Factors affecting the establishment of a classical biological control agent, the horehound plume moth (Wheeleria spilodactylus) in South Australia.

Submitted by

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Preamble

This thesis describes the original work of the author. Information derived from the work of others has been specifically acknowledged in the text. This thesis has not been submitted previously for a degree at any University.

I give consent to the thesis being made available for photocopying and loan.

Saturday, 25 May 2002

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General Summary

Natural enemies have long been used for biological control (Simmonds *et al.* 1976) but predicting optimal conditions for successful establishment is still an uncertain process (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Criticisms of planned introductions include perceptions of unnecessary risks associated with the release of 'another exotic' into the environment (Simberloff and Stiling 1996; M^{c}Evoy and Coombs 1999) and the apparently low success rate achieved by such programs when compared to their cost (Julien 1989; Williamson 1996). On average, one in four agents achieve major impacts on the target species (Julien 1989; Williamson 1996). However, especially in Australia where alternative forms of control can ultimately be more expensive in financial cost and/or in damage to the indigenous environment, or just impractical, biological control may be the only viable option. Consequently, understanding the factors that affect the initial establishment of a biological control agent forms the first step in the process that will lead to improving the success rate for selected agents.

Although every biocontrol program is unique, classical biological control is the ideal ground to test some of the concepts and theories surrounding population establishment. In this project, the horehound plume moth (*Wheeleria spilodactylus* Curits), an agent introduced to control the invasive weed horehound (*Murrubium vulgare* L.), was used as a model system to investigate the following factors believed to influence the successful establishment of an introduced natural enemy:

1. Initial population size, host plant quality and time of release;
2. Interaction of population growth rates with decreased genetic diversity;
3. Expression of outcrossing depression for allopatric lines of horehound plume moth;
4. Expression and/or purging of inbreeding depression.

The final area of investigation was to retrospectively test the use of generic population viability analysis (PVA) and decision making tools for determining optimal release strategies for the

horehound plume moth in South Australia and to compare the outcomes with the empirical data collected during the course of this project.

The effects of initial population size and host plant quality on population establishment and growth were examined using replicates of 4 different founder sizes of Spanish collections of the horehound plume moth released at two different locations within South Australia in the spring of 1999. These locations were characterised by either high or low plant quality areas. Time of release was studied at high plant quality locations only, with releases of 4 different founder sizes made in the spring, summer and autumn of 1999/2000. Census data provided population growth rates for each release and were used to determine the relationship between release size, host plant quality, season of release and successful establishment. The existing hypothesis that there was a positive relationship between release size and likelihood of establishment was supported by the observed results. This was evident for both high and low host plant quality. The quality of the host plant was positively associated with successful establishment of colonies. Because season of release influenced host plant quality this indirectly affected the successful establishment of colonies.

Outcrossing allopatric lines of horehound plume moth was undertaken between cultures originating from France and Spain. The F_1 generation was bred in the laboratory from reciprocal crosses of equal founder size. Pure lines, for use as controls, were also bred using the same numbers of adults as foundation stock. The F_1 offspring were then released in the field and census data after one generation used to determine if outcrossing depression or vigour could be detected. The results suggested that the outcrossing of the allopatric lines produced an effect of vigour.

Individual horehound plume moth larvae were harvested from the field release sites for DNA analysis. Initially, it was hoped these would be analysed using microsatellites isolated and developed from a partial total genomic DNA library as well as with the multilocus technique of amplified fragment length polymorphisms (AFLP). However, during the course of the project analysis of microsatellite DNA was abandoned due to difficulties involving isolation of suitable markers. AFLP analysis was then developed to investigate the genetic diversity for each release

site. The AFLP data suggested that random founder effects and genetic drift were the primary factors in genetic differentiation for all releases. Contrary to what might be expected from the biocontrol literature, there was neither evidence of selection processes, nor any evidence of assortive mating in the outcrossed field population. The data showed that there was a positive association between the number of polymorphic sites and average genetic diversity across the loci and population replacement rates, indicating that slower population growth could be expected to result in lower genetic diversity.

The attempted purging of lethal alleles to eliminate potential inbreeding depression was undertaken with full- and half-sib mating programs for Spanish horehound plume moth. By generation two, there was a decrease of 24% in the number of viable offspring per female when compared to random outcrossed pairs. The number of lethal equivalents per gamete for the horehound plume moth was calculated to be 3.6. This figure is of a similar magnitude to that observed for many mammalian species, as well as for the lepidopteran species, *Dryas iulia* and *Heliothis virescens* (Roush 1987; Haag and Dearaujo 1994; Ralls *et al.* 1988).

PVA was undertaken using a generic software package that contained a component allowing the incorporation of inbreeding effects. Life cycle data and environmental variation were estimated from previously published studies (Weiss and Lippai 1996; Clarke 2001) and from observations made during the inbreeding experiment. The field experiments undertaken over the 2000/2001 spring-to-spring time period provided data for comparison with simulated populations. PVA for simulated populations was run for high and low host plant quality and varying founder sizes, with and without inbreeding incorporated. The predicted outcomes suggested that the inclusion of inbreeding effects for reasonable release numbers had no effect on the likelihood of establishment. Simulations predicted the detrimental effect that large variation in seasonal environmental conditions and host plant quality had on successful establishment. The predicted survival rates of horehound plume moth populations concurred with the observed field data. Using the predicted and observed probability of establishment and the optimal release strategy model developed by Shea

and Possingham (2000) also produced similar decisions on which release strategies would be preferred for high and low plant quality locations.

The main conclusions of this study on the horehound plume moth were:

1. There was a positive relationship between release size and probability of establishment;
2. High host plant quality and spring releases (after winter but while host plant quality was still good) improved establishment;
3. Random founder effects and genetic drift had more influence on the genetic profile of established colonies than selection;
4. The outcrossing of allopatric lines resulted in outcrossing vigor;
5. Inbreeding as a method of purging lethal alleles was unlikely to be successful;
6. Generic PVA reflected the observed outcomes;
7. Decision-making tools to determine optimal release strategies were useful when combined with PVA.

When releasing biological control agents, it was apparent that the economic savings come through a reduction of rearing costs. The establishment of harvestable nursery sites early in the program would be of more benefit than varying the number or size of initial releases. The study indicated that procedures for the successful establishment of the horehound plume moth could be improved. The variation in establishment rate in the different regions with contrasting plant quality showed that predictable local geographical, physical and climatic factors affected plant growth habits and so influenced the establishment of horehound plume moth colonies. This has a direct bearing on the efficacy of the control agent across the target regions. The use of generic software to predict the outcomes of various scenarios, as well as release strategies, for the horehound plume moth indicated that the use of decision-making tools could have enhanced the distribution and spread of this biological control agent. These results provide the impetus for other species to be investigated to determine if there are general rules regarding the regional effects on host quality and timing of release on successful establishment. It is also hoped that the use of predictive decision-making

software will continue to develop, thereby providing improved management tools for future biological control programs.

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1. Background theory, hypotheses and aims

Introduction

Problems faced by managers of classical biological control programs are similar to those affecting managers of many small, isolated populations because in the early stages of the release the aim is to establish viable populations with a limited number of founders. The problems are exacerbated by additional genetic factors where a colony may have been reared in a laboratory over several generations before being released to face a novel environment. This study focused on the classical biological control agent, horehound plume moth, (*Wheeleria spilodactylus* Curtis), introduced into Australia as a natural enemy of the exotic weed, horehound, (*Murrubium vulgare* L.). These two species provided a model system to investigate some of the hypotheses concerning factors affecting the probability of a biological control agent successfully establishing a persistent population. Many factors interact to influence the outcome of successful establishment of a newly released biological control agent. This study focused on the importance of environment, release size and genetic diversity on the population growth rates of the horehound plume moth over a maximum of 4 generations.

The pest weed: horehound (Marrubium vulgare (Laminaceae: Lamiodeae))

Horehound is a perennial herb with an erect growing habit. It originated in Central and western Europe, where it occurs on well-drained calcareous soils (Young and Evans 1986; Sagliocco and Coupland 1995). It was originally introduced into Australia as a medicinal or garden herb and spread rapidly into all states except the Northern Territory (Zeigler *et al.* 1991; Sagliocco and Coupland 1995). The transportation of the burrs and seeds in the wool and fur of animals facilitated its spread (Young and Evans 1986; Sagliocco and Coupland 1995). The results of this transportation can be seen in the patches of horehound that persist along old stock routes, around old shearing sheds and growing on un-used rabbit warrens. Competition with other plants can check the infestation of horehound, but when overgrazing and drought reduced the density of

competing vegetation, it rapidly establishes (Zeigler *et al.* 1991). Established plants lose foliage when water is scarce and juvenile mortality occurs more frequently during exposure to low rainfall and high temperatures, like those experienced in southern Australia (Sagliocco and Coupland 1995). The large seedbank present in soils allows a rapid recovery by this species following drought. Germination is recorded to occur in autumn, but sufficient rainfall at other times also stimulates germination (Zeigler *et al.* 1991). Herbicides such as 2,4-D and MCPA effectively suppress horehound, especially if used in conjunction with pasture improvement programs, but chemical control is often ineffective and undesirable in conservation areas (Sagliocco and Coupland 1995). In addition, horehound is unpalatable to livestock, which results in the overgrazing of other species that could have competed with the weed (Zeigler *et al.* 1991).

In 1991, CSIRO, the Keith Turnbill Research Institute (KTRI) of the Victorian Department of Conservation and Environment, and the Australian Wool Corporation established a program with the aim of providing long-term biological control of horehound. Several natural enemies were identified as potential candidates for such a control program (Zeigler *et al.* 1991, Weiss and Lippai 1996). One of these was the horehound plume moth.

The biological control agent: horehound plume moth (Wheeleria spilodactylus (Lepitoptera: Pterophoridae))

The horehound plume moth was initially targeted as a suitable biological control agent because it is an effective defoliator of leaves, especially the bud tips (Zeigler *et al.* 1991; Weiss and Lippai 1996). In southern Australia, the larvae over-winter in the tips of horehound plants and emerge in late August/September to be seen as small caterpillars on the tips of plants (Weiss and Lippai 1996). The lifecycle is around 6-8 weeks allowing 4-5 generations per year (Weiss and Lippai 1996). The ease of rearing, high number of generations per year, the ability of the larvae to defoliate a plant and host specificity meant that the horehound plume moth was chosen as the first of the biological control agents against horehound to be tested in Australia (Weiss and Lippai 1996). Horehound plume moth was introduced into a host range testing program at KTRI in late November of 1991, underwent the first field releases in 1994 and, since then, dozens of releases have been made in Victoria and South Australia (Clarke 2001). The initial colony consisted of

individuals collected from the south of France. After laboratory colonies had been established, releases in South Australia were made and monitored (Clarke 2001). It was found that successful establishment occurred in regions where the annual rainfall exceeded 400mm, but many releases failed or populations increased very slowly where the annual rainfall was less than 200mm (Clarke 2001). This resulted in a second importation of insects from both the Cape d'Agde (France) and Zaragoza (Spain) to test for improved climatic matching Clarke (2001). Releases of insects from these sources proved to be successful in areas where the annual rainfall was as low as 250 mm, although the improved success appeared to be due to lower inbreeding and less disease rather than climate matching (Clarke 2001). These last two collections provided the founders used for the experiments described below.

