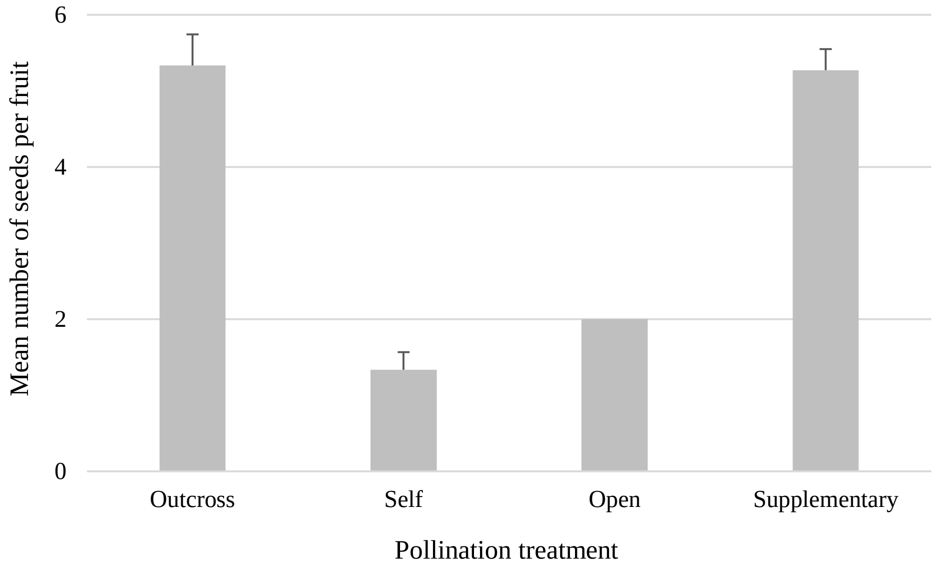

Figure 3.7 The mean (+95% CI) number of seeds per fruit in *T. triandrus* for each pollination treatment.

Table 3.5 GLMM for the mean number of seeds per fruit for each pollination treatment in *T. triandrus* (compared to open pollination)

Pollination treatment	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.69	0.71	0.98	0.33
Outcross	0.96	0.71	1.35	0.18
Self	-0.35	0.74	-0.47	0.64
Supplementary	0.98	0.71	1.39	0.17

Note. The sample size for open pollination is 1 as only one flower set fruit. For outcross: *n*=36, self: *n*=17, supplementary: *n*=31.

T. multiflorus flowering

The greatest mean and maximum number of *T. multiflorus* flowers was observed in Area B. Although Area B is larger, the mean number of flowers per square metre was slightly greater than in Area A (Table 3.6).

Table 3.6 The maximum and mean (±95% CI) numbers of *T. multiflorus* flowers observed in *T. multiflorus* Area A and Area B

	Maximum daily total	Mean/day	Maximum flowers/m^2	Mean flowers/m^2
Area A	590	157 (±50)	1.48	0.39 (±0.15)
Area B	2680	614 (±203)	1.92	0.44 (±0.16)

The peak flowering for *T. multiflorus* occurred at a similar time in both areas; the maximum daily flowering total was obtained in Area B on the 27th of October, and in Area A on the 31st of October (Figure 3.8).

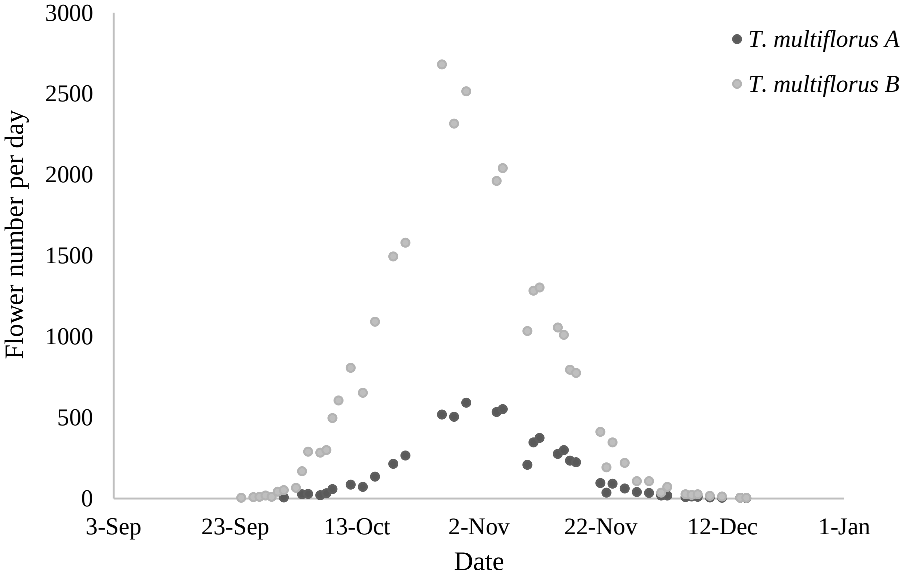

Figure 3.8 Total number of *T. multiflorus* flowers per day in Area A and Area B in 2013.

A comparison of the mean proportion of *T. multiflorus* flowers setting fruit between areas showed that Area A had a greater proportion of flowers setting fruit in 2011, but Area B was greater in 2012 and 2013 (Figure 3.9).

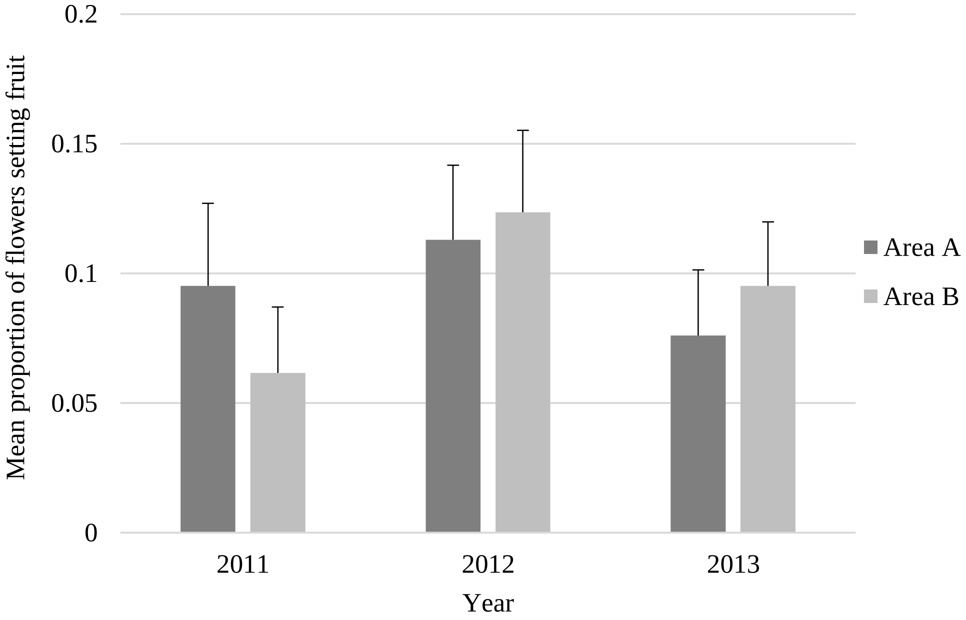

Figure 3.9 Mean proportion (+95% CI) of *T. multiflorus* which set fruit in Area A and Area B from 2011-2013 (data collected by P. Ladd).

However, across years the mean proportion of flowers setting fruit was greatest in 2012, and this was significantly greater than the proportion of flowers which set fruit in 2011. The proportion setting fruit in 2013 was slightly greater than 2011, however this difference was not statistically significant (Table 3.7).

