Dispersal biology of *Orobanche ramosa* in South Australia

Master of Science

Thesis

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Chapter 1. Introduction

This thesis documents experiments investigating two dispersal vectors for *O. ramosa*: sheep and wind. This introductory chapter forms a literature review including a brief introduction to parasitic plants and the *Orobanche* genus, the status of the genus in Australia, and current management practices for the genus around the world. Seed dispersal biology is then discussed, with a particular focus on plants with small seeds.

Parasitic plants

As in the animal kingdom, plants can form parasitic relationships with other plants. It is estimated that approximately 1% of angiosperms, or 3000 species in 17 families are parasitic (Kuijt 1969; Parker and Riches 1993). Classification of parasitic plants relies on two features. The first is the degree to which the parasite relies on its host. Holoparasitic (wholly parasitic) angiosperms have no chlorophyll and rely entirely on the host for their nutrition. Conversely, hemiparasitic (partly parasitic) angiosperms have chlorophyll and are therefore capable of photosynthesis to produce their own carbohydrates, in addition to the contribution from the host. The second feature that is used to classify parasitic plants is the site of attachment to the host. The attachment organ, the haustorium, can be either above the ground on the shoots, or below the ground on the roots.

While there are an estimated 3000 species of parasitic flowering plants, only a handful of genera threaten agriculture and economy. The main genera of interest from an economical standpoint are: *Striga* Lour., the witchweeds, by far the most important genus with 11 species known to parasitise graminaceuos crops including maize, sorghum, millet, rice and sugar cane; *Orobanche* L., the broomrapes, parasites of broadleaf crops, mainly legumes and vegetables; *Cuscuta* L., the dodders, shoot parasites with a wide range of hosts, including legumes and woody crop trees such as citrus and coffee; and *Alectra* Thunb., parasites of legumes and sugar cane (Parker and Riches 1993; Riches and Parker 1995). Mistletoes in the families Viscaceae and Loranthaceae may be considered economically important when parasitising forest, fruit and ornamental trees (Parker and Riches 1993).

Parasitic angiosperms are usually detrimental to the health and vigour of the host, which in agriculture can cause a decrease in crop yield and quality. It is challenging

to accurately assess the impacts of root parasites on yield as parasite-free control plots are difficult to create in order to establish competition studies (Parker and Riches 1993). The seedbank is usually removed by complete fumigation, which may also destroy beneficial soil pathogens, thereby preventing an accurate determination of the effect that the parasite may have on yield. Despite these experimental difficulties, estimates of yield reductions under parasite infestation are possible. For example, *S. hermonthica* caused 21% yield reduction on Sorghum in Ghana (Parker and Riches 1993); *O. ramosa* caused 21-29% yield reduction on tomato in United States (Parker and Riches 1993); and *O. crenata* caused 50% to 100% yield reduction on faba bean in Spain (Mesa-García and García-Torres 1984) and Syria (Manschadi et al. 2001), respectively.

Overview of *Orobanche* spp.

The family Orobanchaceae has 14 genera; all are root holoparasites. Within the family, *Orobanche* is economically the most important genus with over one hundred species, five of which are considered important agricultural pests (Parker and Riches 1993): *O. crenata*, *O. cernua*, *O. cumana*, *O. ramosa*, and *O. aegyptiaca*. Parker and Riches (1993) consider *O. ramosa* to be the most widespread of the pest species.

The lifecycles of *Orobanche* spp. are similar to most of the important agricultural parasitic plants mentioned in the previous section. Joel et al. (1995) outline the importance of recognising two phases in the life cycle; the independent life phase and the parasitic life phase. The lifecycle can also be considered as the above soil phase and the below soil phase. Because they are holoparasites, *Orobanche* spp. are tied to the distribution and success of their hosts, which are mainly herbaceous dicotyledonous plants, including agricultural crops and agricultural weeds.

Orobanche spp. are annual plants, and the lifecycle has been summarised conclusively by Parker and Riches (1993). Individual seeds in the soil require a conditioning period of 1 to 2 weeks to become imbibed, and temperature is important during that stage. Different *Orobanche* spp. have different optimal conditioning temperatures, usually between 13 and 28 °C. Following the conditioning process, each individual seed must receive a chemical stimulant from the host root, to alert the seed that a host root is in close proximity (Parker and Riches 1993; Kroschel 2001). These root exudates are specific for each host species and can be in the form of

gaseous or liquid compounds. Once germination has been initiated by the stimulant, a 3 to 4 mm radicle emerges from the seed, oriented in the direction towards the host root (Parker and Riches 1993; Kroschel 2001). On contact with the host root, the radicle adheres to the surface by sticky papillae and penetration is facilitated by separation of the host root cells, caused by enzymatic activity. Subsequently a connective organ, the haustorium, develops between host and parasite, with cells from each species playing part in the junction (Parker and Riches 1993; Kroschel 2001). The haustorium swells and forms a nodule that after one to two weeks differentiates into a tubercle with shoot bud, and eventually a flowering shoot.

After three to four weeks the flowering spike emerges from the soil, and grows to heights between 10 and 100 cm depending on the species. More than one shoot can emerge per plant, depending on the species. The bisexual flowers (Molau 1995) are insect pollinated (Kuijt 1969) and the resulting seeds are produced in capsules with 800 to 10 000 seeds per capsule (Parker and Riches 1993; Molau 1995). This ability to produce large numbers of seed per plant is the forte of these and similar parasitic agricultural weeds. The seeds are approximately 0.3 mm in length, and 0.2 mm in width, and weigh 3 to 6 µg (Parker and Riches 1993). They are held in the capsule until the capsule dries out and the seeds are released. The seeds are dispersed and incorporated into the soil in a seedbank, where they can remain viable for many years, depending on the environmental conditions. *O. ramosa* has been documented as surviving for 13 years in soil (Parker and Riches 1993).

The range of hosts for *O. ramosa* is extremely broad. Dicotyledonous plants are common hosts, including many broadleaved crops of agriculture and horticulture (Table 1.1). As well as the long list of crop, fruit, vegetable, and pasture hosts, many broadleaved weed species can host *O. ramosa*. Thus, in a non-host crop such as wheat, broomrape can still be present on broadleaved weeds that are within the crop. While this situation will not affect the yield or quality of the crop product itself, the marketability will be affected due to contamination with *O. ramosa* seed, and land use in other crop cycles may be jeopardised.

O. ramosa is an unwanted weed for two reasons: under high levels of infestation, crop yield is diminished due to the competition for water and nutrients that the parasite inflicts on the host (as discussed earlier); and the presence of seeds in the harvest material reduces the saleability of crops. The potential total cost of

O. ramosa on Australian markets has been estimated at $243 million (EconSearch 2003). Managers of *O. ramosa* in Australia are currently more concerned about decreased crop values and resultant limited export markets, rather than yield reduction.

Orobanche spp. in Australia

There are three known *Orobanche* spp. in Australia (Barker 1986). *O. cernua* var. *australiana* Loefl. is a rare native that is not known to attack crops. *O. minor* Sm. is a common but minor weed throughout temperate Australia.

In South Australia *O. ramosa* was first collected in 1911 at Glenelg near Adelaide (Barker 1986). It then disappeared from record until it was rediscovered by chance in 1992 on a property near the Murray Mallee town of Bow Hill, although it had probably been present for some time before it was identified (P. Warren pers. comm., R. Carter pers. comm.). As yet the precise origin of the recent introduction is unknown, although there are some genetic clues that link it with the Mediterranean/Middle East ramosa complex (Cooke 2002).

In 1992 *O. ramosa* was listed as a proclaimed plant in South Australia and a quarantine zone was established around the newly discovered infestations in 1999 (Figure 1.1). The quarantine zone was expanded as new infestations were discovered, and in December 2005 it encompassed 193 100 ha (Warren 2006). The total gross infestation area in 2007 was 7048 ha (Panetta and Lawes 2007). Each spring systematic paddock surveys are performed inside the quarantine zone, within the surrounding district, and elsewhere in South Australia and Victoria (Warren 2006). In the 2005 survey season, surveys for *O. ramosa* covered 308 000 ha (P. Warren pers. comm.).

The maintenance of the quarantine zone is controlled by the ‘Code – Control of branched broomrape’ (hereafter referred to as 'the Code') under the South Australian *Fruit and Plant Protection Act 1992* (DWLBC 2003). The Code outlines procedures for inspection and written approval before fodder, machinery, grain and straw, horticultural crops, livestock, or soil can be moved from a paddock within the quarantine zone. Failure to obtain written approval before moving these items can result in a fine of up to $20 000 (DWLBC 2003).

Table 1.1. Confirmed hosts of *O. ramosa*. # indicates from Parker and Riches (1993). $ indicates from Virtue et al. (2002). * indicates remaining hosts that have been confirmed in South Australia by field observation and/or pot trials (Virtue and De Dear unpubl.). ^ indicates that Virtue et al. (2002) found no attachment of South Australian *O. ramosa* to previously confirmed hosts. Plant nomenclature taken from Shepherd et al. (2001).