The host specificity of horehound plume moth, coupled with the observed life cycle parameters, indicated that it was an ideal species for investigating the interaction of how timing of release, release size, genetic diversity and the environment affected the successful field establishment of an insect biological control agent.

1.2 Biological control: the issues for isolated colonies

1.2.1 Background

The introduction of biological control agents results in the creation of new populations. Under normal conditions, populations of plants and animals fluctuate in size over time, with the dispersal of a few individuals potentially founding new colonies. This results in cycles where the founders must recover from genetic and numeric bottlenecks if they are to survive in their new habitat (Soulé 1986; Caughley 1994; Soulé 1994; Barton and Whitlock 1997; Hedrick *et al.* 1996; Hedrick and Gilpin 1997). The factors causing the major difficulties in persistence of populations are environment, catastrophe, demography and genetics (Caughley 1994).

Despite a long history of the use of natural enemies for biological control (Simmonds *et al.* 1976), predicting the establishment of any introduced agent on a targeted pest is still imprecise (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Additionally, there is often controversy

associated with the further release of what purists call 'another exotic' into the environment (Simberloff and Stiling 1996; M^{c}Evoy and Coombs 1999), because it is argued that 'another exotic' may result in further threatening indigenous species in ways that have not been predicted or foreseen. Even though every biological program presents a unique situation, classical biological control is the ideal ground to test some of the concepts and theories proposed in the disciplines of invasion biology and biological control. Many authors now conclude that the only way to resolve both the controversies and improve the probability of future successful establishments is through a critical assessment of existing knowledge, working hypotheses, and empirical facts supported by the formulation and validation of models that predict possible outcomes or provide general rules determined from rigorous scientific investigation (Julien 1989; Moller 1996; Vanette and Carey 1998; Ehler 1998; Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). The methodology and issues central to classical biological control are outlined in a number of texts (e.g., Huffaker and Messenger 1976, Mackauer *et al.* 1980 and Harley and Forno 1992) and will not be discussed here.

For the introduction of a biological control agent to be termed 'successful', the agent must establish, increase in numbers and go on to show some measurable control, or damage, on the pest host (Ehler 1998). In his book *Biological Invasions*, Williamson (1996) estimated that 61.3% of the insects introduced for the biocontrol of weeds established, and of those 32.4% went on to provide effective control. This equates to one-fifth of the attempted introductions as being effective. In this sequence of events, the probability of establishment becomes not only one of the key criteria for determining the success for a biological control agent, but also the initial criterion. If the agent cannot establish, then subsequent control or damage of the target organism cannot occur. To date, concepts (untested ideas or theories) rather than working hypotheses (tested theories) govern many of the decisions and conclusions made when selecting potential biological control agents or determining why they failed (Ehler 1998). To improve methods used for selection of classical biological control agents, it is necessary to move beyond concepts and theory to working hypotheses that have been tested against empirical data.

Despite the fact that a large number (approximately 40%) of attempts at biological control of weeds have failed to establish populations of natural enemies, comprehensive evaluations as to why failure occurs have not been undertaken (Julien 1989). Studies of population establishment outside of biocontrol examples, as well as studies on biocontrol agents, indicate a series of factors are crucial to the establishment of populations. Suggested causes for failure to establish include poor climate match (Crawley 1986; Clarke 2001; Duncan *et al.* 2001); low genetic variability of the agent (Roush 1990); release method, number and timing (Hopper and Roush 1993); laboratory adaptation (Mackauer 1980) and environmental resistance to establishment, which encompasses the presence of incumbent generalist predators and any host change that has occurred in the new habitat (Elton 1958; Goeden and Louda 1976; Peters 1977; Lattin and Oman 1983; Kim 1983). Only some of these suggested causes have been evaluated for any type of small populations. These include climate matching (Clarke 2001; Duncan *et al.* 2001), genetic variability, including possible laboratory adaptation (Ralls and Ballou 1983; Miller and Hedrick 1993; Miller 1994; Ouberg and Vantreuren 1994; Saccheri *et al.* 1996) and release method (Campbell 1976; Memmott *et al.* 1998; Clarke 2001). However, many of these studies have been retrospective. The study presented here focused on the impact of the environment, release size and genetic diversity in an attempt to determine the primary factors for predicting successful establishment and determining optimal release strategies based on the known biology and ecology of the horehound plume moth.

1.2.2 Environment

Environmental factors that may harm a population encompass disturbances ranging from long term variation (climate changes), short-term fluctuations (seasonal changes) and random changes (catastrophes). When populations are small, it is important that they are capable of withstanding environmental perturbation if they are to survive (Simberloff 1983; Soulé 1986; Caughley 1994; Korn 1994). Species that are near the limits of their habitat spectrum, are at a continually low population density, or have specialised habitats remain vulnerable to extinction when a catastrophe occurs or natural fluctuations exceed the species' limits (Terborgh and Winter 1980; Soulé 1986; Korn 1994; Lacy 1997). Different environments may select for different phenotypes and genes for a particular trait could become depleted or lost from an isolated population (Slatkin 1987; Connor

and Ferguson-Smith 1993). If these genotypes were to be relocated to a novel environment, the adaptations of the population could prove to be detrimental (Lewontin 1974; Futuyma 1979; Lambrechts and Dias 1993; Krogstad *et al.* 1996). However, published studies have not proven this hypothesis unequivocally. For example, Lambrechts and Dias (1993) investigated the differences in onset of egg laying in blue tits (*Parus caeruleus*) between a mainland woodland population and an island population and concluded the differences were mainly genetic. Conversely, Krogstad *et al.* (1996) found no evidence of directional selection when he transplanted house sparrows (*Passer domesticus*) from coastal and inland populations onto an island near a coastal area. However, it is unclear in either of these two studies what level of gene flow or genetic distance already existed between the populations prior to the experiment. Theoretically, for adaptation to new conditions, there must be a level of robustness, or immediate fitness, in the individuals plus the presence of enough genetic variation to allow selection processes (Lewontin 1974; Soulé 1994; Hedrick and Gilpin 1997). The possibility that individuals of the same species, evolving under one set of conditions, could not cope with different conditions is conjectural, unless specifically tested.

Climate matching is assumed to be a key factor for selection of suitable biological control agents for release. There are many historical examples where disparity in climate between the source and release area have been postulated to have resulted in failure or the restriction of an agent to specific conditions (e.g. Messenger *et al.*1976, Flint 1980 and Nealis 1985). However there is some disagreement as to how close climates must be matched in order for success to be achieved (Leen 1991). Leens (1991) analysed 527 records of releases made with 30 species of phytophagous insects conducted worldwide from 1944 to 1986 using mean temperatures and rainfall values. This approach failed to consider differences in climatic extremes that may have existed between the source and the introduced habitats, but concluded that climate has rarely hindered the establishment of phytophagous insects. Duncan *et al.* (2001) reviewed the introduction of 55 bird species introduced to mainland Australia using a climate matching program, CLIMATE (Phelong 1996), and concluded that magnitude of the effort in introducing propagules, habitat matching and previous successful establishment were all strong predictors of success for avian species. This

suggests that, although habitat matching played some part in ensuring successful establishment, it is not the driving force.

A recent study by Clarke (2001) investigated the effect of climate matching on two recently introduced classical biological control agents in South Australia in greater detail. He concluded that for one of the species, horehound plume moth (*W. spilodactylus*), climate matching did not affect the probability of establishment, while for the other species, bitou tip moth (*Cosmostolopsis germana* Prout), the assumption was only partially correct. The primary cause for failure to establish in the second case was predation by ants. However, difficulties in bitou tip moth adapting to the moisture stressed conditions and sub-species of the host plants found in South Australia still suggested that habitat matching was required where the morphology of the host plant was markedly altered by environmental extremes. Clarke (2001) also noted that host plant quality affected horehound plume moth survival. Water stressed horehound plants not only reduced larval survival but also slowed developmental times. Poor resource quality can cause the natural enemy to experience mortality levels outside the range normally experienced and, if another fluctuation occurs within a short enough time interval, result in the population declining even further until extinction occurs. The results observed by Clarke (2001) suggested that while climate matching was not an issue that directly affected the horehound plume moth, the result of long-term poor host plant quality where climatic conditions were poor or extremely variable could lead to declining populations and ultimate extinction of horehound plume moth populations.

For continued survival of a population, there must be adequate habitat available that is capable of sustaining the population over time (Simberloff 1983; Soulé 1986; Shaffer 1987). The quality and quantity of the habitat in the area influence the survival of the individuals within it. Quality includes the physical conditions, the quality of resources such as food, shelter and breeding sites, and the presence of other species that may compete, parasitise or prey upon the population (Quammen 1996; Hastings 1997). Obviously, the quality varies, or fluctuates, around an 'average' condition, but climatic and environmental extremes may be important considerations when planning the release of biological control agents because of their affect on resource availabilty.

Some published examples of manipulating resource quality exist in the literature (Wilson 1960; Room and Thomas 1985; Center *et al.* 2000). Wilson (1960) reported that the population density of a biological control agent increased when fertilizer was applied to chlorotic prickly pear cacti and resulted in increased damage by *Cactoblastis cactorum* L. in Australia. Room and Thomas (1985) reported an example of how poor host quality influenced the establishment of a biological control agent. In this case study, the initial introduction of a beetle (*Cyrtobagous salviniae* Calder and Sands) into Papua New Guinea to control the floating weed (*Savinia molesta* Mitchell) failed to control the weed. However, subsequent releases, where the pest weed was fertilized with nitrogen, allowed populations of the beetle to build up rapidly, establish and go on to damage the floating weed, even when fertilizing ceased. More recently, Center *et al.* (2000) investigated releases of the Australian melaleuca snout beetle (*Oxyops vitiosa* L.) a biological control agent of paperbark tree (*Melaleuca quinquenervia* L.) in Florida. Abundant young foliage facilitated establishment of the snout beetle, while the larval stage predominated during October to May, coincident with the flushes of plant growth. In contrast, only adults remained during summer, except at a site that was periodically mowed. The mowing induced new growth and supported a continuous larval population. It appears resource quality has been recognised as a factor influencing population growth rates of natural enemies, but little has been done in evaluating the importance of host quality on the probability of establishment (Van den Bosch and Telford 1964; Campbell 1976; Harris 1981).