Table 3.7 GLMM of the mean proportion of *T. multiflorus* flowers setting fruit between areas and between years (compared with Area A and 2011)

Area and year	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-2.43	0.13	-19.37	<2e-16 ***
AreaB	-0.02	0.10	-0.23	0.82
as.factor(Year)2012	0.33	0.13	2.51	0.01*
as.factor(Year)2013	0.02	0.13	0.11	0.91

Note. Area A: *n*=2348, Area B: *n*=2563, 2011: *n*=1303, 2012: *n*=1610, 2013: *n*=1998.
* result is significant at p<.05.
*** result is significant at p<.001.

The mean number of seeds per fruit for *T. multiflorus* in Area A and Area B was between 2.6 and 3.3 for all years (Figure 3.10).

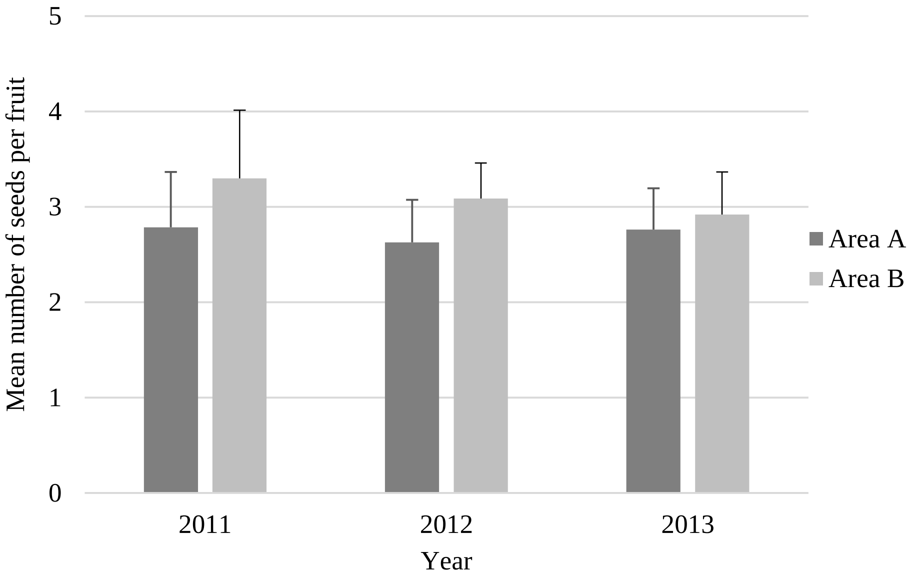

Figure 3.10 Mean (+95% CI) number of seeds per fruit in *T. multiflorus* in area A and B from 2011-2013 (data collected by P. Ladd).

Area B (*T. multiflorus*) had a greater mean number of seeds per fruit (Figure 3.10), and the mean number of seeds per fruit in 2012 was slightly less than 2011. Both results have a low level of statistical significance (Table 3.8).

Table 3.8 GLMM of the mean number of seeds per fruit in *T. multiflorus* between areas and between years (compared with Area A and 2011)

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.15	0.06	18.68	<2e-16 ***
AreaB	0.11	0.06	1.98	0.05*
as.factor(Year)2012	-0.18	0.07	-2.48	0.01*
as.factor(Year)2013	-0.12	0.07	-1.64	0.10

Note. Area A: *n*=211, Area B: *n*=229, 2011: *n*=104, 2012: *n*=175, 2013: *n*=161.
* result is significant at p<.05.
*** result is significant at p<.001.

T. multiflorus floral display size

Breeding systems studies showed that self-pollination in *T. multiflorus* produced fewer fruit, and seeds per fruit, than outcross pollination. These results were used to investigate whether seed set success varied with floral display size. Overall, the *T. multiflorus* samples with large floral displays received more visits, and produced more fruit and seeds than those with small displays (Table 3.9).

Table 3.9 Total and mean number of flowers, visits and the number of fruit and seeds for *T. multiflorus* plants with large and small floral displays

	Total # flowers	Total # visited	Mean proportion visited	Total # fruit	Total # seeds
Large	4938	651	0.13 (±0.05)	260	865
Small	820	143	0.17(±0.05)	137	437

Despite the slightly higher proportion of visited flowers on plants with smaller floral displays, they had a lower proportion of flowers which set fruit (Figure 3.11), however, the difference was not significant (Table 3.10).

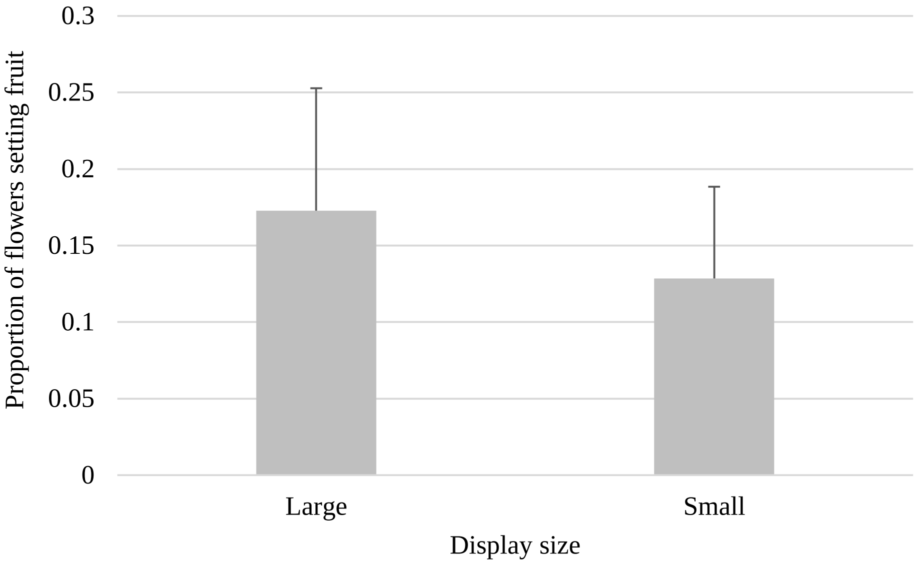

Figure 3.11 The mean (95% CI) proportion of *T. multiflorus* flowers setting fruit for large (>5 flowers) and small (≤5 flowers) floral displays.

Table 3.10 GLMM for the proportion of *T. multiflorus* setting fruit for flowers on plants with a small floral display (in comparison to those with large displays)

Display size	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.64	0.20	-8.18	2.74e-16 ***
Small	-0.40	0.29	-1.57	0.12

Note. *** result is significant at p<.001.

The *T. multiflorus* plants with large floral displays also had more seeds per fruit than small samples (Figure 3.12), but this difference was not statistically significant (Table 3.11).

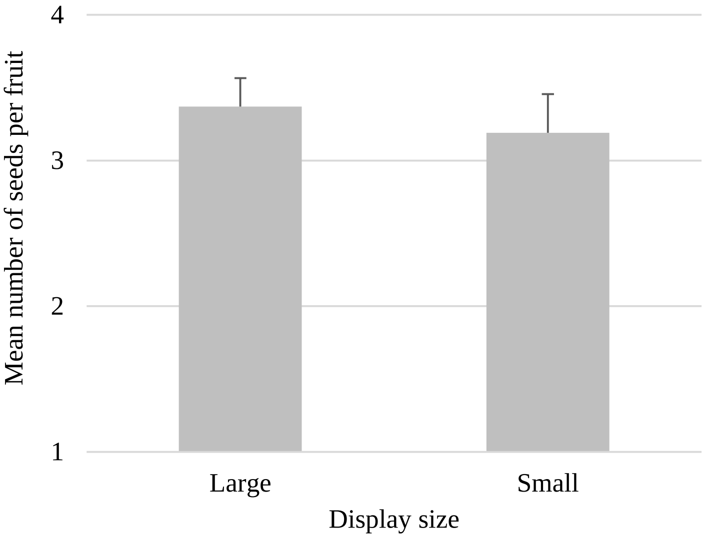

Figure 3.12 The mean (+95% CI) number of seeds per fruit for *T. multiflorus* in large (>5 flowers) and small (≤5 flowers) floral displays.