Plant Type	Plant Variety	Species	Family	Reference
Cropping	Coriander	*Coriandrum sativum* L.	Apiaceae	$
	Safflower	*Carthamus tinctorius* L.	Asteraceae	$
	Sunflower	*Helianthus annuus* L.	Asteraceae	# $
	Canola/Rape	*Brassica napus* L. var *napus*	Brassicaceae	# $
	Hemp	*Cannabis sativa* L.	Cannabaceae	#
	Chickpea	*Cicer arietinum* L.	Fabaceae	# $
	Lentil	*Lens culinaris* Medik.	Fabaceae	#
	Lupin	*Lupinus angustifolius* L.	Fabaceae	$
	Faba/Broad bean	*Vicia faba* L.	Fabaceae	# $
	Common Vetch	*Vicia sativa* L.	Fabaceae	$
	Linseed/Flax/Linola	*Linum usitatissimum* L.	Linaceace	*
	Evening Primrose	*Oenothera stricta* Ledeb. Ex Link ssp. *stricta*	Onagraceae	*
	Tobacco	*Nicotiana tabacum* L.	Solanaceae	#
Fruit & Vegetable	Celery	*Apium* graveolens L.	Apiaceae	#
	Carrot	*Daucus carota* L. ssp. *sativus* (Hoffm.) Arcang.	Apiaceae	# $
	Fennel	*Foeniculum vulgare* Mill.	Apiaceae	#
	Parsnip	*Pastinaca sativa* L.	Apiaceae	#
	Lettuce	*Lactuca sativa* L.	Asteraceae	# $
	Cauliflower	*Brassica oleracea* L. var. *botrytis* L.	Brassicaceae	*
	Cabbage	*Brassica oleracea* L. var. *capitata* L.	Brassicaceae	# $
	Broccoli	*Brassica oleracea* L. var. *italica* Plenck	Brassicaceae	# $
	White Mustard	*Sinapis alba* L.	Brassicaceae	# $
	Watermelon	*Citrullus lanatus* (Thunb.) Matsum. & Nakai	Cucururbitaceae	#
	Rock Melon	*Cucumis melo* L. ssp. *melo*	Cucururbitaceae	#
	Cucumber	*Cucumis sativus* L.	Cucururbitaceae	#
	Marrow/Squash	*Cucurbita pepo* L.	Cucururbitaceae	#
	Groundnut/peanut	*Apios americana* Medik.	Fabaceae	#
	Pea	*Pisum sativum* L.	Fabaceae	# ^
	Onion	*Allium cepa* L.	Liliaceae	# ^
	Capsicum/pepper	*Capsicum annuum* L.	Solanaceae	#
	Tomato	*Lycopersicon esculentum* Mill.	Solanaceae	# $
	Eggplant	*Solanum melongena* L.	Solanaceae	#
	Potato	*Solanum tuberosum* L.	Solanaceae	# $
Pasture	Strand medic	*Medicago littoralis* Rohde ex Loisel.	Fabaceae	*
	Woolly burr medic	*Medicago minima* (L.) Bartal.	Fabaceae	$
	Small burr medic	*Medicago minima* L.	Fabaceae	*
	Annual burr medic	*Medicago polymorpha* L.	Fabaceae	$
	Lucerne	*Medicago sativa* L. ssp. *sativa*	Fabaceae	$
	Disc medic	*Medicago tornata* (L.) Mill.	Fabaceae	$
	Annual white clover	*Trifolium michelianum* Savi	Fabaceae	# $
	White clover	*Trifolium repens* L.	Fabaceae	#
	Shaftal/Persian clover	*Trifolium resupinatum* L.	Fabaceae	# $
	Subterranean clover	*Trifolium subterraneum* L.	Fabaceae	#

Plant Type	Plant Variety	Species	Family	Reference
Weed	Capeweed	*Arctotheca calendula* (L.) Levyns	Asteraceae	*
	Skeleton weed	*Chondrilla juncea* L.	Asteraceae	*
	Cretan weed	*Hedypnois rhagadioloides* (L.) *F.W.Schmidt* ssp. *cretica* (L.) Hayek	Asteraceae	*
	Smooth cat's ear	*Hypochaeris glabra* L.	Asteraceae	*
	Stemless thistle	*Onopordum acaulon* L.	Asteraceae	*
	Common sowthistle	*Sonchus oleraceus* L.	Asteraceae	*
	Tolpis	*Tolpis barbata* (L.) Gaertn.	Asteraceae	*
	Bathurst burr	*Xanthium spinosum* L.	Asteraceae	*
	Corn gromwell/Sheepweed	*Buglossoides arvensis* (L.) I.M.Johnst.	Boraginaceae	*
	Salvation Jane/Paterson's curse	*Echium plantagineum* L.	Boraginaceae	*
	Common heliotrope/potato weed	*Heliotropium europaeum* L.	Boraginaceae	*
	Indian mustard	*Brassica juncea* (L.) Czern.	Brassicaceae	*
	Long fruited/Mediterranean turnip	*Brassica tournefortii* Gouan	Brassicaceae	*
	White mustard	*Sinapis alba* L.	Brassicaceae	*
	Melilotus	*Melilotus* spp.	Papilionaceae	*
	Three-flowered/Cutleaf nightshade	*Solanum triflorum* Nutt.	Solanaceae	*
Native	Variable daisy	*Brachyscome ciliaris* (Labill.) Less.	Asteraceae	*
	Cut-leaf daisy	*Brachyscome multifida* (forms)	Asteraceae	*
	Golden everlasting	*Bracteantha bracteate* (Vent.) Anderb. & Haegi	Asteraceae	*
	Yellow buttons/Common everlasting	*Chrysocephalum apiculatum* (Labill.) Steetz	Asteraceae	*
	Poached egg daisy	*Myriocephalus stuartii* (F.Muell. & Sond. Ex Sond.) Benth.	Asteraceae	*
	Variable groundsel	*Senecio lautus* G.Forst ex Willd.	Asteraceae	*
	Sticky goodenia	*Goodenia varia* R.Br.	Goodeniaceae	*
	Creeping boobialla	*Myoporum parvifolium* R.Br.	Myporaceae	*
	Sturt's desert pea	*Swainsona formosa* (G.Don) Joy Thomps	Papilionaceae	*
	Sweet apple berry	*Billardiera cymosa* F.Muell.	Pittosporaceae	*
Exotic Garden	Sweet pea	*Lathyrus odoratus* L.	Fabaceae	*
	Nasturtium	*Tropaeolum majus* L.	Tropaeolaceae	*

The climate is Mediterranean with warm to hot summers and cool winters. There is a weak winter rainfall dominance and summer rains are associated with thunderstorms (average 10 thunder days per year) (Anon 1995). Specific climate data for the area are shown in Table 1.2.

Current management practices (global and Australia)

Management techniques for *Orobanche* spp. vary throughout the world, depending on distribution, level of infestation, effect on yield, and economic factors. In Australia most infestations are found on broadleaved weeds in non-host crops, and thus the effect on yield is nonexistent but saleability is severely impacted. The Australian infestation has attracted funding from both state and federal governments in the order of tens of millions of dollars. Financial grants are available for infested farms to assist with the cost of farm management activities and chemicals, and other services are available including farming advice and extension, and financial and social counselling.

The predominant management tools for infestations in Australia are fumigation (to destroy seedbank) and herbicides (to control hosts). The main chemicals used for fumigation are methyl bromide, dazomet, and a pine-oil based herbicide used as a soil drench (Warren 2006).

In European and Middle Eastern countries the hosts of *Orobanche* spp. are predominantly vegetable crops, which can be impacted by massive reductions in yield. In poorer African countries where expensive chemical tools are not available, the main management tool is to hand-pull emerged adult plants, a labour-intensive and inexact weed management practice.

Dispersal of plants

Ridley (1930) defined dispersal or dissemination as “the methods by which the plant is diffused or transported from place to place”. The dispersal strategies of plants maximise the chance of their offspring dispersing to good locations (Macdonald and Smith 1990). The dispersal units of plants, the diaspores, can be in the form of spores, seeds and fruits, as well as whole plants or parts of plants (Ridley 1930).

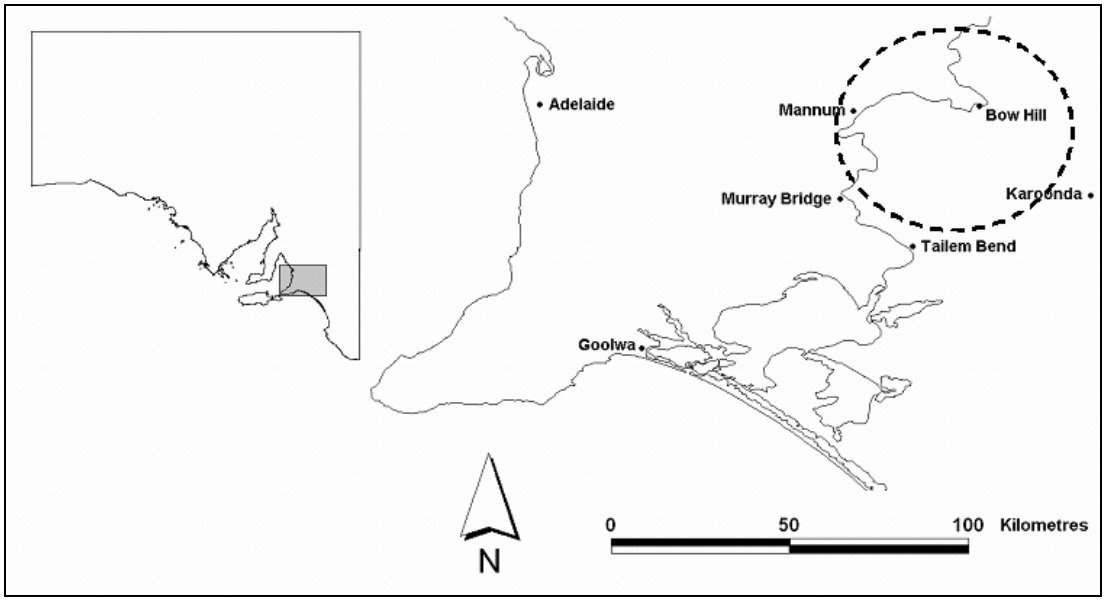

Figure 1.1. Approximate location of the *O. ramosa* quarantine zone in South Australia.

Table 1.2. Climate data for Murray Bridge, South Australia (35.12° S, 139.26° E). Data are averages for years 1971 to 2000 (Australian Bureau of Meteorology 2009).

NOTE:
This table is included on page 9
of the print copy of the thesis held in
the University of Adelaide Library.

In seed dispersal theory, there are two stages of dispersal. Phase one (Chambers and MacMahon 1994), or local dispersal (Cousens and Mortimer 1995), is considered to be the initial separation of the seed from the parent plant and the seed settling onto a surface. Phase two (Chambers and MacMahon 1994), or geographic dispersal (Cousens and Mortimer 1995), is the subsequent movement of the seed from that first surface to another location.

The scales at which dispersal can be studied are: plant level (the initial release of seeds from the parent plant); paddock level (the spread of seeds and therefore plants in a defined paddock); landscape level (the population in a given area or ecosystem); and global level (the spread of a species around the world). Each level has certain considerations, and some dispersal mechanisms may fit into more than one scale level.

Seeds can be self-dispersed or aided by external dispersal mechanisms (Cousens and Mortimer 1995; Cousens et al. 2008). Self-dispersal is a passive process, where the parent plant simply loses its attachment of the seed, and gravity drives the seed to land on a surface (Burrows 1986). The mechanisms for aided dispersal can be divided into two groups: biotic and abiotic. Biotic factors are animals and humans.