Seasonal effects occur on a cyclical basis and weather can influence population growth rates of many species directly (Kamata 1997; Kamata 2000; Ruohomaki *et al.* 2000; Clarke 2001). Apart from the recorded temperature and day length triggers for winter diapause in the horehound plume moth, Clarke (2001) noted that extremes of temperatures and humidity decreased egg viability, decreased larval growth rates and decreased adult survival. In a study on an entomopathogenic fungus (*Cordyceps militaris* L.) which is considered to be an important factor in suppressing population outbreaks of beech caterpillar (*Quadricalcarifera punctatella* Motsch.), Katama (1997) found that the rate of infection of the fungus changed seasonally. In a later study on the beech

caterpillar, *Syntypistis punctatella* L, Katama (2000) concluded that the outbreaks of this species were synchronised with the weather. Ruohomaki *et al.* (2000) investigated the regional cycles of a geometrid moth (*Epirrita autumnata* Bkh.) in northern Europe. These authors claimed that the initiation of the increase phase seemed to coincide with maxima in sunspot activity and that climatic factors associated with the solar cycle could contribute to the large-scale geographical synchrony of outbreaks. Such seasonal and climatic effects may be regulatory and cause little variation in population numbers or may result in varying levels of the 'boom-bust' cycles that have been reported in the literature. Colder temperatures and decreased winter photoperiod may induce diapause, slow growth, reduce reproduction and result in increased mortality in some insect species (Tauber *et al.* 1984; Ruohomaki *et al.* 2000; Clarke 2001). Likewise, extreme summer heat can decrease fecundity and increase mortality for some insects as body temperatures are pushed to the limits of what is physiologically tolerable (Tauber *et al.* 1984; Clarke 2001). Seasonal changes in photoperiod, temperature, food quality and quantity, and moisture act as cues for the organism (Tauber *et al.* 1984). Recognition of these cues may be finely tuned and slight changes could result in a misreading of conditions, which alter the ability of the organism to respond to environmental change. Season influences the host as well as the natural enemy. Additionally, seasonal changes affect entire eco-systems.

It is necessary to ensure host/agent synchrony when introducing biological control agents (Nealis 1985; Hill *et al.* 1991; Van Driesche 1993). Moving agents from the northern hemisphere to the southern, for example, may require management of the agents' life stages to ensure that when field releases are attempted, the host and enemy are matched with respect to the life stages that ensure maximum likelihood of survival and establishment for the enemy on the host. Conditions for diapause, food source, refuges and oviposition must be optimised, even if laboratory manipulation is required prior to release (Nealis 1985; Van Driesche 1993).

Catastrophic events have often been blamed as the final factor that causes the extinction of small populations (Soulé 1986 1994; Caughley 1994; Hedrick *et al.* 1996). Unlike extremes of season, which may occur on a more or less cyclical basis, catastrophic events occur at random and are

usually localised and affect all individuals equally. Small populations, therefore, are susceptible to extinction before they can expand over a sufficient geographical range to avoid localised catastrophes. When biological control agents are first released and few populations are established, catastrophic events could set the program back or even doom it to failure. Consequently determining optimal release strategies to minimize the risk of failure while maximizing the success rates of establishment becomes critical (Memmott *et al.* 1998; Grevstad 1999, Shea and Possingham 2000).

1.2.3 Demography

Demography is the study of factors that relate directly to the population dynamics, i.e., rates of development. In the absence of regulatory factors a population continues to grow (Hastings 1997, Gottelli 1998). Population regulation can result from a number of causes such as competition for food, space and other resources, predation (including cannibalism), parasitism and disease (Korn 1994; Joern and Behmer 1998; Kamata 2000). For example, limited food supply has been known to affect the fecundity and life span of many species (Chippindale *et al.* 1993; Delisle and Bouchard 1995; Delisle and Hardy 1997; McMillan and Wagner 1997; Behmer and Grebenok; 1998; Joern and Behmer 1998; Sgro and Hoffman 1998).

In natural ecosystems, populations may remain relatively stable, fluctuating between upper and lower limits. Such regulation occurs despite the fact that most organisms produce far more offspring than would be required to maintain their populations (Price 1975). Demographic factors only become important to population persistence when the colony size is very low (Soulé 1986 1994; Lande 1988; Ryan and Seigfried 1994) or if the regulatory factors, when populations reach extreme levels, are particularly harsh and produce boom-bust cycles. At low breeding numbers an Allee effect, in the form of mating delays or failure to find mates, influences the reproductive rates and could result in reducing the population below a critical density, from which it cannot recover (Roush and Hopper 1995). The result is that, if demographic factors are altered so that mortality rates are higher than reproductive rates, small populations are unlikely to persist for more than a

few generations because it takes only a single adverse event, such as a severe winter, to cause extinction (Hastings 1997).

1.2.4 Genetics

Low founder numbers and the absence of gene flow are characteristic of many small populations and biological control releases (Hopper *et al.* 1993; Backus *et al.* 1995; Frankham 1995a,b; 1997). This is associated with concerns that, during the initial collections, importations and rearing stages, rare alleles are either not collected or lost via bottlenecks (Roush 1990). Various authors have argued about the importance of genetic diversity in small populations and to what extent it contributes to extinction (Roush 1990; Caughly 1994; Frankham 1997; Harris 1997; Hanksi 1998; Soulé and Mills 1998). Subsequent bottlenecks after field release can result in an even further loss of heterogeneity that may exacerbate any inbreeding depression or loss of fitness (Korn 1994; Berger and Cunningham 1995; Frankham 1995b). Theoretically, the loss of genetic diversity in very small populations results in decreased mean fitness within a population and a reduced potential for responding to changes in the environment (Nei *et al.* 1975; Ewens *et al.* 1987; Soulé 1986; Ryan and Seigfried 1994; Hedrick and Gilpin 1997). If this were true, the assumption that the long-term outcome would be eventual extinction seems natural.

The deleterious effects of loss of genetic diversity are being questioned. Colonies founded by as few as two individuals, and other populations with low genetic diversity, have persisted for long time periods (Newsome and Noble 1986; Ralls *et al.* 1986; Baker *et al.* 1990; Caro and Laurenson 1994; Gleeson 1995; Quemman 1996; Memmott *et al.* 1998). For example, two Australian brush turkeys and four laughing kookaburras established these species on Kangaroo Island (Newsome and Noble 1986), while the first Australian feral rabbit population was founded in Geelong by 20 wild English rabbits in 1859 (Flux 1994), although it would seem logical to assume further introductions supplemented the initial colonization. Two females were all that was required to establish the parasitoid *Apanteles circumscriptus* in Canada (Cameron *et al.* 1986). One of the most recent well-recorded recoveries is that of the Mauritius kestrel (Quammen 1996). In 1971, only four birds could be found. By 1988, with extensive human assistance, there were roughly forty

pairs in the wild and thirty-six pairs in aviaries. Baker *et al.* (1990) suggested that one mating pair of fruit fly would be enough to establish a population of fruit flies in New Zealand. Thus, there are many documented examples of small populations establishing, but perhaps they are exceptional.

Many inbred populations express inbreeding depression or experience higher extinction rates (Ralls and Ballou 1983; Pray *et al.* 1994: Frankham 1995b; Hedrick *et al.* 1996; Frankham 1997). The interaction of environment and high levels of inbreeding have been shown to be more detrimental to persistence in the field than expected from experiments in the laboratory in some cases (Jiménez *et al.* 1994, Miller 1994). Theoretically, the longer a colony persists and the more rapidly it expands, the more likely mutations and recombinations will restore genetic variability, overcome inbreeding depression and restore the potential for selection of suitable phenotypes (Nei *et al.* 1975; Chakraborty and Nei 1977; Saccheri *et al.* 1996, Saccheri *et al.* 1998).

Despite the fact that many colonies with what appears to be reduced heterogeneity have survived over several generations, the only way to determine the importance of heterogeneity is by considering the success and failure of colonies in association with their genetic diversity. Jiménez *et al.* (1994) estimated the effects of inbreeding derived from wild populations of white-footed mice, *Peromyscus leucopus noveboracensis* and that inbreeding had a significant detrimental effect on the survivorship of mice reintroduced into a natural habitat. The effect was more severe than that which had been observed in laboratory studies. Miller (1994) investigated the effect of inbreeding depression on *Drosophila melangoaster* under conditions that imposed environmental stress. The results suggested that inbreeding depression was more severe when environmental stress was encountered. If environmental stress exacerbates the effect of an inbreeding depression, then it is necessary to understand how potential inbreeding depressions and loss of genetic diversity would influence the successful establishment of a biological control agent.

Inbreeding depression is an accepted phenomenon, but the value of outcrossing been questioned in some cases (Legner 1971; Soulé 1986; Berger and Cunningham 1995). Legner (1971) analysed expressions of hybrid vigour in F1 through F3 generations of crosses between strains of

Muscidifurax raptorellus (Hymemoptera: Pteromalidae), a parasitoid of synanthropic flies. Crosses of strains from temperate and tropical areas showed slight negative heterosis in the F2 generation. Soulé (1986) cited an earlier study of an attempted outcrossing between Turkish and Nubian ibex (*Capra ibex ibex*) that led to the resulting fertile hybrids rutting in early fall. Consequently their offspring were born in the coldest month of the year and this caused extinction of the entire population. Berger and Cunningham (1995) reported the consequences of mixing two allopatric lineages of bison *(Bos bison*) and found the growth rates of the F1 inbred lines were higher than those of the outcrossed hybrids. But Berger and Cunningham (1995) cautioned against the conclusion that this was due to outbreeding depression because the males from one lineage failed to mate and provide all possible combinations of F1 generation mixes, which prevented appropriate statistical contrasts. As early as 1930, Fisher suggested that isolated populations may eventually prove to be incompatible with each other because of natural selection for local adaptation. At this point, sub-speciation occurs (Fisher 1930). This may explain observed outcrossing depression, as it appears that outcrossing may affect fitness if the populations have evolved in markedly different environments, or in isolation from each other. The assumption that outcrossing will reverse loss of fitness observed in biological control programs should be evaluated against the knowledge that hybrid vigour is not always guaranteed.

The Hardy-Weinburg equilibrium is based on the assumption that the relative frequency of each genotype remains unchanged over time (Futuyma 1979; Connor and Ferguson-Smith 1993). But several factors can result in the gene, or at least, the genotype, frequencies changing from generation to generation. These include non-random mating, an alteration in the mutation rate, selection, migration and small populations undergoing rapid expansion (Futuyma 1979). In the absence of selection the actual frequencies of alleles may vary widely between generations in a process known as genetic drift as a result of random mating patterns (Futuyma 1979). Purely by chance, one allele may fail to be passed on and undergo extinction. With each generation, the average heterozygosity should, in theory, decrease by a factor of 1/2N, where N is the total number of parents (Crow and Kimura 1970; Nei 1978; Templeton 1980; Douwes and Stille 1987; Connor and Ferguson-Smith 1993; Gilpin and Taylor 1994; Ryan and Seigfried 1994). Eventually, if

mutation or migration does not occur the genetic variation of a population could fall to zero (Hedrick and Gilpin 1997).