Table 3.11 GLMM for the mean number of seeds per fruit in plants with small floral displays (in comparison to large displays)

Display size	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.20	0.07	17.63	<2e-16***
Small	-0.10	0.11	-0.86	0.39

Note. *** result is significant at p<.001.

The proportion of *T. multiflorus* with one seed per fruit was greater for small floral displays than large displays (Figure 3.13). Although the proportion of fruit with one and two seeds (i.e. geitonogamous self-fertilisation) was expected to be greater in the large samples, a chi squared comparison of the number of seeds per fruit for different floral display sizes showed there was no significant difference (χ^2 [5] = 1.60, p>.05).

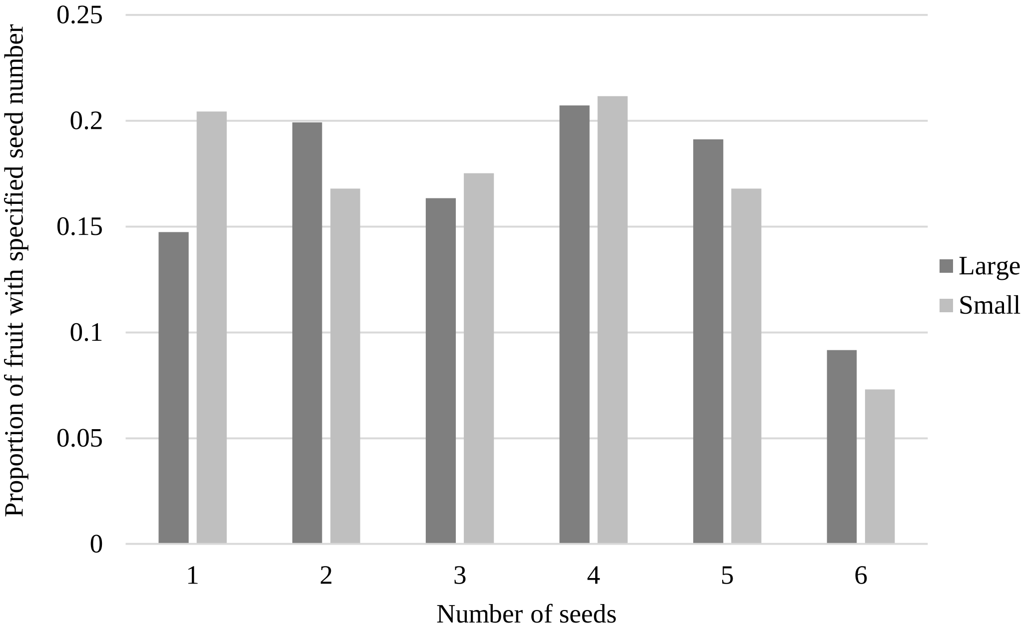

Figure 3.13 Proportion of *T. multiflorus* fruit with specified seed number, for samples with large and small floral display sizes.

T. triandrus flowering

T. triandrus started flowering 40 days later than *T. multiflorus*, and flowered for around 40 days (as opposed to *T. multiflorus* which flowered for about 80 days). The maximum number of *T. triandrus* flowers per plant for both Area A and Area B was obtained on the 18th of November 2013 (Figure 3.14), but total and mean numbers of flowers were greatest in Area B (Table 3.12). Where the two species overlapped, the maximum number of flowers (of both species) per square metre was 5.96, and the mean was 1.56.

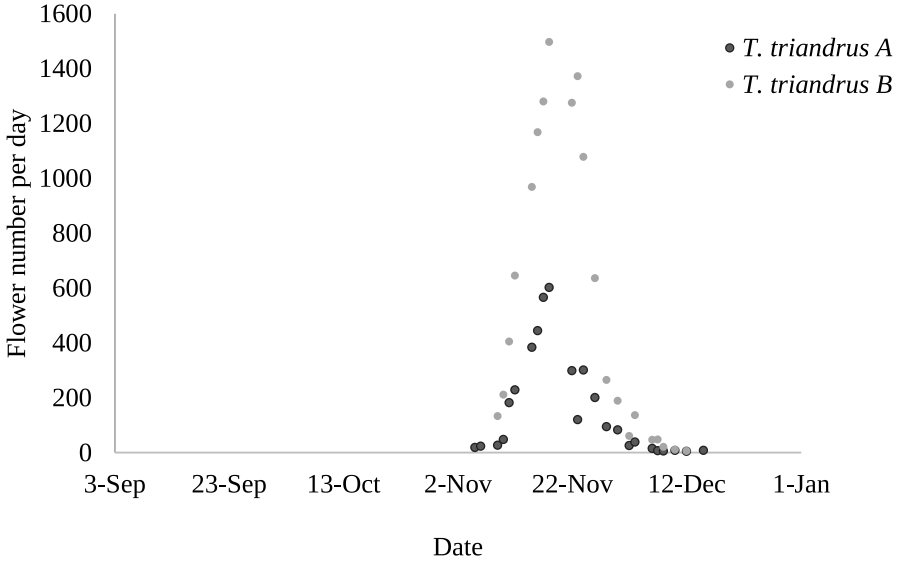

Figure 3.14 The total number of *T. triandrus* flowers per day in Area A and Area B, 2013.

Table 3.12 Mean (±95% CI) and maximum number of flowers observed in *T. triandrus* flowering Areas A and B, and mean and maximum per square metre

Area	Maximum daily total	Mean #/day	Max /m^2	Mean /m^2
A	602	155 (±50)	3.01	0.78 (±0.37)
B	1496	544 (±145)	3.86	1.40 (±0.58)

Like *T. multiflorus*, the proportion of *T. triandrus* flowers setting fruit was greater in Area A in 2011, and Area B in 2012 and 2013 (Figure 3.15).

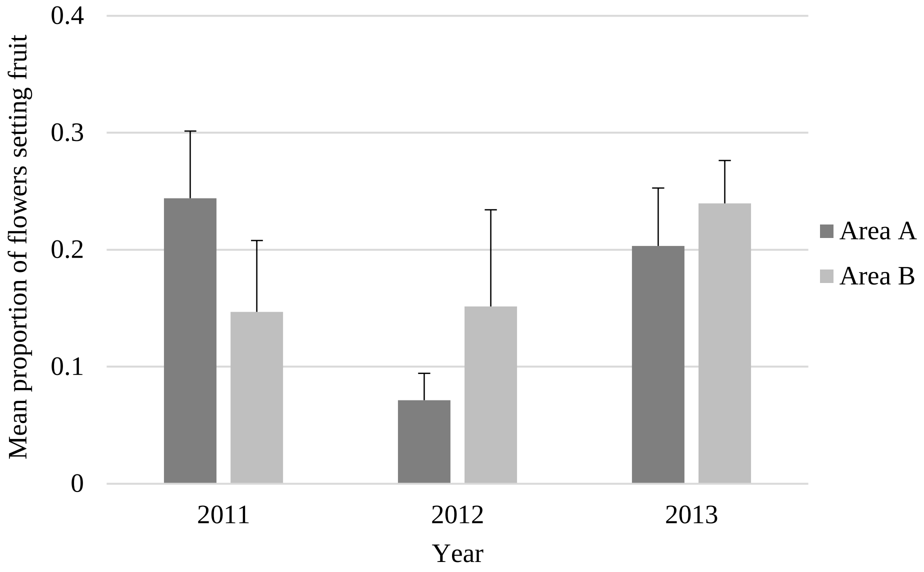

Figure 3.15 Mean proportion (+95% CI) of *T. triandrus* which set fruit in Area A and Area B from 2011-2013 (data collected by P. Ladd).

Overall, the proportion of *T. triandrus* flowers setting fruit in 2012 was significantly lower than in 2011 (Table 3.13).

Table 3.13 GLMM of the mean proportion of flowers setting fruit for *T. triandrus* between areas and years (in comparison to Area A and 2011)

Fixed effects:	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-1.69	0.30	-5.68	1.39e-08***
AreaB	-0.08	0.28	-0.29	0.78
as.factor(Year)2012	-0.95	0.42	-2.27	0.02*
as.factor(Year)2013	0.21	0.32	0.65	0.51

Note. Area A: *n*=2984, Area B: *n*=4218, 2011: *n*=1497, 2012: *n*=1005, 2013: *n*=4700.
* result is significant at p<.05.
*** result is significant at p<.001.