The transport of seeds by animals can be both internal and external (Van der Pijl 1969; Cousens and Mortimer 1995; Cousens et al. 2008). Internal transport can occur by animals eating the stem, fruit or seeds of a plant, and the seeds moving through the digestive system to be defecated at possibly another location. External transport can occur by seeds adhering to fur or feathers of the animal, or by sticking with mud and soil to feet and legs. Darwin (1875) suggested that it was possible for viable seeds to be carried in clods of soil on the feet and legs of migratory birds. Such dispersal would occur on a global scale, but external animal transport could also occur on a local scale.

Cousens and Mortimer (1995) noted that human interaction is usually of high importance for the movement of weed seeds on a landscape or global scale. This human interaction is necessary for weed populations to negotiate the highly fragmented agricultural environment (Opdam 1990). Human transport becomes unique to that of other animals through the use of machinery; farming operations

such as tilling and harvesting are well documented weed seed dispersal activities (Hodgson and Grime 1990; Mayer et al. 1998; Cousens et al. 2008).

Abiotic factors are water (rain and flooding) and wind. Wind is known to transport the diaspores of many plants. Important factors in wind dispersal are the height of release, wind velocity acting on seeds, seed morphology and physical characteristics of the surrounding environment (Van der Pijl 1969; Burrows 1986; Cousens and Mortimer 1995).

Some seed dispersal literature use specific nomenclature associated with the individual vectors involved (e.g. anemochory for dispersal by wind, zoochory for dispersal by animals, etc.) (Van der Pijl 1969; Cousens and Mortimer 1995; Vibrans 1999; Cosyns et al. 2005a), however here I use the terminology of Cousens et al. (2008) and avoid the 'chory' terminology.

Cain et al. (2000) defined long distance seed dispersal as distances over 100 m. They emphasised that it is important to investigate the furthest dispersed seeds, even though it is difficult to do so. Cousens and Mortimer (1995) also acknowledged that estimates of maximum dispersal distance will be inaccurate and that it would be difficult to detect seeds at the tail-end of a species' dispersal distance frequency distribution curve. For wind dispersed seeds, the true maximum dispersal distance may be very large in extreme weather conditions (Cousens and Mortimer 1995).

During a given dispersal period, the dispersal vectors acting on a given group of seeds will vary in strength and direction, depending on environmental and land use factors (Cousens et al. 2008).

Dispersal of small seeded plants

The morphology or syndrome of a seed may indicate its primary mode of dispersal (Ridley 1930; Van der Pijl 1969; Howe and Smallwood 1982). In the case of *Orobanche* spp., the small seed size indicates that wind may be a dispersal mechanism (Howe and Smallwood 1982), although elaborate wings and plumes are not present. Kuijt (1969) suggested that cavities on a seed surface, such as those seen in *Orobanche* spp., may assist to create aerodynamic lift. Dispersal via mud on animals may also be possible, as is the case with small-seeded aquatic plants (Cousens et al. 2008). Seed morphology alone should not be the sole determinate of seed dispersal vectors; syndromes cannot be relied upon outright, and field-based

investigations of the process should also be made (Howe and Smallwood 1982). Recent authors (Tackenberg et al. 2003a; Mouissie et al. 2005; Tackenberg et al. 2006) have found that syndromes do not necessarily indicate seed dispersal method. Similarly, Cousens and Mortimer (1995) suggested that some physical features that researchers assume may aid dispersal, might have evolved for another function. Hence, the physical form of a seed may indicate the more evolutionarily important features and not indicate dispersal attributes at all. Despite these cautions, some authors (e.g. Vibrans 1999) do make assumptions on dispersal method based solely on morphology and disregard certain vectors because of the lack of seed appendages.

Hughes et al. (1994) determined that dispersal mechanisms for small-seeded species (< 0.1 mg) tend to be classed as 'unassisted', although their model also predicted wind as a likely dispersal mode. There are few papers investigating seed dispersal mechanisms for small-seeded agricultural parasitic weeds (*Orobanche*, *Striga*, *Alectra*, etc.). One of the few papers published in this area is by Berner et al. (1994) who investigated seed dispersal mechanisms for *S. hermonthica* in Africa. They found that the main seed dispersal vector was via transport of harvested crop seeds across the landscape, while wind and cattle dung were found to disperse seeds to a lesser extent. Berner et al. (1994) highlighted the importance of investigating the possibility of external animal transport although they did not investigate this mechanism themselves.

For *Orobanche* spp. there are only three papers documenting seed dispersal. Jacobsohn et al. (1987) documented the time taken for seed of *O. aegyptica*, *O. cernua*, and *O. crenata* to pass through sheep, and determined that viable seeds did pass through sheep gut for up to four days. Castejón-Muñoz et al. (1991) documented *O. cernua* seed presence in sunflower achenes, indicating wind dispersal. And Mesa-García et al. (1986) examined *O. crenata* seed distribution in a paddock, and hypothesised about seed dispersal mechanisms, deeming mechanical tillage and harvesting to be the most important vector.

Seed dispersal generally is a well-studied field, particularly for weed species important to primary production. Regrettably, authors often fail to describe the size and/or morphology of the seeds being studied (e.g. Piggin 1978; Johnson and West 1988; Willson 1993; Vibrans 1999; Machon et al. 2003; Cosyns et al. 2005b; Dauer

et al. 2006; Dauer et al. 2007; Will et al. 2007), which leads to uncertainty when assessing methods to quantify dispersal (e.g. seed trap design).

For dust propagules, reduced seed size comes at the expense of seed mass and the provision of resources for the newly germinated seedling. Dust propagules are often seen in parasites or saprophytes (myco-heterotrophs), plants that depend on another organism for nutrition, thus allowing a reduction in seed size and resources (Van Rheede van Oudtshoorn and Van Rooyen 1999). The classic small seeded Orchidaceae counter this lack of resources by forming relationships with mycorrhizal fungi. Similarly, *Orobanche* spp. form relationships with host plants.

Considering seed size and number, parallels can be drawn between *Orobanche* spp. seeds and other 'dust' propagules, particularly orchids. Arditti and Ghani (2000) documented the seed size and fecundity of over 20 genera of orchids, ranging from 0.05 to 6.0 mm in length, 0.31 to 24 µg in weight, and up to 4 000 000 seeds per capsule. These are on a similar scale to the seeds of *Orobanche* spp. which are approximately 0.3 mm in length, and 0.2 mm in width, weigh 3 to 6 µg, with 800 to 10 000 seeds per capsule (Parker and Riches 1993; Molau 1995).

Thesis overview

Despite being the subject of a quarantine zone, very little is known about the dispersal vectors that may be operating for the South Australian population of *O. ramosa*. As a consequence, I investigated the role of two possible vectors: sheep and wind.

Sheep were chosen for investigation as they are the predominant grazing livestock in the area. Chapter two investigates sheep as a dispersal vector, both internally via the gut and externally via the wool and feet.

Seed morphology suggests that wind may be an important dispersal vector for *O. ramosa*. Chapter three outlines a preliminary survey of wind dispersal in a field setting, while chapter four describes experiments conducted using a field-based wind tunnel.

The final chapter is a review of the two vectors investigated, and their relevance to the quarantine and eradication program for *O. ramosa* in South Australia, and elsewhere in the world.

Chapter 2. Sheep as seed dispersal vectors for *Orobanche ramosa*

This chapter reports on three experiments to investigate external and internal sheep dispersal for *O. ramosa* seed. It also provides evidence of the efficacy of a DNA probe for *O. ramosa* that was used to detect seed in soil and manure samples. The experiments were designed to determine whether *O. ramosa* seeds become attached to wool in the field, the length of time that seeds are retained in the wool, and the amount of time that seeds take to pass through the sheep gut. The results will increase knowledge of *O. ramosa* dispersal and assist in the management of sheep within *O. ramosa* infested regions.

Introduction

Orobanche ramosa L. (branched broomrape) is an obligate parasite of, mostly, dicotyledonous plants, including many broadleaved crops of agriculture and horticulture (Parker and Riches 1993; Riches and Parker 1995). It has been present in the Western Murray-Mallee of South Australia since at least the early nineteen-nineties and is the subject of extensive quarantine procedures to prevent its spread.

Sheep husbandry is one of the agricultural activities within the branched broomrape quarantine zone. The Code (DWLBC 2003) dictates the quarantine procedures that livestock are subject to before they can be removed from the quarantine zone. Animals must spend 48 h in a clean holding yard, then be kept for 15 d in a clean paddock before written approval is given for removal. An exception is where animals are sent straight to slaughter, in which case no holding periods apply, but the vehicle needs to undergo approved wash down procedures (DWLBC 2003).

The dispersal of seeds by transport on the external surface of animals (Van der Pijl 1969; Cousens and Mortimer 1995; Cousens et al. 2008), is determined by two factors: the attachment of seeds on the animal, and the period of time they are retained (and thus how far the animal moves before the seed is detached) (Fischer et al. 1996; Mouissie et al. 2005; Will et al. 2007). As described in the introductory chapter, the size and morphology of a seed may in part determine how it is dispersed, including how it is attached and retained on an animal (Ridley 1930; Van der Pijl 1969; Howe and Smallwood 1982; Römermann et al. 2005) or how it survives an

animal's digestive system (Couvreur et al. 2005a). Other factors include the height of seed capsule on the plant, fur type (for mammalian vectors), and the structure of the surrounding vegetation (Mouissie et al. 2005; Römermann et al. 2005).

Studies investigating external animal transport often focus only on seeds with hooks, barbs, panicles or prickles that adhere to fur and hair, rather than the dust-like seeds of *O. ramosa.* Gardener et al. (2003) assessed the dispersal of the seeds of *Nassella neesiana* (Trin. & Rupr.) Barkworth by sheep, and discovered that the seeds attached readily, and remained in wool for up to five months; although half-life was 7.5 days (Gardener et al. 2003). Five months is ample time for the animal to disperse seeds either locally within a paddock or farm, or across the landscape if they are moved for sale.