In the early stages of a biological control program, unassisted arrival of new individuals into a population does not occur. Migrating individuals modify the gene pool of their new population if they successfully breed after arrival. Isolated populations can have high genetic variation between them as a result of different alleles becoming fixed by localized selection processes (Slatkin 1987; Leberg 1991; Mercure *et al.* 1993; Gleeson 1995; Jaarola and Tegelstrom 1995; Kurdyla *et al.* 1995; Hudson and Adams 1996; Johannsen *et al.* 1996; Martínez-Torres *et al.* 1996). This process of fixation by localised selection processes is frequently referred to as a selective sweep. Natural selection for important adaptations may result from differences at relatively few loci with the remaining loci being weakly selected or completely unaffected (Watt *et al.* 1983; Oldroyd *et al.* 1995). Overall, different populations may contain variants that could survive different selection pressures and assist in protecting the species from extinction in the face of catastrophe by allowing localized colonies to persist (Gilpin 1987; Korn 1994; Oldroyd *et al.*1995).

Assuming a strong competitive force operates when sexual selection occurs, it is also valid to assume individuals will alter the pattern and rate of gene flow in a natural population, depending on individual success (Jones *et al.* 1995; Roush and Hopper 1995). Sexual selection in polygamous systems can be intense (Delisle and Boucherd 1995; Kirkpatrick 1987; Jones *et al.* 1995; Emlen and Oring 1997). For example, in non-social insects the trend for last-male precedence through sperm displacement has been observed (Boomma and Sundstrom 1998). Polygamous versus monogamous mating strategies will also influence the pattern and rate of gene flow (Crow and Kimura 1970). Time and space limits the degree with which sexual preference can operate (Emlen and Oring; 1997) but if resources and (or) mates were uniformly distributed, and there were no complications resulting from individual behaviour, then the contribution from all individuals is maximised. However, it seems likely that some individuals could monopolise the available resources and (or) mates and influence the genetic profile of future generations. So assortative mating strategies can promote differences in the genotypes found between populations.

Ouberg and Vantreuren (1995) hypothesised that small populations of the meadow cleary (*Salvia prentis* L.) would have a lower fitness than large populations and investigated the fitness related characteristics and allozyme diversity in order to determine if there was a relationship between fitness and population size. They concluded that none of the differences in fitness could be attributed to population size or level of allozyme diversity. Young *et al.* (1999) examined the genetic diversity of 16 fragmented populations of the Button Wrinklewort (*Rutidosis leptorrhynchiodes* F. Muell.) and concluded that, while genetic variation between the populations was low, neither the genetic diversity not heterozygosity was related to population size. However, Rebordinos *et al.* (1999) examined multilocus heterozygosity and growth rates for the Portuguese oyster (*Crassostrea angulata* L.) and reported positive correlations existed for multilocus heterozygosity and growth rates in the largest weight classes. Reduced population performance in the rare gentian (*Gentianella germanica* L.) was attributed to genetic effects, although environmental variables accounted for significant variation in population growth rates and performance in the field (Fischer and Mattais 1998). A study on topminnows (*Poeciliopsis monacha* L.) found genetic diversity was highly correlated with fitness and the detrimental effects were reversed when genetic diversity was reintroduced (Vreinhoek 1998). Consequently, associations between genetic diversity and fitness appear to be inconclusive. If such an association existed, and the presence of genetic variability were a requirement for successful establishment of a biological control agent, what magnitude of loss would limit the colony's ability to establish and/or adapt to a novel environment?

Kimura suggested that random chance played a much larger role than natural selection in evolution (Kimura 1968, 1983). When a new allele is created through a mutation three possible fates exist. The first is that random chance and/or natural selection would eliminate the allele from the population. The second is that the allele may increase in frequency until it is fixed. Fixation occurs when the allele increases in frequency until every member of the species has two copies of the new allele. Or finally, that the allele would stabilize at some intermediate frequency (Kimura 1968, 1983). Kimura's view divided modern scientists but has shown that the assumption that natural

selection can fix only beneficial mutations is incorrect. In small populations, random chance can play a much larger role than natural selection (Kimura 1968 1983; Ohta 1973 1992; Kreitman 1996). Even in large populations, alleles with no selective advantage can become fixed (Kimura 1968 1983; Ohta 1973 1992; Kreitman 1996; Akashi 1999). The introduction of biochemical and molecular techniques into population genetics more than 25 years ago revealed vast stores of genetic variation within populations, and this together with the discovery of coding and non-coding regions, suggested that many mutations would occur in regions that are neutral, or nearly neutral with respect to natural selection processes (Connor and Ferguson-Smith 1993; Kreitman 1996; Akashi 1999). While evidence indicates that the neutral theory of evolution cannot explain key features of protein evolution or patterns of biased codon usage in certain species (Kreitman 1996), the applicability of the neutral or nearly neutral theories have explained the largest range of phenomena (Skibinksi *et al.* 1993; Ohta and Gillespie 1996).

From the perspective of this study, the main difference between the neutral (or nearly neutral) and the traditional selection theory is that the neutral theory predicts rapid molecular evolution in small populations, while selection theory predicts rapid molecular evolution in large populations as a result of positively selected alleles sweeping through a population (Ohta 1996). It is hypothesised that positive selection for populations released in similar locations would result in the detection of non-neutral alleles increasing, or decreasing, across all populations. This assumes that the allele was included in the genetic composition of the original founders for all populations.

1.2.5 The founder effect

The number of individuals available for release can limit the success of a biological control program (Memmott *et al.* 1998). This means that management decisions on the size and number of releases are critical when optimising the release strategy (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Releasing small numbers of individuals may mean that populations will be eliminated by demographic and/or environmental stochasticity before inbreeding becomes a problem (Lacy 1997; Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). While there is evidence that larger releases establish more successfully (Hopper and Roush 1993;

Memmott *et al.* 1998; Duncan *et al.* 2001; Forsyth and Duncan 2001), Cameron *et al.* (1993) reviewed biological control programs in New Zealand, and found that small importations were not necessarily detrimental to success. In fact they suggested there was little correlation between the numbers of an agent imported and the degree of success. The most recent study to investigate the relationship between release size and establishment of a biological control agent was undertaken by Memmott *et al.* (1998). This study determined that the size of the propagule was positively associated with successful establishment. The key question for managers becomes "should few large or several small releases be made?" Or should management decisions be flexible, depending on insect availability and the probability that environmental stochasticity or catastrophe will prevent successful establishment?

The only study to investigate the initial genetic diversity of the founders and to compare this to the final diversity in persisting populations was the investigation of the exotic ladybird beetle, *Propylea quatuordecimpunctata* (Coleoptera: Coccinellidae). This biocontrol agent was introduced to specific areas of USA and allozyme analysis was performed on feral populations from the USA and Europe and seven cultured populations (Krafsur and Obrycki 1996). All populations showed similar levels of heterozygosity, but massive releases of the cultured populations failed to establish. Factors other than genetic paucity were assumed to have caused the failures but there was no data to determine what did cause the failures. Memmott *et al.* (1998) investigated the importance of releasing differing founder numbers with respect to the probability of establishment. This study concluded that a larger number of the smaller populations became extinct and that there was a positive relationship between the size of the release and the probability of establishment, but it did not evaluate the levels of genetic diversity in, or between, the surviving populations. These few investigations do not allow any definitive conclusion on any impact loss of genetic diversity of environmental fluctuation may have on successful establishment.

1.2.6 Summary of key environment, demographic and genetic factors

For useful predictions about the success or failure of an organism's ability to establish, general rules must be developed that can be applied across a wide variety of prospective biological control

agents (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). It is unlikely that normal demographic factors will cause colony extinction because the reproductive rates of a species are normally in excess of mortality rates unless the population densities are being controlled by extrinsic interactions and regulations (Hastings 1997; Gotelli 1998).

Concerns about genetic issues arise because the importation, rearing and release of agents involve small founder numbers and bottlenecks with the prospect of inbreeding depressions and (or) the loss of individual fitness (Roush 1990; Hopper *et al.* 1993; Roush and Hopper 1995). Theory and empirical evidence suggest that organisms with rapid growth rates are capable of recovering from serious bottlenecks while still maintaining a high genetic diversity (Nei 1978).

The founder effect is a complicated interaction of the demographic parameters for each individual, the environmental fluctuations, catastrophic events and, finally, the genetic composition of each of the founders that formed the population. Field experiments using a biological control agent, like the horehound plume moth, allow these relationships between founders, environment and the influence of genetic diversity to be elucidated. They also provide empirical data that can be used to determine the usefulness of population viability analysis (PVA) and simple models of population management to be evaluated as management tools (Lacy 1992; Shea and Possingham 2000).

1.3 Molecular methodology used in the project

1.3.1 Background

In 1966, the use of allozyme electrophoresis revolutionized evolutionary studies by providing a tool to detect genetic variation in samples of individuals from different populations (Harris 1966; Lewontin and Hubby 1966). New tools that directly measure DNA variation at the molecular level have since been developed, and offer increased resolution in determining the genetic structure of populations (Avise 1994; Hoelzel 1998). These tools include analysis of microsatellite DNA and amplified fragment length polymorphisms (AFLP). When such techniques are integrated with information from field studies, comparative morphology and systematics, the results provide a powerful tool for the study of populations, evolution and adaptation (Baker 2000). The major

advantage in using molecular data is that DNA is inherited from both parents, the exception being mitochondrial DNA, which is inherited maternally (Connor and Ferguson-Smith 1993; Avise 1994) and can be more definitive than comparative morphology. More importantly, the genes and alleles controlling morphological, physiological and behavioural traits cannot be altered by non-genetic influences because the genetic bases and mode of transmission are fixed (Connor and Ferguson-Smith 1993; Avise 1994). This stability prevents phenotypic plasticity exhibited by the species under differing environmental conditions from confusing analysis of population structure.

Most populations have had their genetic structure influenced by demography and environment so genetic molecular markers can also provide information on geographical origins, mating systems and gene flow (Douwes and Stille 1987; Leberg 1991; Grant and Leslie 1993; Bacus *et al.* 1995; Hedrick 1995; Jaarola and Tegelstrom 1995; Jones *et al.* 1995; Pogson *et al.* 1995; Dowdy and MacGaughley 1996; Hedrick *et al.* 1996; Garciaramos and Kirkpatrick 1997; Tek Tay *et al.* 1997). Additionally, highly polymorphic molecular markers provide tools for assessing genetic identity and parentage (Avise 1994). The genetic basis of fitness is difficult to define and complicated demographic and behavioral factors can take precedence in determining fitness, but molecular markers may assist in clarifying the relationship between these factors (Slatkin 1987; Avise 1994; Remmert 1994; Hedrick and Gilpin 1997; Jones *et al.* 1997).