The mean number of seeds per fruit in *T. triandrus* was quite similar between years (Figure 3.16), however, it was significantly greater in Area B (Table 3.14).

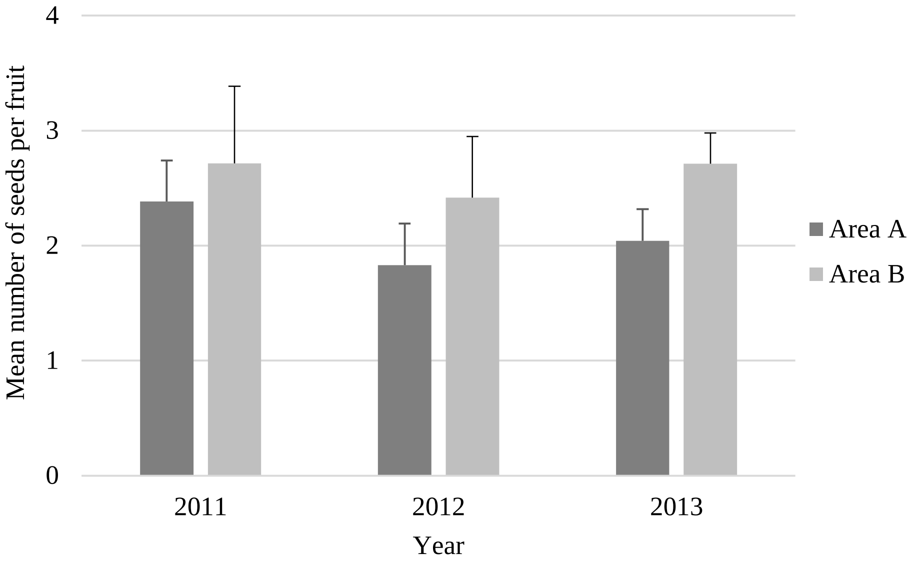

Figure 3.16 Mean (+95% CI) number of seeds per fruit in *T. triandrus* in area A and B from 2011-2013 (data collected by P. Ladd).

Table 3.14 GLMM of the mean number of seeds per fruit for *T. triandrus* between areas and years (in comparison to Area A and 2011)

Fixed effects:	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	0.77	0.09	8.88	< 2e-16 ***
AreaB	0.18	0.08	2.19	0.03*
as.factor(Year)2012	-0.19	0.14	-1.37	0.17
as.factor(Year)2013	-0.01	0.09	-0.06	0.95

Note. Area A: *n*=528, Area B: *n*=869, 2011: *n*=296, 2012: *n*=92, 2013: *n*=1009.
* result is significant at p<.05.
*** result is significant at p<.001.

T. triandrus floral display size

For 30 *T. triandrus* plants with different floral display sizes, the lowest maximum number of flowers recorded was three, and the highest was 343. The maximum number of flowers per plant was most frequently under 30. Only five of the 30 plants had a maximum of more than 61 flowers (Figure 3.17).

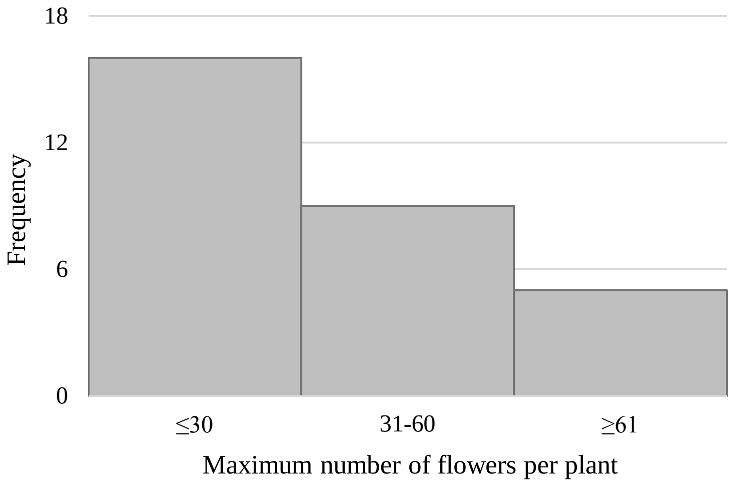

Figure 3.17 The frequency of *T. triandrus* plants with the maximum number of flowers specified.

For the 30 *T. triandrus* samples under natural conditions, the proportion of flowers that set fruit was generally greater for plants that had a lower maximum number of flowers (Figure 3.18). This was significantly greater for plants with a maximum of 30 (or fewer) flowers than those with 31-60 flowers (Table 3.15).

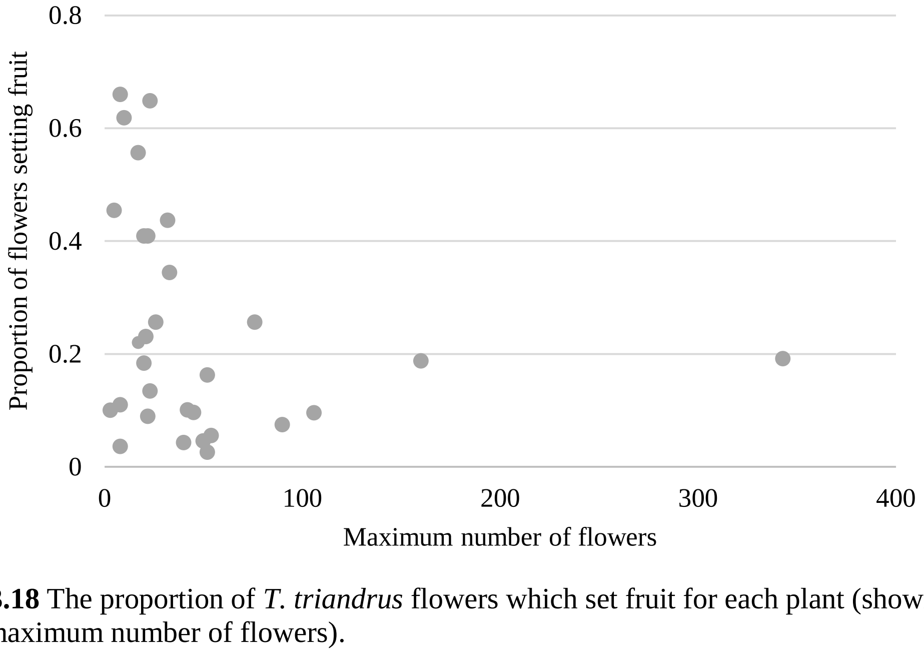

Figure 3.18 The proportion of *T. triandrus* flowers which set fruit for each plant (showing each plant’s maximum number of flowers).

Table 3.15 GLMM for the proportion of flowers setting fruit for *T. triandrus* plants with different size maximum floral displays (compared to the ≤30 group)

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.93	0.26	-3.56	0.00***
31-60	-1.22	0.43	-2.81	0.00**
≥61	-0.83	0.53	-1.58	0.11

Note. ** result is significant at p<.01.

*** result is significant at p<.001.

For the display size investigation in *T. triandrus*, one seed per fruit was the most often observed number (for all three size categories). A test for goodness of fit showed that the difference between the distributions of seed numbers for different sized plants was significant ($\chi^2[10] = 25.3$, $p<.05$). The overall pattern suggests that as the floral display size increased, the proportion of fruit with more than two seeds decreased (Figure 3.19), which supports the hypothesis for more geitonogamy in plants with large floral displays.