Characteristics of *O. ramosa* plants and seeds indicate that transport by a range of animals is possible. A persistent seedbank can be associated with external transport (Couvreur et al. 2005a) and also indicates that seeds are likely to survive gut passage (Cosyns et al. 2005b). Density of seeds is correlated with attachment (Mouissie et al. 2005), and small seed size has been found to correspond with longer retention times in wool and fur (Couvreur et al. 2004; Tackenberg et al. 2006), and a high probability of external (Hughes et al. 1994; Fischer et al. 1996; Couvreur et al. 2005a; Couvreur et al. 2005b; Tackenberg et al. 2006; Will et al. 2007) and internal transport (Couvreur et al. 2005a).

Based on studies with other plants, sheep are a likely vector for *O. ramosa* because seeds not showing the typical attachment morphology can still be transported by sheep wool (Fischer et al. 1996; Couvreur et al. 2004; Mouissie et al. 2005). Sheep wool can also carry a wide variety of seed morphology types (Fischer et al. 1996), and has been shown to carry more seeds than cattle and deer hair (Couvreur et al. 2004; Will et al. 2007). Furthermore, seeds are more likely to survive gut passage through sheep, compared with rabbit, cattle, horse and donkey (Cosyns et al. 2005b).

Römermann et al. (2005) modelled the attachment potential of seeds to sheep wool and cattle hair, using 166 plant species with many different seed morphologies; flat appendages, elongated appendages, balloon structures, hooks, and plain seeds. They concluded that for sheep wool, smaller seeds with hooks or elongations were more likely to attach, while in cattle hair, seed mass alone was predictive of seed

attachment, with smaller seeds attaching more readily than larger ones (Römermann et al. 2005). Of the 166 species investigated, 10 were of a comparable size (< 90 μg) to *O. ramosa*, and also lacked appendages (Römermann et al. 2005). Attachment potential for these 10 species was on average 90.0% for sheep wool and 50.9% for cattle hair. If a seed mass of 0.0045 mg, typical of *O. ramosa* seed, is entered into the model of Römermann et al. (2005), it predicts attachment potential of 100% for sheep wool and 64.3% for cattle hair.

Transport of seeds by animal ingestion is another possible vector for *O. ramosa* dispersal. Animal gut passage time has been determined for a range of plant and animal species (Piggin 1978; Gardener et al. 1993; Heap and Honan 1993; Gardener et al. 2003; Cosyns et al. 2005a; Cosyns et al. 2005b; Michael et al. 2006) with a classic pattern emerging: a peak in seed excretion within the first few days, and a tail of fewer and fewer seeds excreted over time (i.e. right-skewed normal distribution). While dispersal via ingestion for weeds generally is relatively well studied, only one paper exists for the *Orobanche* genus. Jacobsohn et al. (1987) investigated the time taken for seed of *O. aegyptica*, *O. cernua*, and *O. crenata* to pass through sheep. The study used rumen-cannulated rams (i.e. sheep with tubes surgically inserted into the rumen of the gut that are open to the outside environment) allowing for direct addition of seeds into the rumen. This bypassed the mouth and enzymatic action of the upper digestive tract, and thus may not be a realistic representation of what occurs when an animal actually consumes a seed in the field. To detect the presence of viable seeds in manure of the experimental sheep, host pot-tests were used (Jacobsohn et al. 1987). Dried and ground manure was added into the soil of pots where hosts (sunflower and tomato) were grown. The host root exudate stimulated germination and the number of subsequent root attachments were counted. This method did not allow for differentiation between seeds not surviving gut passage, and seeds present but not successfully undergoing germination, attachment and emergence. Jacobsohn et al. (1987) themselves point out that using host pot tests as a bioassay for the presence of *Orobanche* spp. seeds is imprecise and subject to variability due to environmental conditions. This method is actually a measure of seeds passing through only the lower intestine and subsequent germination, attachment and emergence, rather than true seed passage through the gut. Jacobsohn

et al. (1987) found a sheep gut passage pattern with a peak in germinating seeds at day 2 and zero viable seeds at day 4 for *Orobanche* spp. seeds in their experiment.

Methods

These experiments were approved by the University of Adelaide animal ethics committee (science); project number S-101-2004.

Field site

The study site was located 71 km east of Adelaide, in the Western Murray-Mallee of South Australia (34.91 °S, 139.40 °E), on a privately owned property within the branched broomrape quarantine zone.

Experiments were conducted in February 2005. No rain fell at the site during the experiments.

Animals

Twenty-four, unshorn, 8-month-old Border Leicester/Merino cross sheep (*Ovis aries* L.) were purchased from outside the quarantine zone and kept on a private farm outside the quarantine zone near Bordertown, South Australia, (36.30 ºS, 140.77 ºE) for two weeks. During that time the sheep were housed in a large shed and acclimatized to the food rations that were to be used during the experiment. It was assumed that sheep were free of *O. ramosa* on arrival at the field site. Each sheep was weighed on arrival at the field site, and again after the experiment had concluded. At the conclusion of the experiment, wool samples were sent to a wool-testing laboratory (Classings Limited Murray Bridge, South Australia) to assess wool fibre characteristics. Appendix 1 reports the body weight and wool fibre data. At the conclusion of the experiment, the animals were transported to an abattoir for slaughter as permitted under the Code (DWLBC 2003).

Feed and water

Feed and water regimes were identical during the acclimatisation period and the experimental period. Sheep were fed daily with 1.5 kg of pelleted ration (‘Ewe and Lamb Nuts’ Laucke Mills, Daveyston South Australia, protein 15% minimum, fat 2% minimum, fibre 15% maximum) and a handful of lucerne chaff per animal. Each pen was fitted with self-filling water bowls providing fresh water. Animals were tended to daily.

DNA probe for detection of O. ramosa *seeds*

The South Australian Research and Development Institute (SARDI) Diagnostics Group developed a DNA probe by real-time quantitative polymerase chain reaction (PCR) to detect *O. ramosa* DNA in samples. Originally it was devised to detect *O. ramosa* DNA in soil samples, with an accuracy of 1 seed (equivalent DNA) in 200 g of soil (A. McKay pers. comm.). The probe had previously been shown capable of assessing *O. ramosa* DNA in sheep manure rather than just in soil (Secomb 2003) although further calibration was recommended by the SARDI diagnostics group (A. McKay pers. comm.).

DNA probe calibration for *O. ramosa* seeds in sheep manure and soil

Spiked samples were produced by adding known numbers of *O. ramosa* seed to clean samples of soil or dried sheep manure. *O. ramosa*-free soil was weighed into 100 g samples (to the nearest 0.01 g). *O. ramosa* seeds were counted out under a dissecting microscope. Zero, 5 and 50 seeds were added to soil samples, with 10 replicates of each number of seeds. Samples were sealed in plastic bags and labelled.

O. ramosa-free manure was collected from outside the quarantine zone (Roseworthy campus of the University of Adelaide, South Australia, 34.528 ºS, 138.686 ºE), where different sheep were fed a similar diet to the experimental sheep. Manure was air dried for several days in a well-ventilated shed. Manure was weighed into 100 g samples (to the nearest 0.01 g). Seeds were counted out under a dissecting microscope. Individual pellets were pierced with sharp tweezers and set numbers of seeds were inserted into the pellets with a piece of fine wire. The hole was then closed over with manure material. These spiked pellets were returned to the 100 g samples. Five replicates of zero, 5 and 50 seeds were prepared. Samples were sealed in plastic bags and labelled. To assist in the physical grinding of the manure pellets, 100 g of *O. ramosa*-free soil was added to each manure sample during processing. Extra soil was not added to the spiked soil samples.

Testing the samples for *O. ramosa* DNA was conducted by the SARDI Diagnostics Group. Methods for the DNA extraction and the quantitative PCR are the intellectual property of SARDI and have not been provided to the author. The analyses were conducted on a fee-for-service basis.

Analysis

A two-factor analysis of variance (2-way ANOVA) was used to assess differences between seed estimates at the three spiked levels on the two media types. Data were analysed (JMP IN Versions 3.2.6 and 8) with $\alpha = 0.05$. Means were compared with Tukey-Kramer honestly significant difference test (Tukey's HSD).

Experiment 1: Attachment of *O. ramosa* seeds to the external surface of sheep

This experiment created an artificially enhanced seedbank, and then used pens to ensure sheep remained in contact with the seedbank for up to 7 days. It is assumed that the sheep pens restricted the natural movement and behaviour of the sheep somewhat, but it was important to restrict the sheep to the area of ground with the enhanced seed bank in order for attachment to be detected.

Pens

Sheep pens were constructed using transportable gates and fences. Three, 3 x 3 m pens were set up with a 1 m gap between them (Figure 2.1). Each pen had a shade tent erected over it, three food troughs and a water bowl. These pens held three sheep each, giving a total of nine sheep for the indirect attachment experiment. The sheep were in direct contact with the soil, which had been prepared as described below.

Wool marking

Before the experiment commenced, sheep were marked with wool dye ('Heiniger Scourable Sheepmarker' Heiniger Australia Pty. Ltd., Bibra Lake, Western Australia), both to identify individual sheep and to mark areas where they would eventually be clipped. Sheep were restrained by hand and a 10 cm diameter, stainless steel ring was placed against the wool and traced around with wool dye on each of the two locations on the thigh and the belly (Figure 2.2).

Soil coring and preparation

The *O. ramosa* seedbank of the topsoil was assessed three times. The initial, natural seedbank was assessed by soil cores (as described in the following paragraph) on 17 February, before the experiment began (day –1). On 18 February (day 0), seeds were added directly to the soil as described below, and soil cores taken again to quantify the enhanced seedbank. The sheep were introduced to the pens immediately

after the seed addition on 18 February. After the last sheep was sampled on 25 February (day 7), a third and final set of soil cores were taken to estimate the remaining seedbank population.

Seedbank populations were quantified by soil cores and DNA testing. Thirty soil cores (5 cm diameter x 1.5 cm depth) were taken from each pen using a metal cylinder and trowel. The core samples for each pen were pooled, weighed to the nearest gram and sieved, discarding particles greater than 300 μm to remove rocks and plant material, and weighed again. These samples were sub-sampled from a homogeneous mix in order to obtain samples of a suitable size for DNA testing (i.e. less than 400 g). Samples were weighed again, bagged and labelled, and sent for DNA testing by the SARDI Diagnostics Group as described previously.