Markers must be evolving at an appropriate rate for the problem under investigation (Baker 2000). Choosing techniques that depend on highly conserved regions of DNA to detect variation is inappropriate when studying young populations (Baker 2000). Most studies of plants or animals below the level of genera will require markers such as amplified fragment length polymorphisms (AFLPs), microsatellites or allozymes (Baker 2000), which provide detailed data for estimating genetic diversity and homozygosity in newly released populations. The last two markers have the advantage of showing co-dominance, while AFLP techniques, although assumed to be dominant, can show many loci.

1.3.2 Microsatellites

In humans, most polymorphisms occur in the 97% of the human genome that encodes no proteins, which has been referred to as "junk" DNA (Avise 1994, Baker 2000). Such non-coding regions of DNA may be located within genes (where they are called "introns") or in between genes Avise 1994, Connor and Ferguson-Smith 1993). These regions do not code for proteins so it is assumed that variations within them are functionally inconsequential and hence well tolerated during evolution (Baker 2000). This has allowed tremendous genetic diversity to develop in these regions. Much of this non-coding DNA consists of highly repetitive segments of DNA consisting of several iterations of a specific sequence known at "DNA repeats". Such sequences, called Variable Number Tandem Repeats (VNTRs), are unique to each person and are the basis for the precise DNA fingerprinting used in forensics. One such class of sequences in humans consists of simple tandem repeats (STRs), often a dinucleotide (sometimes tri- or tetra-) repeat consisting of AC (adenosine and cytosine) on one DNA strand and GT (thymine and guanine) on the other (1994, Baker 2000). Such repeats of 2-5 nucleotide segments are known as microsatellite DNA and can be used with a single pair of PCR oligonucleotide primers, that surround such sequences, to produce variably-sized DNA fragments, depending upon the number of repeats in the microsatellite sequence (Baker 2000).

Microsatellites have been embraced as the marker of choice for many empirical studies because of their co-dominance and rapid rate of mutation (Scribner and Pearce 2000). There are several excellent reviews of the use of microsatellites in conservation biology (Bruford and Wayne 1993), genetic relatedness and paternity (Strassman *et al.* 1996), population genetics and evolutionary biology (Bruford and Wayne 1993; Blouin *et al.* 1996; Jarne and Lagoda 1996), and phylogenetic inference (Goldstein and Pollock 1997).

The function and evolutionary significance of microsatellites is unknown. It is believed they arise from DNA slippage during replication and, as a result, have high mutation rates (Levinson and Gutman 1987; Dallas 1992; Dib *et al.* 1993). They are also assumed to be selectively neutral, unless occurring within or adjacent to expressed loci (Litt and Lutty 1989; Sutherland and Richards

1995). One problem with micorsatellites is homoplasy, or the same sized fragments caused by mutations occurring at different sites within the fragment. Size homoplasy is common (Estoup *et al.* 1995; Shriver *et al.* 1995) and sequencing is the only way to differentiate such differences. Homoplasy can make statistical summaries difficult unless sequencing clarifies whether mutations are occurring at the same site or not.

If microsatellite loci are not already known, they can be found by standard cloning methodology from genomic libraries (Sambrook *et al.* 1989; Hoelzel 1998; Baker 2000). Several protocols are available which outline the isolation, primer development and PCR optimization and methods for the analysis of microsatellite DNA (Sambrook *et al.* 1989; Hoelzel 1998; Baker 2000). The polymorphisms are co-dominant and scored on the basis of fragment size for the alleles (Strassman *et al.* 1996). However, additional complications can arise because of non-amplification (null alleles) resulting from a mutation in one of the primer sites (Callen *et al.* 1993; Pemberton *et al.* 1995). The mutational model used to measure most genetic distances is based on the infinite allele model. There is some debate that this may not be the most appropriate model for microsatellite data, because mutations may occur in a manner that requires two or more step changes (Kwiatkowski *et al.* 1992; Weber and Wong 1993).

The abundance of microsatellites across the genome, high level of mutation and assumed lack of physical linkage between loci make them particularly useful for ecological applications. Microsatellite loci can differentiate the individual and population-level demographic processes and because they are co-dominant also indicate homozygous and heterozygous states (Hoelzel 1998; Baker 2000). Despite the problems of homoplasy and null alleles, and the questions of which models should be used in analysis, the detection of polymorphic variation has led to microsatellites becoming widely used in studies of kinship, population variation and evolution. Statistical methods of analyzing the results are continually being refined (Excoffier *et al.* 1992). They are particularly useful in detecting fine population structure, e.g. immigrant ancestors up to two generations in the past and parentage, even when overall differentiation of allele frequencies is low within the population (Hughes and Queller 1993; Rannala and Mountain 1997). Entire populations have been

analysed using microsatellites (Bruford and Wayne 1993) and estimations of effective population sizes have been made (Saccheri *et al.* 1998). Microsatellites were chosen for this study because they were co-dominant and provided detailed data, despite the fact they would have to be developed from scratch.

1.3.3 AFLPs

AFLPs were introduced by Vos *et al.* (1995) and are based on the detection of DNA fragments that have been formed by cutting total DNA with specific restriction enzymes. Subsets of these fragments are amplified using polymerase chain reaction (PCR) technology. AFLPs are used in population genetics for basic diversity and genetic variation studies, and are becoming popular because they can be developed rapidly without the need for complex isolation procedures. Vos *et al.* (1995) argued that AFLPs combined the reliability of restriction fragment length polymorphisms (RFLPs) with the power of rapid and relatively simple PCR techniques. RFLPs involve the cutting of DNA into thousands of different fragments at sites recognized by the restriction enzymes used. The different fragments are then resolved by gel electrophoresis because the fragments migrate at different rates, depending on their size. AFLPs involve the PCR amplification of a subset of these fragments, which still provides many loci but the banding patterns are more reliable and many more bands on the gel can be scored (Baker 2000). Powell *et al.* (1996) suggested that AFLPs provided high levels of resolution to allow delineation of complex genetic structures, whilst Winfield *et al.* (1998) concluded that AFLPs were reliable and informative multilocus probes. In general, AFLPs are well suited to molecular ecological studies, because they produce many polymorphic loci, but unlike conventionally developed microsatellite loci require no prior sequence knowledge and have minimal developmental time (Vos *et al.* 1995; Krauss and Peakall 1998). Other advantages include reproducible results with very low levels of genomic DNA required for analysis (Vos *et al.* 1995).

AFLP markers are thought to be largely dominant, with polymorphisms detected as either band presence or absence, and there may also be many alleles per locus (Hoelzel 1998; Kraus and Peakall 1998; Baker 2000). This may be a disadvantage because dominant markers are not as

efficient at detecting homozygosity as co-dominant markers for population genetics studies (Lewis and Snow 1992; Lynch and Milligan 1994). Lynch and Milligan (1994) estimated that 2-10 times more individuals need to be sampled per locus for dominant markers compared to co-dominant markers, but Krauss and Peakall (1998) suggest that this disadvantage may be overcome because of the large number of polymorphisms generated when using AFLPs.

Homology, like homoplasy in micorosatellites, is perhaps the greatest problem in AFLP analysis (Kardolus *et al.* 1998). Co-migrating bands are often assumed to be homologous, though there is no *a priori* reason to accept this. Furthermore, a particular sized band may consist of bands from different regions of the genome. Kardolus *et al.* (1998) argued that the chance that two co-migrating AFLP fragments do not represent identical alleles of one locus is small. This conclusion is based on the highly selective amplification and sharp resolution of polyacrylamide gel electrophoresis utilized in AFLP analysis. Given the large number of potential polymorphic loci, homology, unlike homplasy in microsatellites, may not bias the final conclusions about the genetic profile of an individual when compared to other individuals within the analysis.

Investigations using AFLP include studies into introgression, hybridization and population differentiation (Giaotto *et al.* 1997; Kraus and Peakall 1998; Krauss 1999; Reineke *et al.* 1999; Rieseberg *et al.* 1999). Reineke *et al.* (1999) used AFLPs to detect the genetic diversity within and between gypsy moth (*Lymantria dispar*) populations. The results indicated that a certain degree of genetic variability between European gypsy moth populations was explained by the accumulation of polymorphisms resulting from both historical population bottlenecks and adaptation to different environmental conditions. Reiseberg *et al.* (1999) looked at the hybridization and introgression between cultivated sunflowers and a sympatric wild sunflower *Helianthus petiolaris* (Asteraceae). The AFLP technique allowed the identification of 27 hybrid individuals and estimated low levels of introgression in the 4 sympatric populations studied. Beismann *et al.* (1997) examined the distribution of two willow (*Salix*) species and their hybrid and concluded that three distinct groups existed plus hybrids between the groups. AFLP markers have also been used at the level of the individual, for use in paternity analyses and gene-flow investigations. For example, Krauss &

Peakall (1998) analyzed paternity in natural populations of *Persoonia mollis* (Proteaceae), a long-lived fire-sensitive shrub from southern Australia. Krauss and Peakall (1998) achieved a level of polymorphism sufficient to assign paternity unambiguously to more than 99% of all seed in experiments. These studies demonstrated that AFLP analysis is a sensitive technique for distinguishing genotypes from different geographic origin and differences between local populations so could be used to identify population divergence, hybridization and paternity.

AFLPs are rapid to develop, produce individual fingerprints, are reproducible and require a low amount of DNA for the analysis (Vos *et al.* 1995; Beismann *et al.* 1997; Krauss and Peakall 1998; Reineke *et al.* 1999; Reiseburg *et al.* 1999). While there are problems with the technique, such as homology and dominance rather than co-dominance, it has been effectively used in many population studies. Given the simplicity of the technique and the advances in statistical analysis that can be utilized for this methodology (Excoffier *et al.* 1992; Schneider *et al.* 2000), AFLP analysis was used in analyzing populations in this study in order to determine genetic diversity within and between field releases.

1.3.4 Molecular data analysis

The advent of techniques such as microsatellites and AFLP allow the identification of multi-locus markers that provide vast variation, even in small populations. Both microsatellites and AFLP techniques allow the assessment of "genetic diversity" within and between species, cultivars and populations. The frequency of AFLP loci or microsatellite allele frequencies are used to calculate the levels and patterns of diversity between individuals. The markers are usually treated as independent, with diversities calculated using similarity measures between loci or alleles (Russell *et al.* 1998), Shannon's measure (Maughan *et al.* 1996; Zhu *et al.* 1998), or using analysis of molecular variance, AMOVA (Excoffier *et al.* 1992; Travis *et al.* 1996). Statistical procedures have been developed to utilise such data. These range from maximum likelihood approaches that assign individuals to populations, although these can miss individuals with rare alleles, to alternative methods of clustering algorithms based on genetic distances and shared alleles, such as those used to investigate human migration (Davies *et al.*, 1999).