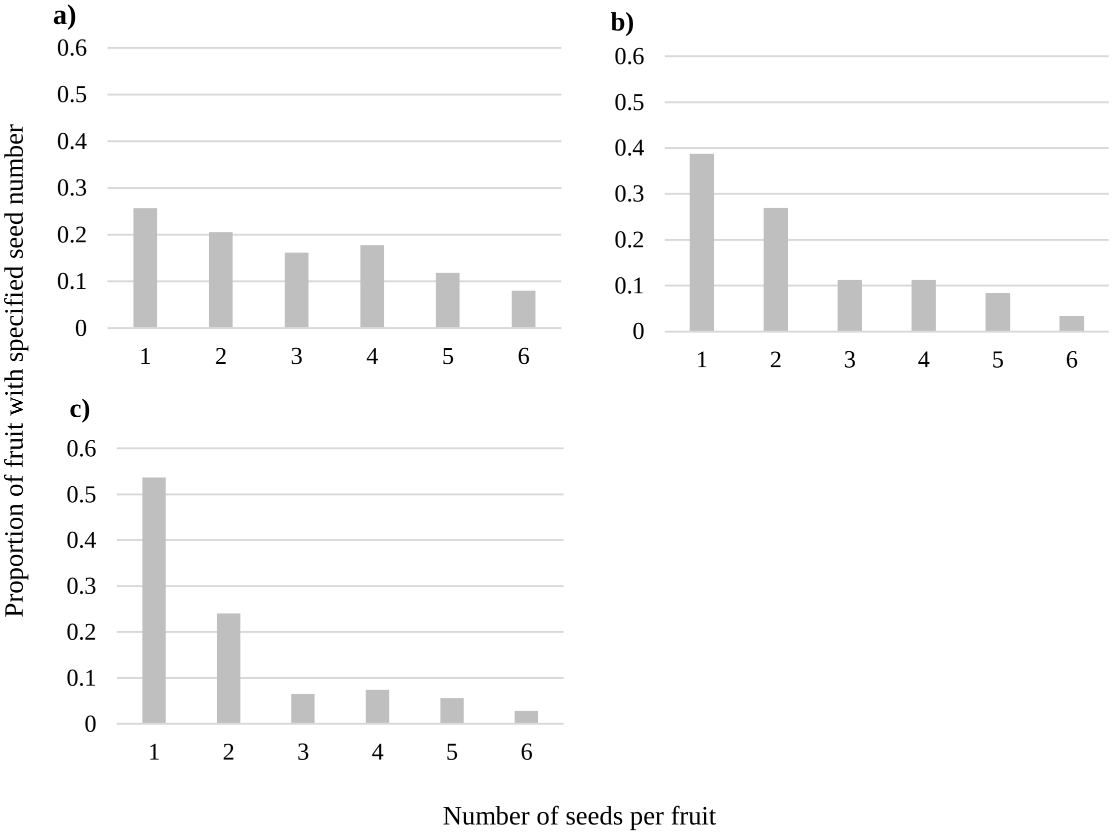

Figure 3.19 The proportion of fruit with the specified seed number for *T. triandrus* samples with different numbers of flowers (a. ≤30, b. 31-60, c. ≥61).

The mean number of seeds per fruit was greatest in plants which had a maximum of ≤30 flowers (Figure 3.20).

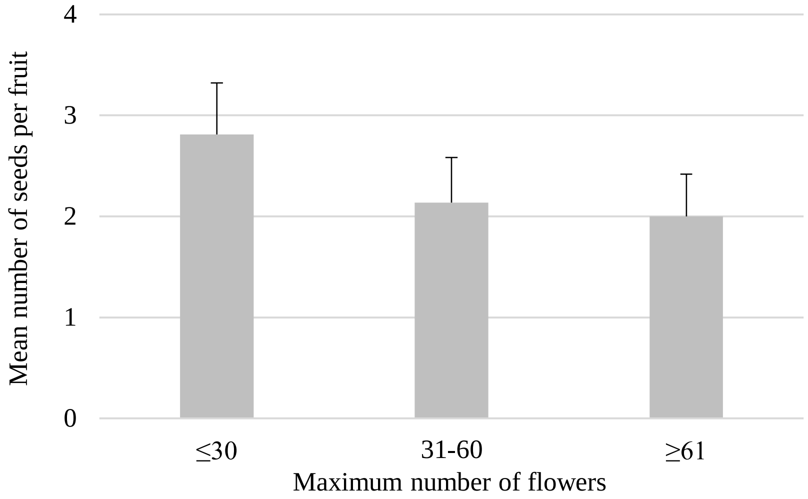

Figure 3.20 The mean (+95% CI) number of seeds per fruit for *T. triandrus* plants with the maximum number of flowers specified.

The mean number of seeds per fruit was significantly less in the 31-60 and ≥61 groups than it was for the ≤30 group (Table 3.16).

Table 3.16 GLMM of the mean number of seeds per fruit for different maximum display sizes in *T. triandrus* plants

Fixed effects:	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	1.05	0.06	16.22	<2e-16***
31-60	-0.26	0.12	-2.22	0.03*
≥61	-0.38	0.14	-2.84	0.00**

* result is significant at p<.05.
** result is significant at p<.01.
*** result is significant at p<.001.

Pollen yield

The mean number of pollen grains calculated per flower for each species showed that *T. manglesianus* had the lowest mean number of pollen grains per flower (Figure 3.21). This difference was statistically significant in comparison to the other two species (*F*[2,289]=17.91, *p*<.001). *T. multiflorus* and *T. triandrus* did not differ significantly from each other (*F*[1,210]=0.19, *p*>.05).

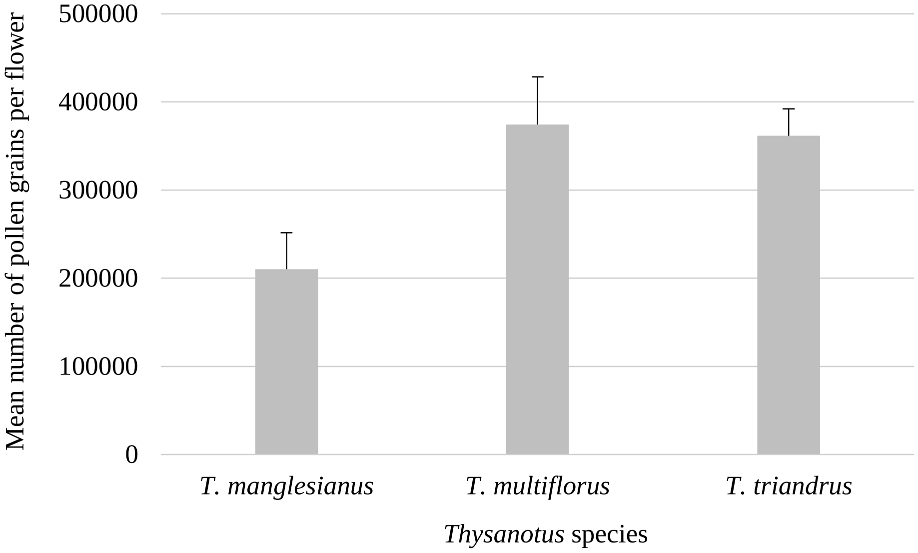

Figure 3.21 Mean (+95% CI) number of pollen grains per flower determined for each species.

Pollinator observations and flower colour

Only one insect visitor was recorded on *T. manglesianus* from a total of 190 minutes of observations, and one on *T. multiflorus* from 130 minutes of observations. From the 11th -18th of November, pollinator observations in Area A (where *T. multiflorus* and *T. triandrus* flowered together) occurred daily. Plants were watched for a total of 500 minutes (in 50 ten minute intervals). In total, 16 blue banded bees (*Amegilla chlorocyanea*) were observed, 14 of which were seen visiting a flower. Of these, one visited *T. triandrus* only, and two visited *T. multiflorus* only. The other 11 bees were observed flying directly between *T. multiflorus* and *T. triandrus* (or vice versa), on one or more instances. Interspecific pollen transfer between the two species was therefore likely. In addition, spectrometer analysis of *T. multiflorus* and *T. triandrus* flowers showed they have very similar reflectance at different wavelengths (Figure 3.22).