The seedbank was enhanced by the addition of extra *O. ramosa* seeds that had been collected the previous season by harvesting mature plants from the field site where they were parasitising *Hedypnois cretica*. *O. ramosa* seed pods were ground and sifted and seeds retained. Approximately 9.0×10^4 *O. ramosa* seeds were measured by volume and mixed with 10 ml of sand. The sand/seed mix was divided into nine parts and each part was broadcast uniformly via a stainless steel shaker over each 1 m^2 section of pen (i.e. the equivalent of 10 000 seeds m^{-2} were added to each pen).

Wool clipping

One sheep in each pen was sampled on 21, 23 and 25 February (Figure 2.3); day 3, 5, and 7. Sheep were caught and restrained by hand. The two pre-marked areas, one on the thigh and one on the belly of each sheep, were carefully clipped using electric hair clippers (Tiffany, GAF Electrical Pty. Ltd. Altona North, Victoria). Samples were stored in sealed plastic bags for transport to the laboratory. In the laboratory, samples were carefully shaken and teased apart and the seeds were collected. Wool samples were individually washed with water and detergent, agitated and rinsed in a series of clean water baths. The material that washed out was sieved and the seeds caught were counted as part of each individual wool sample. Several wool samples were thoroughly checked after washing to ensure the washing process was suitably removing seeds. No residual seeds were found caught in the wool after washing, and all following wool samples were simply washed to remove seeds without further

checking of the wool. The collected seeds were tested to determine germination rates (see below).

Foot cleaning

On the day of sampling for each sheep, one foot was brushed down with a small paintbrush to dislodge soil and seeds, the toenails were clipped, and the hair on the foot/hocks were clipped and collected. These foot samples were visually checked for *O. ramosa* seeds in the laboratory.

Seed germination testing

Seed germination ability was assessed following methodology outlined by Kroschel (2001). Seeds were collected from the wool samples under microscope and a subsample was reserved for germination testing. Seeds were surface sterilized with 2% NaOCl for 5 minutes, rinsed, then dried at room temperature. Seeds were stored in a dark room and incubated at 20 °C for 14 days in 250 µl of 0.02% Thiram in reverse osmosis water. Samples were incubated for 7 days at 20 °C in 250 µl of GR24 (a synthetic germination stimulant). After 7 days, germination was assessed by the presence of emerged radicles, returned to incubation for 7 further days, and germination assessed again.

Analysis

A two-factor analysis of variance (2-way ANOVA) was used to assess differences between seed counts on different days and body parts. Data were analysed (JMP IN Versions 3.2.6 and 8) with $\alpha = 0.05$. Means were compared with Tukey-Kramer honestly significant difference test (Tukey's HSD).

Experiment 2: Retention of *O. ramosa* seeds on the external surface of sheep

This experiment documented the retention of seeds once deliberately added to the sheep wool. Sheep were kept in individual pens, raised off the ground with trays underneath, in order to catch all material falling from the sheep over 7 days. It is assumed that the pens restricted the natural movement and behaviour of the sheep, probably more than for the larger pens used in experiment 1, thus reducing the likelihood of the seeds being dislodged from the wool. However, the use of the pens allowed for material falling from the sheep to be collected every 24 hours thus illustrating the patterns of retention and detachment.

Pens and trays

Two rows of six, 1.5 x 1.0 m pens were erected using transportable gates, corrugated iron, wooden planks and metal grills for the floors (Figures 2.4 and 2.5). Three shade tents were set up over each row of six pens, providing total cover. Each pen held one sheep, giving a total of 12 sheep in the experiment.

Wooden trays measuring 1.5 x 0.9 m were placed under each pen, and calico fabric, cut to size (1.5 x 1.0 m) was stapled to each tray (Figure 2.5). The fabric collected manure, soil, spilt food and any other material that fell through the metal grill floors of each pen.

Wool marking, seed addition and wool sampling

On 18 February (day 0), each sheep was marked on the thigh and belly with wool dye as described in Experiment 1 above. Before the metal ring was removed, approximately 1.0 x 10^4 *O. ramosa* seeds, measured by volume and mixed in 10 ml of sand, were sprinkled onto the wool at each of two marked sites on the body. The ring was then removed and the sheep released into its pen.

Three sheep were sampled once on 19, 21, 23 and 25 February; day 1, 3, 5, and 7 (Figure 2.6). Wool samples were taken and seeds removed and counted as described in Experiment 1.

Calico sheets

Seeds shed from the sheep were collected using the trays and calico sheets. Every 24-h the soiled sheet was replaced with a clean sheet, and the soiled sheet was bundled up to retain its contents, dried and stored in a plastic bag in a freezer until processed. Several days later, sheets and their contents were thawed and air-dried under cover. Following air-drying, the manure was transferred into aluminium baking trays and dried until weight-loss ceased. Once dry, each sample was sieved with a 1000 μm sieve to remove surface dust (which was retained) and sub-sampled to produce manure samples of 100 g each. These were transported to the SARDI laboratory where they were combined with 100 g of *O. ramosa*-free soil. *O. ramosa* seed content was quantified for each sample using the DNA technique described above.

The calico sheets were hung vertically on a rack in a sink, beneath which was a series of sieves (1000, 500, and 150 μm) and a 50-L-capacity plastic crate. Water was applied under pressure via a pump and carefully washed over the sheet in a downward motion. Each sheet was rinsed twice. The sheet was then removed and the rack and sink were rinsed out with water. Material in the 1000 and 500 μm sieves was discarded while material in the 150 μm sieve was transferred onto a small piece of wire mesh and air-dried. Once dried, the material was combined with the dust from the manure sample and transported to the SARDI laboratory for DNA analysis as described above.

Analysis

A two-factor analysis of variance (2-way ANOVA) was used to assess differences between seed counts on different days and body parts. Data were natural log transformed to meet assumptions of normality and homoscedasticity. Data were analysed (JMP IN Versions 3.2.6 and 8) with $\alpha = 0.05$. Means were compared with Tukey-Kramer honestly significant difference test (Tukey's HSD).

Experiment 3: *O. ramosa* seed through sheep gut

Eight sheep from Experiment 2 were used concurrently for Experiment 3. On 18 February (day 0), approximately 1.0×10^5 *O. ramosa* seeds were measured by volume and mixed with approximately 50 ml of water and drenched via a large, plastic plunger down the throat of each sheep.

The same wooden drawers and calico sheets outlined for the previous experiment allowed accurate manure collection every 24-h (Figure 2.7). Four sheep (drench group 1) had manure collected daily over 7 days, and the other four sheep (drench group 2) had collections over 9 days. Sheets were bundled up and stored in a freezer, then thawed and dried as outlined for the previous experiment. Each sample was sieved with a 1000 μm sieve to remove dust, sub-sampled to 100 g and then transported to the SARDI laboratory where they were combined with 100 g of *O. ramosa*-free soil and tested for *O. ramosa* DNA by the SARDI Diagnostics group as outlined above.

The germination viability of the seeds that had passed through the gut was not tested.

Analysis

The difference in total seed count between days (gut passage time) was analysed using a one-factor analysis of variance (1-way ANOVA). Data were analysed (JMP IN Versions 3.2.6 and 8) after log x+1 transformation and with $\alpha = 0.05$. Means were compared with Tukey-Kramer honestly significant difference test (Tukey's HSD). Data were log (x+1) transformed to meet assumptions of normality and homoscedasticity.

Results

DNA probe calibration for *O. ramosa* seeds in sheep manure and soil

A strong linear relationship was found between the number of *O. ramosa* seeds added to spiked samples and the number of seeds detected using the DNA probe, although approximately twice the number of seeds were detected in soil samples compared with manure samples: for manure samples $y = 1.05x$ and $r^2 = 0.99$; for soil samples $y = 2.2x$, $r^2 = 0.99$ (Table 2.1).

The reason for this overestimate of seed number in soil samples compared with manure is not known. Despite these variations, the DNA probe was considered sufficient to allow the following experiments to proceed.

Experiment 1: Attachment of *O. ramosa* seeds to the external surface of sheep

Analysis of soil cores showed that samples taken from the topsoil had *O. ramosa* seeds present, however there was no significant difference between the sampling dates at the $\alpha = 0.05$ level (1-way ANOVA, $F_{2,9} = 1.08$, $P = 0.40$) (Figure 2.8).

Approximately 10 000 seeds m^{-2} were added to the sheep pens. However, on average, only 2028 seeds m^{-2} were detected in the second sampling (Figure 2.8).

Figure 2.1. Pens and nine sheep used for 'attachment experiment' (Experiment 1) on site at Bowhill Road, Mannum South Australia, February 2005. Three pens each held three sheep. Food, water and shade were provided as shown. Sheep were in direct contact with the topsoil seedbank which was enhanced by the addition of extra *O. ramosa* seed to supplement the natural seedbank, before the sheep were introduced.