Sampling error can cause a bias resulting in individuals being mis-assigned (Baker 2000). Recent advances in kinship studies are addressing the problems of sampling error and the possibility of more than one source population (Rannala and Mountain, 1997; Marshall *et al.* 1998). As fixed differences may not exist between recently isolated populations, or where immigration and migration occur at high levels, assignment tests are limited to the population level. The effect of genetic drift is not currently considered by this approach, but maximum likelihood methods of assigning individuals to populations may be useful for investigating recently founded populations, particularly when equilibrium may not have been reached.

There have been several statistical tests developed to examine whether molecular polymorphism can be used to investigate the fine structure of populations. Many of these are designed to elucidate the problems of population bottlenecks, sub-division or selective sweeps for alleles favourable to the specific conditions (Simonsen *et al.*, 1995). They all make a number of assumptions before they can be used with confidence (Lynch 1990). The first, a purely technical consideration, is that adequate controls are run to ensure against errors in assigning fragment identity across gels. Secondly, all individuals are assumed to be randomly selected from the population. Thirdly, co-migration of fragments can be resolved by band intensity or from other information. Fourthly, the same sets of loci are assayed. And finally, the principles of Hardy-Weinburg equilibrium hold within and between loci and the loci are not linked. But all five assumptions may not be valid. The first four are based on technical and controllable aspects but the fourth is not. Hardy-Weinburg equilibrium cannot always be assumed. Certainly, most of the assumptions of population equilibrium (e.g. Hardy-Weinburg principle) associated with the theory of population genetics do not hold for rapidly expanding populations (Excoffier *er al.* 1992).

Arlequin Ver 2. is a software package developed by Schneider *et al.* (2000) and designed to provide the user with a comprehensive package of methods and statistical analysis for a range of molecular technologies. It is available free from the Internet (http://www.anthro.unige.ch/arlequin) together with a detailed manual and explanation of the tests available. Both microsatellite and AFLP data

(entered under the RFLP category) can be analyzed with a variety of methods, such as genetic diversity and AMOVA within and between populations. This software package was used for the bulk of the statistical analysis on the DNA data obtained in this study. Details of tests used are outlined in the materials and methods section of the relevant chapters.

1.4 Predicting the success of establishment and release strategies for horehound plume moth

1.4.1 Predicting establishment and survival using population viability analysis (PVA)

Ecologists, including investigators in biological control, are concerned with predicting the interactions between species adaptations to habitat and persistence (Shaffer 1987; Soulé 1994; Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Predictive models require knowledge of the parameters that are critical in describing and extrapolating the patterns that contribute to the dynamics and long-term persistence of a population. Numbers required for persistence may be much higher than previously thought when genetic factors are considered together with the demographic and environmental factors (Lande and Barrowclough 1987; Shaffer 1987; Nunney and Campbell 1993; Korn 1994; Miller 1994; Saccheri *et al.* 1996; Lacy 1997; Soulé and Mills 1998). Historically, deliberate inbreeding may have been overestimated as a method for removing deleterious alleles in small populations (Willis and Weise 1997). In some studies the loss of genetic variation has been shown to result in an increased susceptibility to environmental fluctuation and catastrophe (Korn 1994; Willis and Weise 1997). Other studies indicated that there was an environment by genotype interaction (Miller and Hedrick 1993; Miller 1994; Savolainaen 1996; Koh *et al.* 1997), which supports the concept that selection operates at a localized level. A drawback from these conclusions is that even if the level of genetic diversity could be used to predict successful establishment, the fact that environment by genotype interaction occurs would mean that results for one site could not be used to predict the result at another. Additional information about the nature of such an interaction would be required before genetic diversity could be used for predicting establishment.

The assumption that demographic factors were more important than genetic issues in population persistence has been long accepted (Lacy 1997; Saccheri *et al.* 1998; Soulé and Mills 1998). Even

so, some authors considered the possibility that information on the long-term persistence of a species could be obtained from genetic marker data (Milligan *et al.* 1994; Saccheri *et al.* 1998). Pal (1996) used a bit-string model of biological aging to show that the extinction probability of a small population was preceded by a decline in genetic fitness. In their empirical studies on the Glanville fritillary butterfly (*Melitaea cinxia*) using eight polymorphic loci, Saccheri *et al.* (1998) concluded the extinction risk increased significantly with decreasing heterozygosity, even after accounting for the demographic, ecological and environmental factors. This provided support the notion that molecular markers could be used to determine the genetic status of a population and this might help estimate the probability of continued survival for that population.

Currently, genetics and demographic forces seem to be treated as distinct concepts in most models. Given the fact that final conclusions reached by simulations using one concept or the other may be similar, is it necessary to include genetic parameters in models at all (Ewens *et al.* 1987; Nunney and Campbell 1993)? The time period over which the probability of survival is considered also alters the likelihood of extinction (Soulé 1994; Hastings 1997). This means that it is increasingly complex to adequately model biological reality, especially if fine details are to be included, unless general rules can be applied (Levins 1969; Hanski and Gilpin 1997). The timeframe of simulation also affects predicted genetic diversity. For example, the longer the time frame, the more likely new mutations would appear in the population and, if neutral or favourable, contribute to increased genetic diversity.

The first point of interest for those managing recent introductions of biological control agents is whether populations establish and grow to a point where only a large-scale catastrophe event would drive them to extinction (Shaffer 1981; Soulé 1987; Wissel *et al.* 1994; Memmott *et al.* 1998; Shea and Possingham 2000). For example, given the risk of extinction over a certain time period, can a population level be determined that will ensure, within set probability limits, continued survival? Determining such population levels could assist managers in determining release sizes for biological control agents. If levels of genetic diversity were found to influence establishment, then including genetic diversity as a parameter in computer simulations would assist in management

decisions such as active intervention via re-introduction of individuals or the establishment of mating programs to preserve rare alleles. The assumption is that once the population reaches a certain level, or critical mass, the probability that extreme environmental or demographic fluctuations will wipe it out is extremely low (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000).

Overall, the main factors, including the variation surrounding them, which must be considered in any model, include:

- The probability that a certain number of individuals produce in a certain number of adult offspring i.e. birth rates, mortalities,
- Density dependence i.e. Allee effects, resource competition, carrying capacity.

Genetic differences among individuals introduce complications that do not operate in isolation. The differences may influence the population through a variety of characteristics, such as disease resistance or reproductive success, i.e., by affecting the demographic characteristics (Hedrick *et al.* 1996; Lacy 1997). This means that that many of the genetic factors affecting fecundity and survival could possibly be incorporated into existing models via the demographic parameters, such as births and mortalities. But what of the possible increasing effect of more and more deleterious alleles becoming homozygous as population numbers continue to decline? For example, Tanaka (1997) modelled the effects of deleterious mutations by decreasing the growth rate and increasing the carrying capacity of a small population. The result was the prediction of rapid extinction of the population due to the synergistic interaction between inbreeding depression and declining population size. This may mean that the only way to address the problems of deteriorating genetic diversity is to ensure they are part of the model structure, assuming there was reasonable evidence that inbreeding depression was likely to occur.

The effect of genetic stochasticity on populations is more complex than the variation due to the environment and demographic stochasticity (Lande and Barrowclough; 1987; Lacy 1997). This is because genetic variation continues to be regarded as essential for both viability and continued

adaptation in a changing environment, but the exact measurement of such interactions have not yet been quantified (Gilpin 1987; Lande and Barrowclough 1987; Shaffer 1987; Lindenmayer *et al.* 1994; Frankham 1995b). The combination of inbreeding, demographic data and environment introduces complexity that is difficult to simulate. Generic PVA software programs already exist, but only one (VORTEX), has been designed to incorporate genetics and inbreeding along with demographic and environmental stochasticity (Lacy 1992). This program has been retrospectively tested using empirical data available for several species with varying degrees of success (Lindenmeyer *et al.* 1994; Lacy and Lindenmeyer 1995; Novellie *et al.* 1996; Brook *et al.* 1997b). But how useful are such existing PVA packages as management tools for predicting successful establishment of a biological control agent and does the inclusion of a genetic parameter influence the prediction outcome?

Among those cases in which PVA using VORTEX has been applied is the Lord Howe Island Woodhen (Brook *et al.* 1997b). Brook and his co-workers (1997b) noted that it was necessary to use density dependence in order to obtain results that corresponded to empirical observations and concluded that the synthesis of genetic and demographic influences required more realistic interpretation of events. A study of the Mountain Brushtail Possum used PVA to examine the effect of demographic fluctuations, genetic drift and the interaction between these two factors (Lacy and Lindenmeyer 1995). The key findings in this study, as far as the use of VORTEX was concerned, was that it was extremely important to estimate the size, number and isolation of the sub-populations targeted or results were unrealistic. Novellie *et al.* (1996) used VORTEX to simulate the long-term re-introduction of an endangered large mammal. The authors concluded that the predictions reached using VORTEX were similar to those observed in the real populations.

It has been suggested that using existing packages, retrospectively tested, may be preferable to writing a one-off simulation, which could not be tested retrospectively and may never be used again (Lindenmayer *et al.* 1993; Brook *et al.* 1997b). VORTEX has not been used to retrospectively test the likelihood of successful establishment for the release of an insect biological control agent and the data obtained for the field releases of the horehound plume moth in this study will provide

an opportunity to determine if VORTEX can adequately simulate the probability of establishment for an insect and to evaluate the importance of the genetic component of the program.

1.4.2 Planning optimal release strategies for biological control agents using stochastic dynamic programming (SDP)

Two recognised factors to improve the likelihood of success exist; these are increasing the size of the release and making more releases (Beirne 1985; Hopper and Roush 1993). However, decisions on release strategy are constrained by the ease of rearing the agent as well as the total number available for release (Memmott *et al.* 1996; Grevstad 1999; Shea and Possingham 2000). SDP is a formal mathematical approach to assist decision-making and is being used increasingly to investigate such problems at the population level (Clark 1990; Shea and Possingham 2000).

Shea and Possingham (2000) used an SDP approach to calculate an optimal strategy set, which would provide rules of thumb for decision making when deciding on strategies for releasing classical biological controls. The state transitions for a possible release site were used to build a simple model to calculate release strategies. Shea and Possingham (2000) reported that one of the most important considerations was the relationship between the release size and the probability of establishment. As a result, Shea and Possingham's (2000) investigation of the parameters of importance for the model showed that parameter, *a*, which defined the slope on the probability of establishment equation, defined the shift in which optimal release strategy would be the most suitable to ensure success of a release (Shea and Possingham 2000).