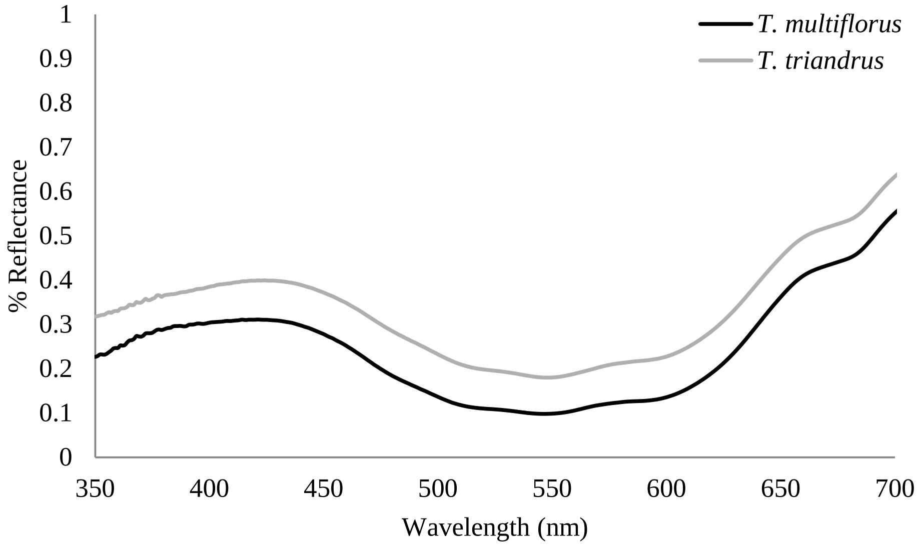

Figure 3.22 Spectrometer reading showing the % reflectance for *T. multiflorus* and *T. triandrus* across the (human) visible light spectrum.

Interspecific pollen transfer (*T. triandrus*)

The presence of heterospecific (*T. multiflorus*) pollen on *T. triandrus* did not prevent fruit set with later conspecific (*T. triandrus*) outcross and self-pollination. The proportion of outcrossed flowers setting fruit in the interspecific pollen transfer (IPT) experiment was only slightly lower than in the breeding system experiment (Figure 3.23), and almost identical for selfing. The frequency of flowers setting fruit in the breeding systems experiment was not significantly different to the IPT experiment ($\chi^2[1] = 0.29$, $p>.05$).

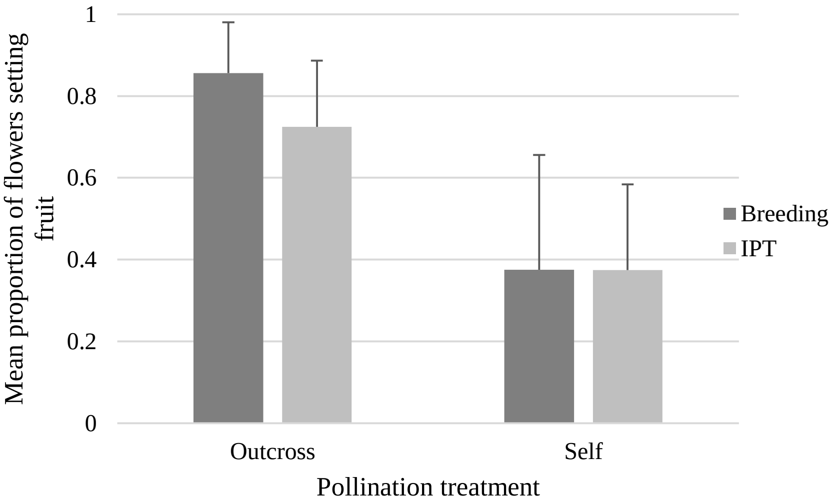

Figure 3.23 The mean proportion (+95% CI) of *T. triandrus* flowers setting fruit for self and outcross pollination for breeding system and IPT experiments.

The mean number of seeds per fruit in *T. triandrus* for the IPT experiment is not significantly different from that of the breeding systems experiment ($\chi^2[1] = 0.86$, $p>.05$) for outcross and self-pollinations (Figure 3.24). For both the mean proportion of flowers setting fruit and the mean number of seeds per fruit, the result is greater from outcross pollinations.

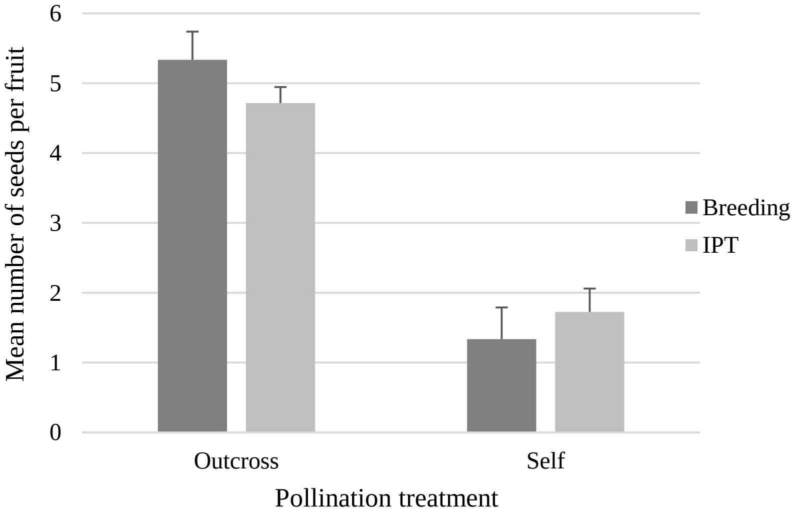

Figure 3.24 The mean (+95% CI) number of seeds per fruit for the *T. triandrus* breeding system and IPT experiment, for both outcross and self-pollination.

Like the breeding systems experiment, self-pollination in the IPT experiment often resulted in fruit with one or two seeds, whereas five or six seeds per fruit was the most frequent result of outcross pollination (Figure 3.25). A chi squared test between the number of seeds per fruit for outcross pollination showed there was no significant difference between the IPT experiment and the initial breeding experiment ($\chi^2[5] = 8.50$, $p>.05$). Although the number of seeds per fruit produced by self-pollinations (in the breeding system experiment) was often too low for statistical analysis, the distribution of fruit with one and two seeds was not significantly different between experiments ($\chi^2[1] = 0.45$, $p>.05$).