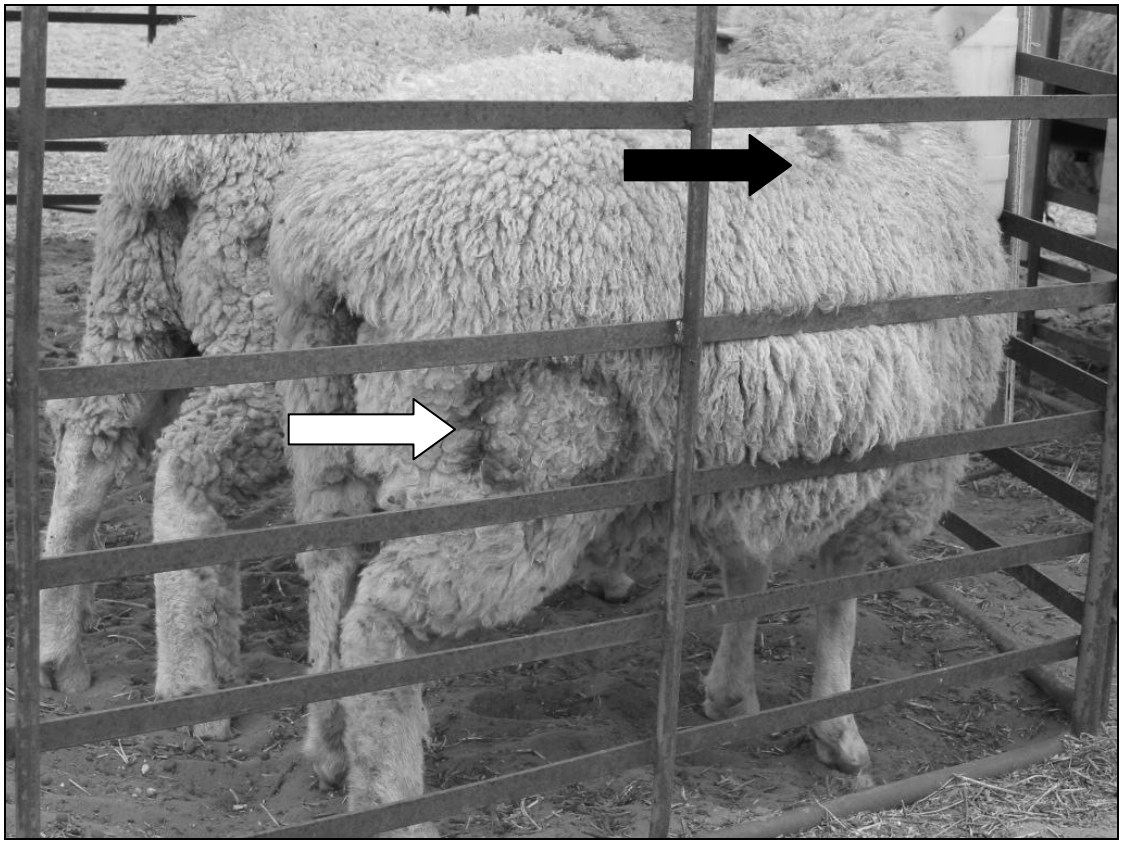

Figure 2.2. Sheep during 'attachment experiment' (Experiment 1), showing wool dye markings for identification (black arrow) and to mark out the experimental clipping area (white arrow). In this picture only the thigh sampling area is visible, but a belly area was also marked by a paint circle. The paint circle indicates the area which would be targeted for clipping after the sheep had been exposed to the seedbank containing *O. ramosa* seeds for up to 7 days.

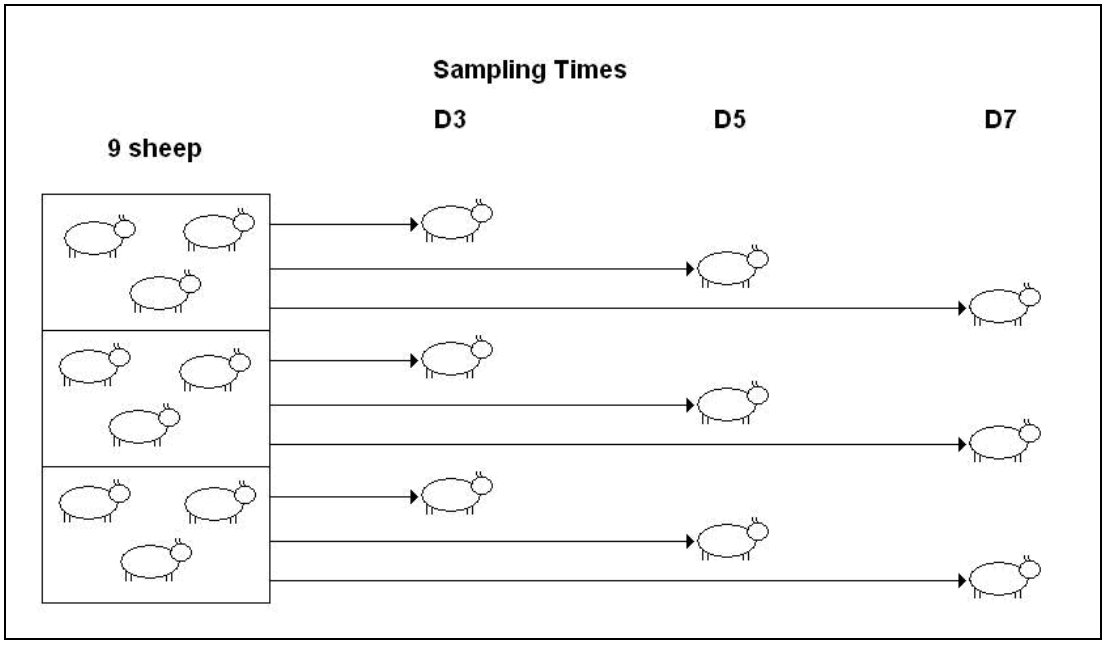

Figure 2.3. Experimental design for 'attachment experiment' (Experiment 1). From day 0 sheep were in direct contact with the seedbank containing *O. ramosa* seeds and one sheep from each pen was sampled on day 3, 5 and 7. Sampling involved clipping pre-determined areas of wool from the thigh and belly of each sheep and counting the numbers of *O. ramosa* seed found in each wool sample.

Figure 2.4. Pens and 12 sheep used for ‘retention experiment’ and ‘gut-passage experiment’ (Experiments 2 & 3). Sheep were raised above the ground in individual pens, so as not to come into contact with the soil. Wooden drawers lined with calico underneath each pen allowed for collection of manure and other material falling from each sheep (see next two figures for more detail).

Figure 2.5. Floors and draws for ‘retention experiment’ and ‘gut-passage experiment’ (Experiments 2 & 3). The metal grill allowed for manure, soil and seeds to fall through the floor, and the wooden drawers under each pen allowed for collection of the material. The drawers were lined with calico cloth that was removed and replaced every 24-h.

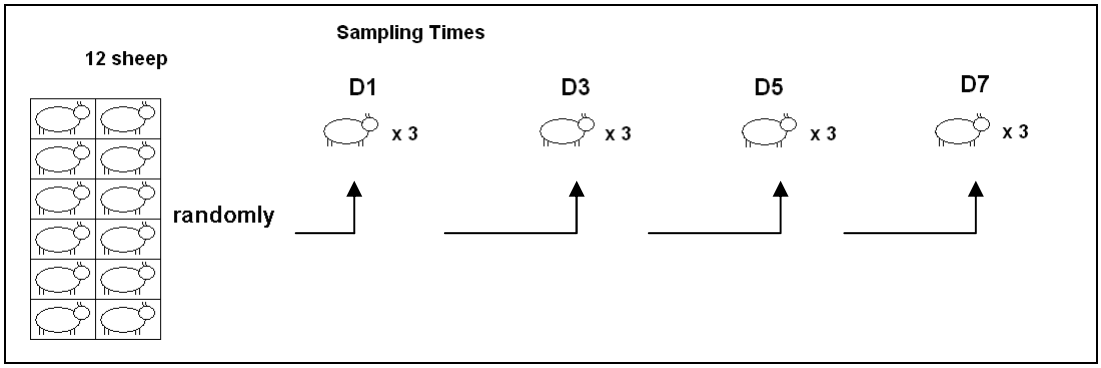

Figure 2.6. Experimental design for 'retention experiment' (Experiment 2). Seeds were added to all sheep on belly and thigh areas on day 0. Calico sheets under pens were replaced every 24-h to collect all material dropping off the sheep. Three sheep were sampled by clipping the marked wool areas on days 1, 3, 5 or 7.

Figure 2.7. Wooden drawers covered in calico sheets which were used for ‘retention experiment’ and ‘gut-passage experiment’ (Experiments 2 & 3). Sheets were stapled to the wooden drawers with metal staples at the edges. These collected manure and dust which fell through the grills on the floors of the sheep pens. Sheets were removed by pulling staples out, bundling up sheets, and replacing with new ones every 24-h during days 0-9.

After spending 3, 5 and 7 days on *O. ramosa* infested seedbank, seeds were found to have attached to samples taken from sheep. However, there was no statistically significant difference in the mean number of seeds between body parts (2-way ANOVA, $F_{3,24} = 3.00$, $P = 0.07$) at $\alpha = 0.05$ level (Table 2.2). Most seeds were found in toenail clippings and clippings of the hair on the feet (combined in 'foot' sample, Table 2.2), although there was no significant difference in mean number of seeds on different sampling days (2-way ANOVA, $F_{3,24} = 0.72$, $P = 0.50$) indicating that short amounts of time (< 7 days) will not influence the number of seeds attached to sheep. Of the nine sheep sampled only one had three seeds in the belly wool sample. Results indicate that the thigh sample area more readily picked seeds up from the soil seedbank compared to the belly sample area, perhaps due to the posture used by sheep when sitting on the ground. Meanwhile the foot samples (nail clipping and toe hair) picked up the highest number of seeds g^{-1}, probably due to the feet making contact with the soil while the sheep were standing. Casual observation indicated that sheep were most often standing in the pens during daylight hours, rather than sitting on the ground.

Seeds removed from the wool samples had a similar germination rate (69%) as seeds from a control group (71%).

Experiment 2: Retention of *O. ramosa* seeds on the external surface of sheep

The number of seeds collected in trays under the sheep decreased over time, with significant differences in the decline for days 0, 1, 2 and 3-7 (2-way ANOVA, $F_{7,72} = 26.56$, $P < 0.0001$) (Figure 2.9). However, there was no significant difference in the number of seeds found in the wool samples over time (2-way ANOVA, $F_{3,24} = 2.81$, $P = 0.07$, Table 2.3), thus these two measures of seed retention did not have complementary results.

As an aside, there were significantly more seeds found in the belly samples than the thigh samples (2-way ANOVA, $F_{1,24} = 5.60$, $P = 0.03$), however the interaction of *day x sample location* was not significant (2-way ANOVA, $F_{3,24} = 0.70$, $P = 0.57$, Table 2.3).

Experiment 3: *O. ramosa* seed through sheep gut

DNA analysis of sheep manure after drenching revealed a classic gut passage time pattern. Seed numbers detected in manure peaked on day 2, and then dropped

sharply, declining to zero from day 8 onwards (Figure 2.10). The difference in number of seeds by day was highly significant (1-way ANOVA, $F_{8,64} = 73.79$, $P < 0.0001$); day 2 was significantly different from days 1 and 3, which were in turn significantly different from days 4 to 9 (Figure 2.10). The last seeds were detected on day 7, with zero seeds detected on days 8 and 9.

On average 27 700 seeds were recovered from each sheep (range: 16 000 to 51 000). This represents only 27.7% of the 1.0×10^5 seeds given to each animal on day 0.