Shea and Possingham (2000) defined the probability of establishment, P(x), as:

$$p_m(1-e^{-ax^2})^2 \qquad (1)$$

where p_m is the maximum probability of establishment, x is the number of founders and a is a constant. This generates a sigmoidal curve and represents an increasing probability of establishment with increasing founder size until a maximum probability of establishment is achieved (Shea and Possingham 2000). In other words, as the value of the constant, a, increases, smaller founder sizes are possible that still provide a maximum probability of establishment.

Combining PVA and SDP to predict the outcome of field releases for a biological control agent has not been tested with empirical data. This seems surprising given that the probability of establishment, or population survival, is recognised as the most important initial step in the success of a classical biological control program (Hopper and Roush 1993; Memmott *et al.* 1996; Grevstad 1999) and that this step was also the most important parameter for determining optimal strategies for release (Shea and Possingham 2000). The data obtained in this study would allow the retrospective testing of PVA and SDP models, such as Shea and Possingham's (2000), to determine if this approach would accurately predict potential release strategies and the successful establishment of biological agents like the horehound plume moth.

1.5 Aims of the project

The release of a biological control agent is associated with high costs (Memmott *et al.* 1998). These costs include identification, testing, importation, captive breeding and ultimately the release and monitoring of an agent in the field (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Predicting the minimum number of individuals that can be released and still achieve high rates of establishment is an important goal for biological control practitioners (Memmott *et al.* 1998, Grevstad 1999; Shea and Possingham 2000). It is also important to remember that immigration of individuals, bringing new genetic material, will not occur unless managers decide to supplement the colony with further releases. Consequently inbreeding depression, bottlenecks and population isolation may also affect the likelihood of successful establishment (Roush 1990; Hopper *et al.* 1993; Roush and Hopper 1993). The implications of re-releasing allopatric lines should also be considered given that outcrossing has been observed to cause an outcrossing depression in some cases (Legner 1971; Soulé 1986; Berger and Cunningham 1995). An understanding of how the relationships between the environment, demographics, founder numbers and genetic deterioration hamper or assist population establishment is required before robust decisions on release sizes can be made.

Management decisions on release strategies become a juggling act. For each potential release site releasing too many or too few individuals becomes a waste of resources. If the number of individuals being released can be reduced then the number of planned releases can be increased to more rapidly spread and saturate the pest population over a wider region and reduce the costs associated with laboratory rearing. The dilemma for management is that the cost of effective releases must be balanced against the knowledge that:

- Smaller releases may have a higher risk of extinction in the early stages (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000) and also take longer to achieve population numbers where control or damage of the target species can be measured because release size may be related to population growth rate (Roush, pers. comm.),
- Many releases could assist in buffering neighboring colonies from local extinction by providing the opportunity for re-colonization or immigration (Hanski 1998; Saccheri *et al.* 1998),
- Isolated, large releases can be wiped out by localized catastrophe (Soulé 1986; Quammen 1996; Saccheri *et al.* 1998),
- High levels of inbreeding could affect population fitness to a level where population numbers decline and eventual extinction results (Ralls and Ballou 1983; Hedrick 1986; Pray *et al.* 1994: Frankham 1995b; Hedrick *et al.* 1996; Frankham 1997; Frankham 2000).

The role of a biological control program is to establish populations that are capable of persisting and increasing without further manipulation in order that the target species is controlled at some detectable level. Some of the issues concerning managers of biological control programs were examined in this study, which used spatio-temporal census data collected over 12 months for varying release sizes to address some of the theory surrounding the founder effect, environmental and seasonal influences on population persistence. The release number, plant quality at release sites

and timing of releases of horehound plume moths were examined to determine the influence each parameter had on the probability of establishment.

The census data were augmented with molecular information obtained from the DNA of harvested individuals and used to investigate if, and how, decreasing genetic diversity was correlated with population growth rates for the horehound plume moth. Empirical data regarding genetic diversity and population establishment are necessary in order to test the current theories that overall population fitness and/or possible future adaptation in a novel environment is dependent on avoiding inbreeding at all costs (Roush 1990; Hopper *et al.* 1993). Increased understanding of the relationship between genetic diversity and population establishment for the horehound plume moth will improve the understanding of how genetic factors influence problems, not only for rearing laboratory colonies, but for determining strategies that will increase the probability of successful releases of the horehound plume moth. It is hoped such information can start extrapolation to provide general rules of thumb that can be applied to other biological control agents.

The assumption that outcrossing between two different collections of the same species would restore genetic variation, improve individual fitness and the flexibility for coping with environmental challenges may not always be valid (Legner 1971; Berger and Cunningham 1995). Two laboratory populations of horehound plume moth existed. These originated, and were collected, in different geographical areas in Europe and were used to determine if an outcrossing depression could be detected for the horehound plume moth colonies released in the field.

The effectiveness of deliberately purging a population of any deleterious genetic load, so that the long-term fitness is not compromised, appears not to have been investigated for a biological control agent. The success of such a program to purge the genetic load from individuals without increasing the probability of extinction is of major concern (Legner 1971; Hedrick 1994; Berger and Cunningham 1995). The horehound plume moth was used to investigate if laboratory purging of lethal alleles was possible and beneficial.

The final focus of the investigation centred on the use of PVA and determining optimal release strategies for the horehound plume moth. Ecologists and conservation managers have embraced the use of predictive models for PVA, but such approaches have lagged in the area of biological control (Shaffer 1987; Soulé 1994; Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Despite the fact that biological agents are carefully selected for a specific purpose, they are potentially invasive colonisers of a novel environment. While each agent would have to be treated on a case-by-case basis because of some uncertainty about how they will react to a novel environment, or what new conditions they will encounter, predictive decision making tools like PVA and SDP may provide useful rules of thumbs if they can be shown to be based on robust rules that can be applied across a number of species. The empirical data from all chapters of this study provided a unique opportunity to retrospectively test the use of existing generic models via a practical and direct comparison of observed and simulated results.

2. Development of release strategies for classical biological control agents

2.1 Introduction

Many plant species that have been accidentally, or deliberately, imported into Australia have managed to successfully invade natural eco-systems. Not only do they threaten native habitat but they also affect agronomic systems, often costing many thousands of dollars per year in terms of lost productivity or environmental degradation. Managing such invasions using herbicides and other methods is often extremely difficult. Classical biological control offers an alternative approach. Initially, these programs seem costly but biological control agents can form one part of an integrated management system that eventually allows the pest to be reduced to densities where they can be controlled relatively cheaply and easily or tolerated. Indeed, in some cases where the weeds have a wide distribution over land with a low economic value, biological control is probably the only viable option. Some of the major theoretical issues surrounding the selection, rearing and release of biological control agents are still being debated (Ehler 1990; 1998). More importantly, conservation concerns and the expense of biological control programs have resulted in increased demands for a more predictive approach to the introduction of natural enemies (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). Greater attention to establishing the validity of these hypotheses is required to ensure that predictive approaches to classical biological control are based on sound scientific foundations and not poorly validated generalizations.

Waage (1990) argued that failure of initial establishment was as important a reason for the failure of biological control programs as the agents' inability to reduce host densities. In recent years several studies have been undertaken to provide empirical data that will shed light on the debate and issues surrounding initial establishment. These include investigations into release strategy

(Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000), release number (Hall and Ehler 1979; Pimm 1991; Cameron *et al.* 1993; Hopper and Roush 1993; Krafsur and Obrycki 1996; Memmott *et al.* 1998), inbreeding (Krafsur and Obrycki 1996), island versus mainland establishment (Greathead 1971; Hall *et al.* 1980; Greathead and Greathead 1992), geographical and climatic origin of the agent (Hokkanen and Pimental 1984; Stiling 1990, Clarke 2001), habitat stability (Hall and Ehler 1979; Hall *et al.* 1980) and resource/host quality (Wilson 1960; Van den Bosch and Telford 1964; Harris 1981; Room and Thomas 1985; Center *et al.* 2000). Many of these studies are retrospective and opportunistic, but still provide useful conclusions about the validity of the hypotheses under investigation.

Given the requirement for successful establishment before spread or damage can occur, the paucity of published field tests specifically designed to investigate release number and time (i.e season) of releases is disappointing. Grevstad (1999) and Shea and Possingham (2000) used computer simulations and models to investigate release strategies. Grevstad (1999) concluded that, where environmental variability was high, a large number of very small releases would maximise the chance of overall establishment but when environmental variability was constant and an Allee effect occurred a single large release was optimal. Shea and Possingham (2000) concluded that the key factor in determining release strategy was the shape of the curve that defined the probability of establishment for various founder sizes and suggested that various release strategies existed, depending on whether or not there were existing successful sites. The only experiments that have investigated release size and strategy for biological control agents were conducted by Campbell (1976) and Memmott *et al.* (1998) with Memmott *et al.* (1998) the only researchers who have recently undertaken an experimental field release with the deliberate aim of optimising the number and size of releases required to ensure successful establishment. The authors found that the size of the release was positively correlated with successful establishment. However, unlike Campbell (1976), Memmott *et al.* (1998) did not include the issue of timing releases in optimising the probability of establishment.

The following study was designed along a similar approach to that of Memmott *et al.* (1998), with the addition that several releases were made at different times of the year. The effect of release size, plant quality over a 12 month study period and how season of release impacted on population establishment of horehound plume moth were investigated. The empirical data obtained was also examined in the context of conclusions reached by Gravstad (1999) and Shea and Possingham (2000). It is hoped the results will be of wider relevance in biological control and related disciplines by elucidating the interaction of these key factors on the likelihood of successful establishment and highlighting important parameters for determining optimal release strategies.

2.2 Materials and methods

Replicated sets of field releases using treatments varying in founder number were made between spring 1999 and spring 2000 (Table 2.1). Fifth instar larvae from the Zaragoza laboratory colony were placed onto large healthy adult horehound plants at selected sites in the Flinders Ranges (FRNP) and Coorong (CNP) National Parks (Table 2.2, Figure 2.1). The FRNP region was designated as a low plant quality area, while the CNP was designated as a high plant quality area on basis of temperature variation (Figure 2.2), rainfall (Figure 2.3) and host plant quality. The criteria used to differentiate plant quality were visual assessment of: the size and lushness of the individual leaves, the amount of foliage, the frequency of flowers and evidence of die back. The plants in the FRNP showed signs of prolonged water stress with small, pale, hirsute leaves. In contrast, the plants in CNP carried heavy foliage, even in high summer, and the leaves were lush, green and less hairy.

Table 2.1. Summary of the treatments and number of replicates for horehound larvae released in high (Coorong, CNP) and low (Flinders Ranges, FRNP) plant quality areas.