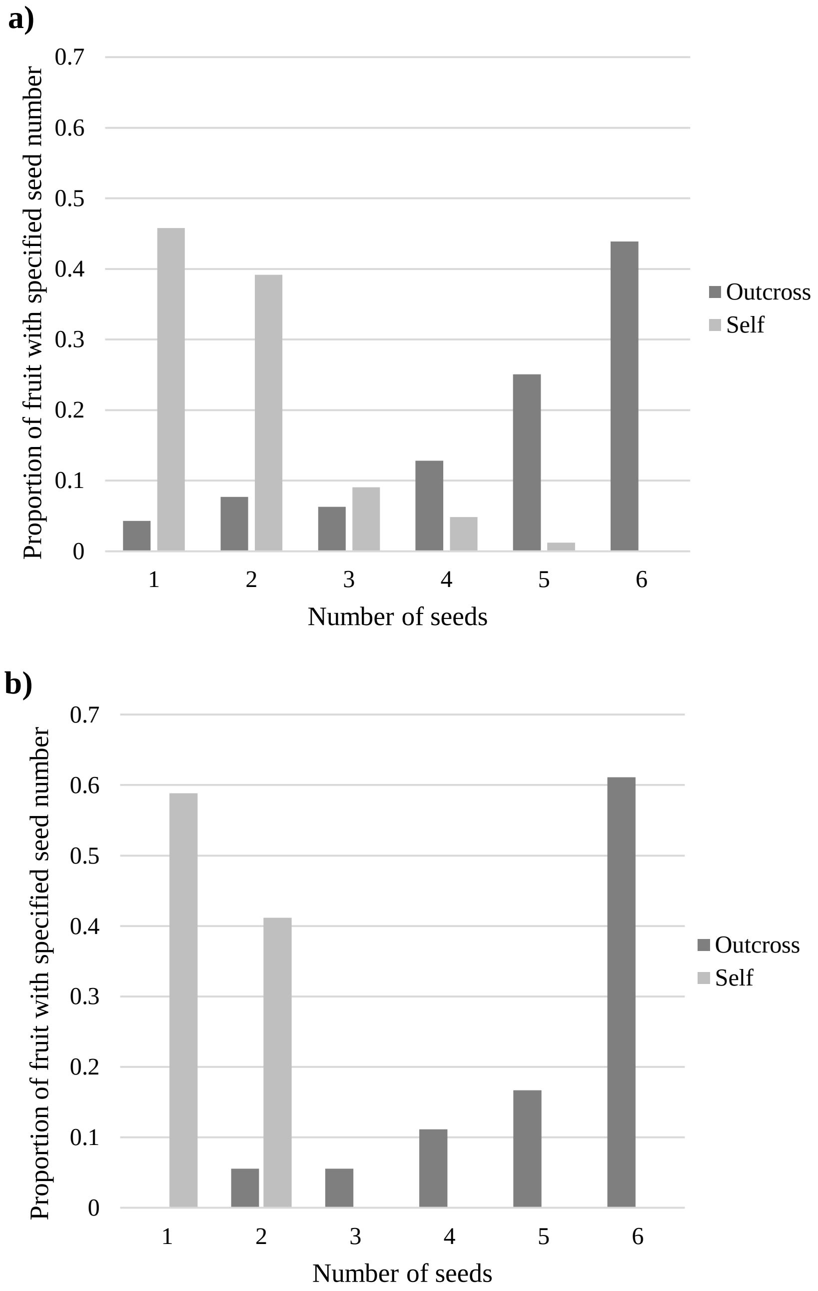

Figure 3.25 The proportion of *T. triandru*s fruit with the specified seed number from a) interspecific pollen transfer experiment b) original breeding system experiment.

Discussion

This study found that the three focal species each have a mixed mating system, however, outcross pollen is more successful than self-pollen in *T. multiflorus* and *T. triandrus*. In *T. multiflorus*, a greater proportion of flowers set fruit on plants with large floral displays, however this was not statistically significant. For large floral displays in *T. multiflorus*, geitonogamous selfing did not increase. On the other hand, large *T. triandrus* displays set proportionally less fruit, and showed the expected increase in geitonogamous selfing. Finally, the presence of *T. multiflorus* pollen on *T. triandrus* did not have any effect on later conspecific *T. triandrus* fertilisation.

T. manglesianus

T. manglesianus has a mixed mating system and produces the same proportion of fruit and seeds per fruit from outcross pollen as it does from self-pollen. Despite a consistent herkogamy distance of 1-2mm between the anther pores and the stigma while the flower is open (P. Ladd, unpublished data), a low level of autogamy occurs. However, autogamy produced far fewer seeds per fruit than manipulated selfing. This is because unassisted contact between anthers and the stigma in a flower is unlikely to occur until the flower closes, which may result in only a few pollen grains adhering to the stigma in some flowers. Therefore, the species is likely to conform to Lloyd’s category of delayed selfing, i.e. autonomous self-pollination without pollen discounting (Harder and Routley 2006; Morgan 2006), allowing at least some seeds to be produced by some of the flowers that are not visited. The greater fruit and seed set for geitonogamous hand pollination most likely resulted because the treatment ensured pollen was placed on the stigma, whereas not all un-manipulated flowers would have received pollen on the stigma from the anthers as the flowers closed. There was no difference in fruit set or seeds per fruit for outcrosses using long and short anthers. Long and short anthers also have approximately the same pollen content, thus the short anthers do not seem to be ‘feeding anthers’ as has been found in some other buzz pollinated species with dimorphic anthers (e.g. Dulberger and Ornduff 1980).

T. manglesianus is clearly pollen limited as supplementary outcross pollen resulted in the highest fruit set. However, the fruit produced in the open pollinated flowers were the sum of both fruits from visited flowers and the fruits produced by autogamy. The lower (although not significant) mean number of seeds per fruit for open pollinated flowers implies that some autogamy may have occurred. This autogamy is not consistent, and in a pollen limited situation few fruit are produced.

At the study site, *Sowerbaea laxiflora* (a buzz-pollinated species which flowers at the same time as *T. manglesianus*) produced no fruit in 2013 (n = 20 inflorescences sampled, unpublished data). In addition, almost no bee visitors were observed, so it is likely there was a low abundance of suitable pollinators. In *Dianella revoluta* (a buzz pollinated lily with somewhat different flower form to that of *Thysanotus*), Duncan *et al.* (2004) showed that a similar proportion of fruit was set in open pollinated flowers, however the mean number of seeds per fruit was much lower. This was attributed to considerable self-pollination. Even from self-pollinations, *T. manglesianus* had a greater number of seeds per fruit than *Dianella,* indicating a greater degree of self-fertility and thus a greater degree of reproductive assurance; this is beneficial for *T. manglesianus*, which has a shorter lifespan than *Dianella*. Unlike *Dianella*, *T. manglesianus* produces a mass display of flowers each day, so a great deal of geitonogamous pollination is likely if there are flower visitors. The overall *T. manglesianus* fruit production was lower in 2006 than 2005 and 2013, so 2006 may have been a poor year for pollination. Although the fruit set is consistently pollen limited, the lack of large variation across the nine year period indicates little variation in pollinator effectiveness during this time.

T. multiflorus and *T. triandrus* breeding systems

Both *T. multiflorus* and *T. triandrus* have mixed mating systems, however cross pollen resulted in greater fruit set and seeds per fruit than self-pollen, thus there is considerable inbreeding depression. Supplementary pollination of uncovered *T. triandrus* flowers produced similar fruit and seed set to outcross pollination in the breeding experiment, indicating the species was pollen limited in 2011, and it is highly likely this was also the case in 2012 and 2013. Although there was no supplementary pollination of *T. multiflorus* at the time of the breeding experiments, it is likely that this species is also pollen limited. As with *T. manglesianus,* mixed mating systems are considered to provide reproductive assurance (Eckert *et al.* 2006), particularly in situations where seed set is pollen limited. In the three *Thysanotus* species, seed set is commensurate with other studies of buzz pollinated species in Australia (Houston and Ladd 2002; Duncan *et al.* 2004) and Brazil (Brito and Sazima 2012), but lower than records for the northern hemisphere (Larson and Barrett 1999b; Usui *et al.* 2005; Gao *et al.* 2006).

Flowering

A difference in flowering phenology is commonly identified as a mechanism which allows species to avoid reproductive interference (Kudo 2006). The buzz pollinated species at the study site have a sequential flowering sequence, with peak flowering times for each species progressing through the spring and early summer (as one species declines, another takes over). *T. manglesianus* is the first to flower and its declining flowering overlaps with the start of flowering for *T. multiflorus. T. multiflorus* flowers for longer than *T. triandrus,* and in 2013 its peak flowering preceded that of *T. triandrus* by 15 days. A strong flowering peak produces a disadvantage to out-of-season individuals (Fenner 1998), possibly due to pollination reduction in an outcrossing species.

Flowers of *T. multiflorus* and *T. triandrus* are almost identical in form and colour as demonstrated by the colour spectrum analysis. It would therefore be expected that flower visitors would have difficulty in distinguishing between them. Both species are likely to provide almost identical reward for effort, as these species were both found to produce similar amounts of pollen, therefore flower visitors have no incentive to distinguish between the flowers of each species. Thus interspecific pollination is likely where the flowering time of the species overlaps.