Discussion

External transport

Transport of seeds on the external surface of animals has not been quantified for any of the *Orobanche* genera, and only recently has it been comprehensively studied for any non-prickle type of weed seed (Mouissie et al. 2005; Römermann et al. 2005). Several authors differentiate between two separate phases in seed dispersal via animal coats: attachment and retention (Fischer et al. 1996; Mouissie et al. 2005; Will et al. 2007).

The attachment experiment (Experiment 1) clearly showed that it is possible for seeds of *O. ramosa* to attach to sheep from a topsoil seedbank. Under the model devised by Römermann et al. (2005), *O. ramosa* seed of weight 4.5 μg has a predicted attachment potential of 100% for sheep wool and 64% for cattle hair. Indeed, Will et al. (2007) showed that more seeds attach to sheep wool, compared with cattle and roe deer hair, regardless of the seed size and morphology.

Table 2.1 Seed detection rates of the DNA probe when testing spiked seed samples in manure and soil. Seed detected are means (μ), *n* = 5 (manure), *n* = 10 (soil), CV is coefficient of variation. Same letters are not significantly different at α = 0.05 level, according to Tukey's HSD test.

Media	Seeds added	Seeds detected	CV	Tukey's HSD
Manure	0	0.06	2.24	a
	5	1.37	0.45	a
	50	52.69	0.41	b
Soil	0	0.00	-	a
	5	8.87	0.25	a
	50	110.26	0.18	c

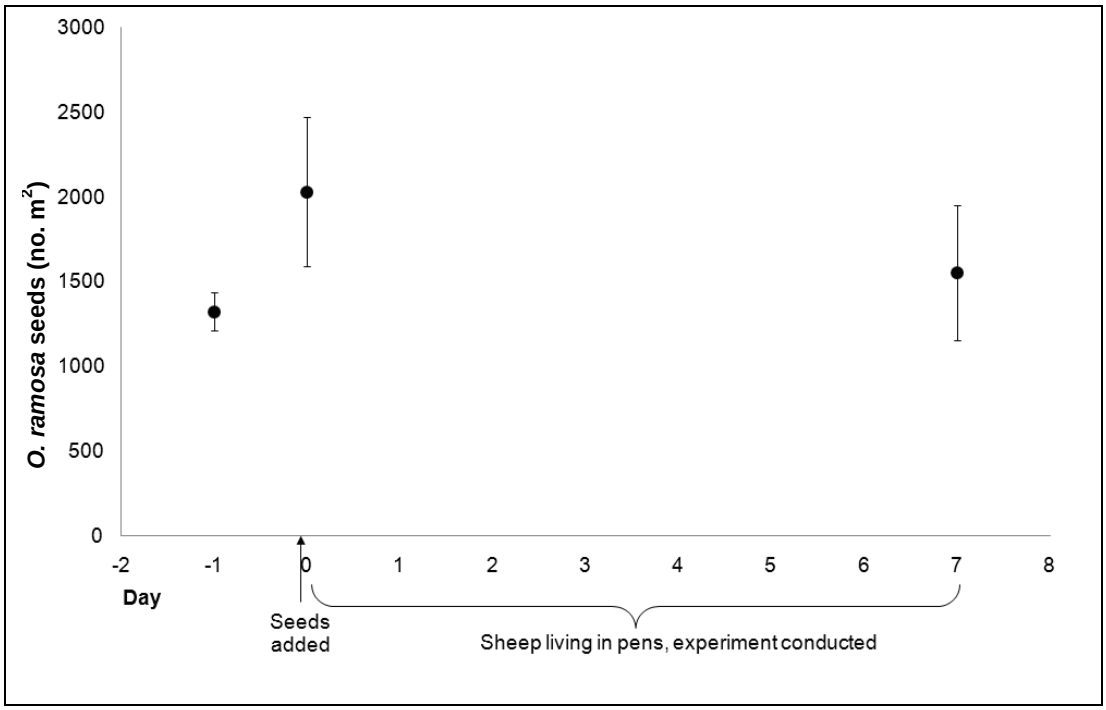

Figure 2.8. Topsoil seedbank for sheep pens used in 'attachment experiment' (Experiment 1), $n = 3$. On day –1 the natural seedbank was measured. On day zero, 9.0×10^4 seeds were added to each 9 m^2 pen. The seedbank was then immediately sampled again, the sheep were then introduced to the pens, and the experiment conducted. On day 7 the experiment concluded, sheep were sampled and removed, and the seedbank assessed.

Table 2.2. Mean number of seeds found in wool samples from belly and thigh samples over 3, 5, and 7 days in the 'attachment experiment' (Experiment 1). *n* = 3 for each day of sampling. Data are mean number of seeds per g of sample (wool or nail+toe hair), ± SE.

Day	Foot	Thigh	Belly
3	5.72 ± 5.33	0.19 ± 0.09	0.00 ± 0.00
5	1.29 ± 1.14	0.66 ± 0.03	0.08 ± 0.08
7	7.57 ± 6.67	0.60 ± 0.03	0.00 ± 0.00

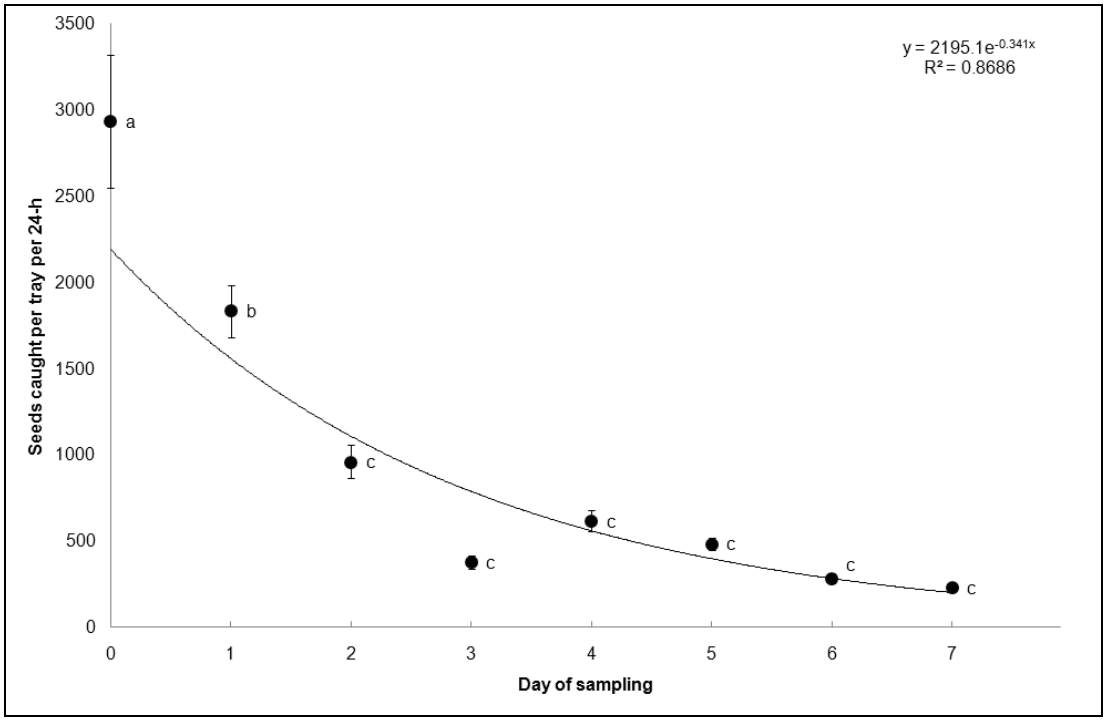

Figure 2.9. *O. ramosa* seeds caught on trays under sheep in 'retention experiment' (Experiment 2). Seeds added to sheep wool on day 0, trays sampled every day to day 7. Data are means ± SE. Day 0 to 3, $n = 12$. Day 4 to 5, $n = 8$. Day 6 and 7, $n = 4$. Line indicates exponential relationship between number of seeds caught and day of sampling, goodness of fit $r^2 = 0.87$.

Table 2.3. Mean number of seeds found in wool samples from belly and thigh locations over 1, 3, 5, and 7 days in the 'retention experiment' (Experiment 2). *n* = 3 for each day of sampling. Data are mean number of seeds per g of wool, ± SE.

Day	Thigh	Belly
1	35.5 ± 3.71	44.33 ± 1.51
3	29.4 ± 8.91	67.51 ± 21.14
5	43.2 ± 8.80	61.56 ± 10.75
7	60.7 ± 17.34	118.04 ± 40.80

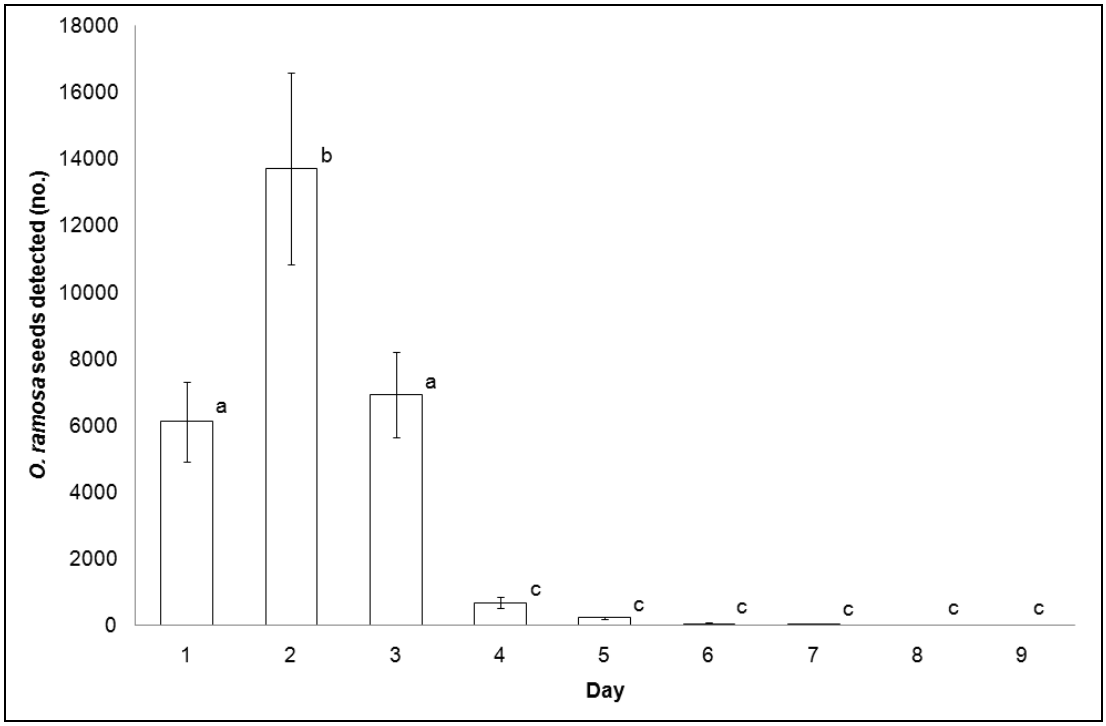

Figure 2.10. Gut passage time for *O. ramosa* seeds through sheep (Experiment 3). Sheep were drenched with 1.0 x 10^5 seeds each on day 0. Data are means ± SE. Day 1 to 7, $n = 8$. Day 8 and 9, $n = 4$. Same letters are not significantly different at $\alpha = 0.05$ level according to Tukey's HSD test.