T. multiflorus and *T. triandrus* floral display size

Although the proportion of *T. multiflorus* flowers visited was greater for small samples (though not statistically significant), it is thought that displaying many open flowers simultaneously will increase a plant's attractiveness to pollinators (Willson and Price 1977; Gerber 1985; Schmid-Hempel and Speiser 1988; Brody and Mitchell 1997; Harder *et al.* 2004; Valdivia and Niemeyer 2006). The contrary findings from the present study are likely to be a result of the small samples having a maximum of only five flowers (i.e. the small number of visits on plants with few flowers is a higher proportion than the same number of visits would be on a plant with more flowers). This idea is reinforced by the higher proportion of flowers which set fruit in the large *T. multiflorus* displays despite the lower proportion of visits.

Despite the non-significant results obtained for the proportion of *T. multiflorus* flowers setting fruit, almost double the amount of fruit and seeds were produced by the group of plants with a large floral display size. This points towards the idea that increased display size increases reproductive success (Schmid-Hempel and Speiser 1988; Harder *et al.* 2004), and pollen removal (Willson and Price 1977; Schmid-Hempel and Speiser 1988). It is likely that these results occurred because of the large displays produced more flowers, thereby increasing the chance of visitors during the overall flowering period of the plant (Firmage and Cole 1988). Although this may account for the tendency for a greater number of flowers which set fruit in plants with large floral displays, *T. triandrus* showed the opposite. For both proportion of flowers setting fruit, and the mean number of seeds per fruit, the result was generally greater for *T. triandrus* plants with smaller floral displays. From the breeding trials it is clear that from outcross pollination, *T. triandrus* had a better fruit set than *T. multiflorus*. If smaller inflorescences are more likely to receive fewer geitonogamous pollinations then it is likely *T. triandrus* will have higher fruit set than *T. multiflorus*. It is also noteworthy that in the three years of open pollinated fruit set data, *T. triandrus* always had greater fruit set than *T. multiflorus*, which may in part be due to fewer other species flowering later in the season, and therefore less competition for pollinator attention.

The hypothesis that larger floral displays would have more geitonogamy was not supported for *T. multiflorus*, as there was no significant difference of number of seeds per fruit between large and small floral displays. In addition, the distribution of fruits with one or two seeds did not increase for the specimens with large floral displays. This may be the result of flowers on large floral displays being visited by more individual pollinators than those on small displays, so the diversity of sires is greater (Schmid-Hempel and Speiser 1988). In addition, the distinction between number of seeds produced by self and outcross pollinations is less precise in *T. multiflorus* than in *T. triandrus* so it is more difficult to see any significant difference. However, the results for *T. triandrus* support the geitonogamy hypothesis, as the proportion of *T. triandrus* fruit with more than one or two seeds decreased in plants with large floral displays.

Interspecific pollen transfer

The study of the effect of heterospecific pollen on *T. triandrus* was intended to examine the question of the extent of reproductive isolation between two species that seemed to be closely related and had similar flowers. In many recent studies there has been considerable attention given to how a change in floral morphology interacting with a specific pollinator leads to reproductive isolation between an ancestral species and one derived from it (e.g. Johnson 2010; Forest *et al.* 2014). The deposition of heterospecific pollen on *T. triandrus* made no difference to fruit and seed set when conspecific pollen was later applied. The proportion of flowers setting fruit after receiving heterospecific pollen was greater for cross pollinated plants than self-pollinated plants, as was found in the breeding experiment. Other studies have also found no detectable effect after heterospecific pollen deposition (e.g. Kwak and Jennersten 1991; Caruso and Alfaro 2000). In contrast, experiments on the effect of heterospecific pollen deposition in *Mimulus guttatus* using a closely related congener (*Mimulus nudatus*) showed the *M. nudatus* pollen mimicked the pollination reaction of the conspecific pollen, but seed set was reduced due to ovule usurpation (Ashman and Arceo-Gomez 2013). In *T. triandrus* there is no evidence of such a deleterious effect. Unpublished data reported by Brittan (1981) suggested that *T. triandrus* and *T. multiflorus* are capable of interbreeding. However, tests for hybridisation with plants from the study site (P. Ladd, unpublished data) showed that *T. triandrus* success with *T. multiflorus* pollination is very low (in 39 trials, one fruit was produced with one seed).

Reproductive isolation cannot be effected by morphological differences between the flowers of the two species and pollinators have not been observed to distinguish between them. Unfortunately it was not possible to examine pollen tube growth in the styles as aniline blue-stained pollen tubes do not fluoresce under ultraviolet light, but it is likely that heterospecific pollen either does not germinate on the stigma, or that pollen tube growth is prevented in the style or at the micropyle. It seems that sympatric speciation could not lead to the origin of these two species and that they would have to have arisen allopatrically. Genetic drift is likely to have led to reproductive isolation that now prevents interbreeding where they have subsequently come into ecological contact. It is noteworthy that there are only five sites known where the two species occur together (Brittan 1981).

As heterospecific pollen had no effect on fruit and seed set in *T. triandrus* it might be expected that the co-flowering of the two species in Area A would enhance the reproductive output of *T. triandrus* (as this species begins flowering when *T. multiflorus* declined) by providing a greater display to attract pollinators (e.g. Yang *et al.* 2013). However, there is no evidence that this has occurred. Over the three years considered here, fruit set was only greater in Area A in 2011, and this was not significant. It is likely that under the conditions of pollen limitation that seem to apply to the *Thysanotus* species growing at the study site, pollinators are just insufficient to facilitate high fruit production. It may be possible this is the result of a reduced pollinator fauna in an isolated urban reserve, however baseline data from a time when the natural bushland area was widespread is not available for comparison, so this cannot be confirmed.

Limitations and recommendations

Consideration should be given to the limitations of the present study when interpreting the results. First, the lack of disparity between the floral display size categories in the present study may have reduced the sensitivity to differences between them. For example, a plant with five flowers was classed as small, but a plant with six flowers was classed as large, even though the two would have little biological difference. To improve this research, a greater difference between the categories of floral display size would have been beneficial. Applying categories with a greater difference (such as ≤5 and >15) would increase sensitivity, and would also eliminate the need to class plants after data collection, as was necessary for *T. triandrus*. The ability to detect differences associated with changes in floral display size may also have been enhanced by the inclusion of more display size categories. For example, small, medium, and large, as opposed to just small and large.

For the interspecific pollen transfer experiment, it may have been useful to also transfer pollen the opposite way (i.e. to determine whether the presence of *T. triandrus* pollen had any effect on the reproductive success of *T. multiflorus*). This was not done in the present study as *T. multiflorus* flowered earlier, and the most accessible plants were already being used for the floral display size experiment by the time *T. triandrus* began flowering.

Finally, increasing the observations of pollinators would be beneficial, as the native bees do not appear to be abundant, which could be a result of the fragmented landscape. To provide a better idea of pollinator abundance, observations could involve watching a larger sample of plants throughout the full duration of their flowering time. Recording data such as plant size, distance to the nearest *Thysanotus* plant (in the case of *T. multiflorus* and *T. triandrus*), and whether flowers are obscured by other plants would also help to gain a better understanding of pollinator behaviour.

Conclusion

This study aimed to provide information on the breeding systems and floral phenologies of the chosen *Thysanotus* species. It also investigated the influence of floral display size and interspecific pollen transfer, two factors which affect reproductive success (i.e. the production of fruit and seeds). This study successfully determined that *T. manglesianus*, *T. multiflorus* and *T. triandrus* all possess a mixed mating system, as they set fruit from both self and cross pollinations. This study also provided some insight into the influence of display size, however the two focal species showed differing results. *T. multiflorus* plants with larger floral displays did not increase geitonogamy. On the other hand, *T. triandrus* plants with large floral displays set less fruit, and geitonogamy increased. This study also showed that heterospecific pollen transfer from a close relative did not reduce reproductive success in *T. triandrus*. These findings may provide a basis for future research into the reproductive biology of other native, buzz pollinated genera.

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