The retention of *O. ramosa* seeds in sheep wool was assessed in Experiment 2. The results of the assessment of seeds retained in wool samples (belly and thigh) were inconclusive: there was a trend (statistically not significant) for increasing seed count over time (presumed to be a sampling error rather than a biological event), there was high variability, and sample size was limited to $n = 3$. However, the test did show that some seeds were retained in wool after 7 days. Assessment of the number of seeds collected on trays under sheep showed a significant decrease in the number of seeds collected over the first two days of measurements. Presumably, many seeds detached during the first two days following contact with the wool, but a large number of seeds remained lodged in the wool for at least 7 days, and possibly much longer.

This hypothesis is aligned with the findings of Tackenberg et al. (2006) who determined that smaller seeds had significantly higher retention potentials, in both sheep wool and cattle hair. Gardener et al. (2003) showed that 25% of the barbed seeds of Chilean needle grass *Nassella neesiana* remained in sheep wool for five months, and hypothesised that the seeds probably stayed there until the fleece was shorn. In the present study, *O. ramosa* seed attachment occurred mainly on the feet and to a lesser extent on the thigh and belly, but usually in relatively low numbers. Nevertheless, it is clear that seed transport on the external surface of sheep is likely and therefore a management issue for *O. ramosa* dispersal.

In a field situation, surrounding vegetation and landscape features will aid in the detachment of seeds from animals (Mouissie et al. 2005; Römermann et al. 2005). However, in Experiments 1 and 2, the sheep were contained in pens, and thus detachment assisted by vegetation did not occur. Another limitation of both experiments is the possibility of seeds entering the pens via wind. Certainly the results of the next chapters show that seeds are readily transported by wind, and the pens were located in an infested paddock within the quarantine zone. In retrospect, the experimental design should have included a number of control sheep housed in similar pens but with no seeds initially added to them, thus controlling for the effect of seeds being blown in on the wind.

While 10 000 seeds m^{-2} were measured by volume and added to each pen before the start of the experiment, on average only 2028 seeds m^{-2} were estimated by the DNA probe to be in the seedbank. This five-fold discrepancy may actually be greater, in

view of the DNA probe detecting on average twice as many seeds as were added to the spiked samples. Further inaccuracy may lie in the estimate of seeds by volume before addition to the pens (which relies on seeds being of consistent size), or through accidental non-random sampling of the seed bank.

Regardless, the results show that *O. ramosa* seeds on the ground can attach and be retained in sheep wool. The likelihood of seed bearing plants actually coming into contact with the wool is unknown. Fischer et al. (1996) showed that the height at which seeds are located on plants is an important factor in dispersal via wool, and that low-growing plants (< 0.3 m) were unlikely to come into contact with the body of sheep grazing in a paddock. *O. ramosa* is a small herb, approximately 0.1 m in height. Seed-bearing capsules are unlikely to come into contact with sheep wool unless the animal happens to wallow where the plant is located, or on ground where the seeds have fallen. However, sheep feet could easily come into contact with *O. ramosa* seed capsules. Plant density in the paddock will also determine likelihood of dispersal via wool (Fischer et al. 1996). In the South Australian branched broomrape quarantine zone where seed set is minimised through paddock management, the density of emerged plants should be low.

Overall these results indicate that once adhered, *O. ramosa* seeds can remain in sheep wool for at least seven days. The attachment and retention attributes indicate that seed transport on the external surface of sheep is a distinct possibility for the dispersal of *O. ramosa* should the seeds come into contact with the wool in the field.

Transport via ingestion

The gut-passage experiment (Experiment 3) revealed that *O. ramosa* seeds pass through sheep within 7 days, with a peak in seed excretion on day 2. This is comparable to the findings of Cosyns et al. (2005b) who reported a mean retention time of 2.5 days for sheep on a range of seed species, though none on the size scale of *Orobanche* spp. Michael et al. (2006) reported that the highest excretion of *Malva parviflora* seeds through sheep occurred between 1.5 and 3 days after ingestion. Similarly Jacobsohn et al. (1987) showed a peak in *Orobanche* spp. excretion on day 2 after introduction of seed to the rumen, however seed was not detected in the sheep manure (presence/absence determined by host pot testing) after day 4. Jacobsohn et al. (1987) pointed out that using host pot tests as a bioassay for the presence of

Orobanche spp. seeds was imprecise and subject to variability due to environmental conditions (Jacobsohn et al. 1987). Seed may not have germinated through insufficient imbibing or conditioning, or may not have been close enough to the host root to have sufficient germination stimulation, giving false negative results. Conversely, the DNA probe used in the present experiment may show false positives; DNA could be present but not necessarily in the form of viable seed. In addition, there is a possibility that viable seed could still be excreted long after the experiment was finished, as reported for *Solanum elaeagnifolium* through sheep; small numbers of viable seeds were still excreted for up to one month after addition, despite having had peak output at 2-3 days (J Heap pers comm. reported in Cousens et al. 2008).

The gut passage pattern that Jacobsohn et al. (1987) found for *Orobanche* spp. seeds through sheep was a peak in excreted seeds at day 2 and zero seeds at day 4. This quicker gut passage time may be due to the use of rumen-cannulated rams, bypassing the upper digestive tract and removing the time taken to traverse that section of the digestive system.

Passage through the gut can reduce germination success in a variety of plant species (Gardener et al. 2003; Cosyns et al. 2005a), however Pakeman et al. (2002) showed that small seeds (< 1 mm width) do not get broken down by the gut-passage process and remain viable once excreted. Pakeman et al. (2002) suggested that adaptations that enhance seed longevity in soil may also allow seeds to withstand the chemical and mechanical processes of passage through an animal gut. As *O. ramosa* seeds are long lived in soil (Parker and Riches 1993) it is predicted that seeds could withstand the gut passage process, although viability was not tested in this experiment. As well as size, shape of the seed may affect its ability to survive passage through the gut. Gardener et al. (2003) showed that panicle seeds with a protruding callus and awn were more likely to be damaged and unviable on excretion, compared with the smaller and less adorned cleistogene seeds of *Nassella neesiana*.

Sheep are foregut fermenters and seeds passing through a sheep will typically have lower germination success than seeds passing through other herbivores (Cosyns et al. 2005b). Cosyns et al. (2005a) assessed relative germination success of 19 plant species (including one on a similar size and mass scale as *O. ramosa*; *Centaurium erythraea*) through the gut of rabbits (caecum fermenters), cattle and sheep (foregut fermenters), and horses and donkeys (hindgut fermenters). Seeds

passing through rabbits had higher germination success, which coincided with the shortest gut retention time for that animal (Cosyns et al. 2005a). Thus it is conceivable that *O. ramosa* seeds passing through rabbits may be another consideration for quarantine managers.

Seed transport while in the gut is determined by several factors: the number of seeds consumed, the effect of mechanical and chemical breakdown on the seeds, the gut retention time, and the transport or dispersal of the animal across the landscape. This paper has addressed gut retention time for *O. ramosa* in sheep, indicating that maximum gut retention time is 7 days. The literature suggests that the mechanical and chemical factors associated with internal transport will be negligible for small seeds with high longevity (Jacobsohn et al. 1987; Pakeman et al. 2002; Cosyns et al. 2005b), and Jacobsohn et al. (1987) reported viable seeds for up to four days in the gut of sheep. The first factor however, the number of seeds consumed, remains unquantified for *O. ramosa* in the South Australian quarantine zone. Sheep may consume seeds of *O. ramosa* when feeding on the seed-bearing plant in the paddock. Certainly in drought years, such grazing animals will indiscriminately eat all plant material in a paddock (pers. obs.). Hay that is baled while seed-bearing *O. ramosa* plants are present in the crop could pose the possibility of sheep (and other livestock) consuming seeds when subsequently fed the hay. Furthermore, it also highlights the threat of *O. ramosa* seeds being spread across the landscape via anthropogenic vectors such as feed transport.

Management

In Australia at present, the Code (DWLBC 2003) requires that animals grazed on infested paddocks must spend 48 h in a clean holding yard and then be kept for 15 d in a clean paddock, so that seeds within the digestive system can be passed. The results from this study suggest that this period of time is ample to allow for gut passage of seeds, and in reality almost all seeds have passed through the gut within eight days. However the issue of seed attachment and retention on the wool has yet to be addressed by the management protocols.

As demonstrated here, external attachment on sheep is possible, and so wool may be a vector for dispersal of *O. ramosa* seeds. This chapter has confirmed the process of attachment and has shown that retention of seeds remains for at least seven days.

Further work may be required to assess the exact retention period in order to refine the quarantine protocols of the Code. It is possible that once seeds are attached to wool they may be retained indefinitely, until shearing or slaughter. Quarantine procedures may need to be applied to shearing sheds, wool processing operations or abattoirs, to ensure that *O. ramosa* seeds eventually detached from fleeces do not enter the environment and establish new populations.

This study demonstrated that seeds were found in higher numbers on the feet, much higher than in the wool of the belly and thigh. Seeds transported on the feet may have a much shorter retention period, as the foot hair is different from the main body wool. Foot hair was shorter, straighter and less dense, compared with the wool on the body, which was dense and curly. Quarantine managers may wish to investigate the length of time needed for seeds to fall from the feet naturally, or investigate the use of foot baths to ensure that feet are clean before transport. A survey of the feet of sheep at the end of the 17 day quarantine process may be required to determine the probability of seed retention and the need for a foot bath.