Treatment	No. replicates	No. larvae released	Plant quality	Location
1	9	10	High	CNP/Pitlochry
2	9	30	High	CNP/Pitlochry
3	9	90	High	CNP/Pitlochry
4	7	270	High	CNP/Pitlochry
5	6	10	Low	FRNP
6	6	30	Low	FRNP
7	6	90	Low	FRNP
8	6	270	Low	FRNP

A set of releases was also made at another area in the Coorong region, on a very lightly grazed property ("Pitlochry") adjacent to the National Park (Table 2.2). The FRNP releases were all made in the spring of 1999, but the CNP releases were staggered over the year to correspond with the spring, summer, autumn and winter seasons in South Australia. In the high plant quality areas (CNP and "Pitlochry"), 5 releases of all treatments were made in spring 1999, followed by 2 releases of all treatments in summer of 1999 ("Pitlochry") and 2 releases of all treatments, except that of 270 individuals (due to the high success rate of establishments, even at 30 and 90), in autumn 2000 ("Pitlochry"). Sites for all treatments within a replicate were separated by 0.5 km so that over the 12-month experimental period, no overlap of populations at release sites was expected. The distance between releases was based on Clarke's (2001) estimation of dispersal rates of around 50 m per generation.

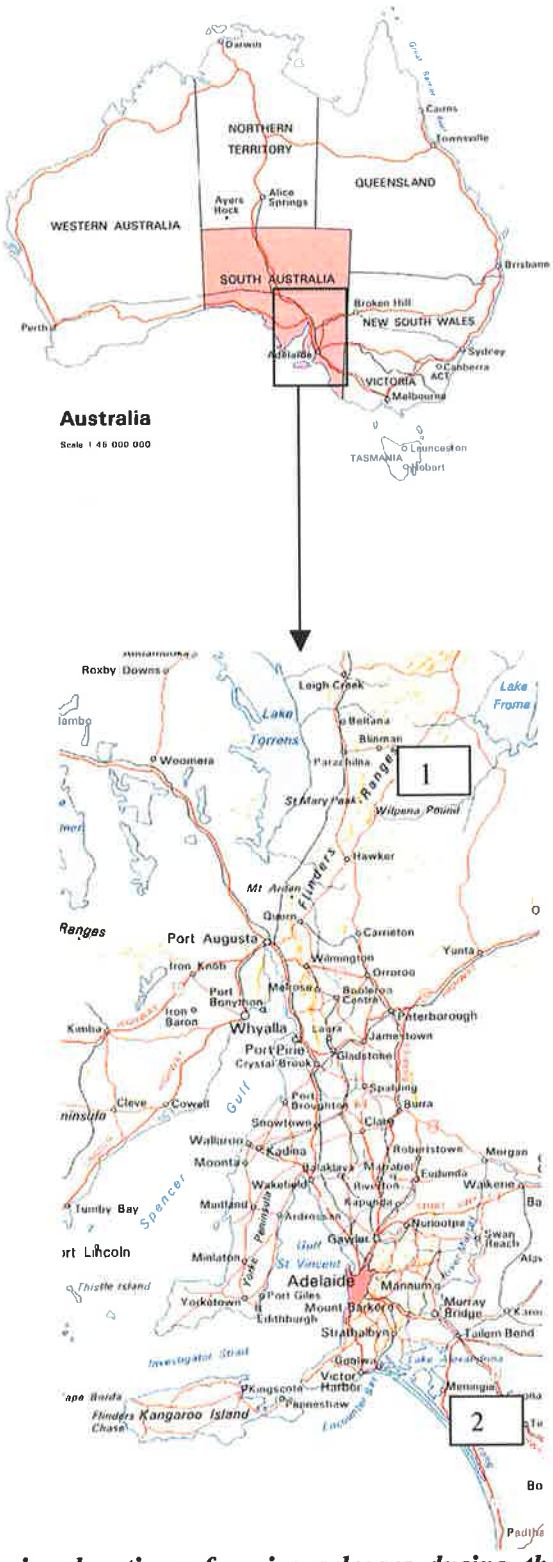

Figure 2.1. Map showing location of major releases during the course of the experiment. Location 1 = founder size and timing of release, Coorong/Pitlochry. Location 2 = founder size, Flinders.

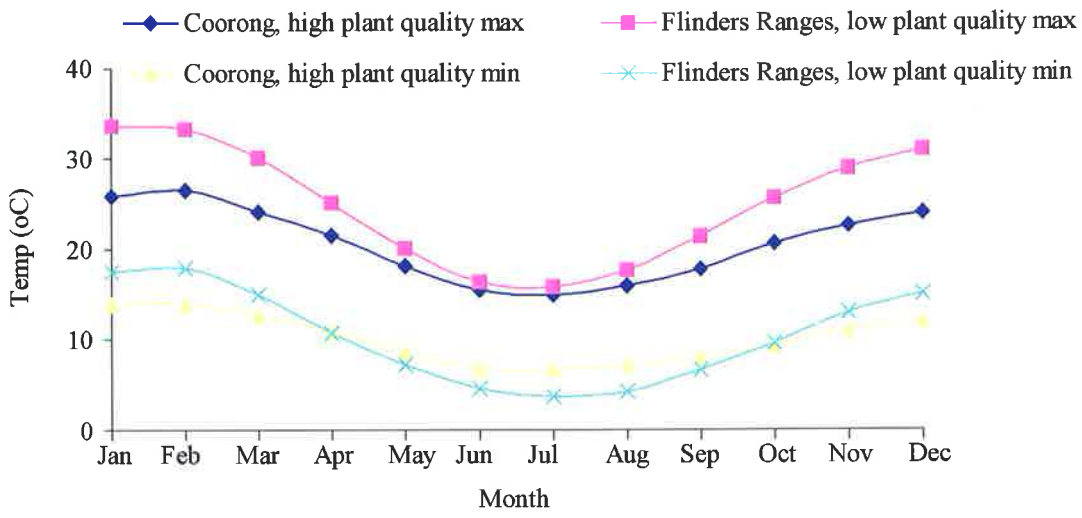

Figure 2.2. Mean average temperatures over 12 months for Coorong (high plant quality) and Flinders Ranges (low plant quality) release areas (source of data: Bureau of Meteorology).

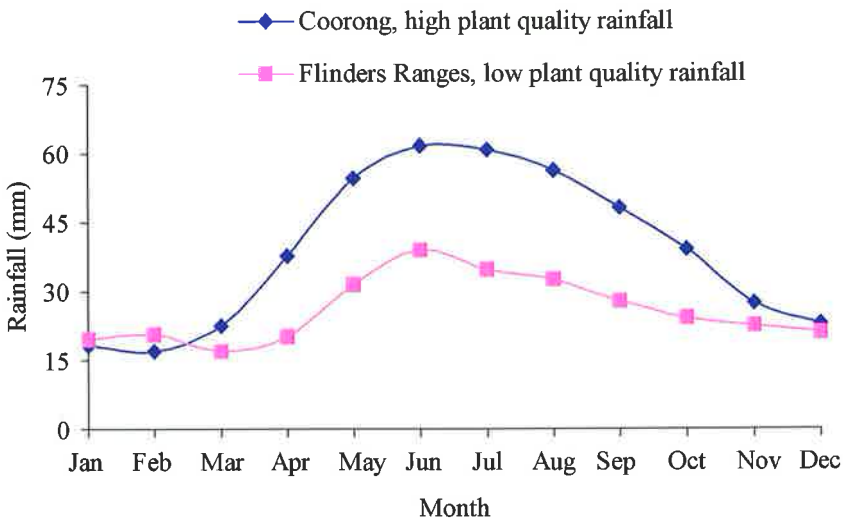

Figure 2.3. Mean average rainfall over 12 months for Coorong (high plant quality) and Flinders Ranges (low plant quality) release areas (source of data: Bureau of Meteorology).

Table 2.2. Co-ordinates for the central release points for major releases during the course of the project. References for central points are provided because it was assumed that after 4 generations overlapping of release sites would occur.

Region	Australian Geographical Map Number	Location	Northing	Easting
Flinders Ranges	6635-2 54JTL	Mt. Billy Creek	65245	2718
	6635-3 54JTL	Dingley Dell	65288	2816
	6635-3 54JTL	Brachina	65307	2725
	6635-3 54JTL	Yanyanna	6524	2718
	6635-3 54JTL	Bunyeroo	6563	2732
	6635-3 54JTL	Jones Camp	65151	2758
Coorong	6825-IV 54HUE	Parnka Point	60279	3558
	6825-IV 54HUE	Treloars 1	60045	3755
	6825-IV 54HUE	Treloars 2	60044	3754
	6825-II 54HUE	Pitlochry	59809	3876
	6825-II 54HUE	Cantara	5977	3867

Percent horehound cover was calculated for all sites where 270 individuals were released and was determined using the Levy point quadrant method (Levy 1927; Crocker and Tiver 1947) during spring 1999, when larvae were first released, and again in the spring of 2000, 12 months later. A total of 1 000 points were recorded in order to estimate the percentage horehound cover.

Total population numbers of live insects were also estimated periodically over the 12-month period, with the exception of winter (when diapause was assumed) (Table 2.3). The quadrant point samples were taken by measuring lines marked by points of the compass (i.e., north, north east, east, south east, south, south west, west and north west) and using a 0.5 m^2 square placed every 2 m along the lines until 4 consecutive readings of no insects were noted. Insects in the quadrants were

counted and used to estimate the total population at each release site. Estimates of total insect density were averaged for all quadrants recorded at the same distance. The total area between each set of equidistant quadrants was used to calculate the area in each 'ring', i.e., the 2m quadrant readings were used to estimate the number of insects in the centre circle with a radius of 2m, the 4m quadrants used to estimate the number of insects in the 2-4m ring and so on by using the area of each ring to multiply the average number of insects/m^2. This gave population estimations over a 12-month period for releases made in spring 1999 and a lesser time period for the other releases, depending on time of release.

Table 2.3. Release and census date for releases used in the development of release strategies for the horehound plume moth.

Release site	Location	Plant quality	Treatments released	Release Season	Release Date	Season and date surveyed			
						Spring '99	Summer '99	Autumn '00	Spring '00
Pitlochry	Coorong	High	1-4	Spring '99	24-Aug-99	12-Oct	12-Dec	07-Mar	21-Aug
Pitlochry	Coorong	High	1-4	Summer '99	10-Dec-00			07-Mar	21-Aug
Pitlochry	Coorong	High	1-4	Summer '99	10-Dec-00			07-Mar	22-Aug
Pitlochry	Coorong	High	1-3	Autumn '00	7-Mar-00				22-Aug
Pitlochry	Coorong	High	1-3	Autumn '00	7-Mar-00				22-Aug
Parnka Point	CNP	High	1-4	Spring '99	12-Aug-99	24-Sep	10-Dec	08-Mar	14-Aug
Mulberry 1	CNP	High	1-4	Spring '99	12-Aug-99	24-Sep	10-Dec	08-Mar	15-Aug
Mulberry 2	CNP	High	1-4	Spring '99	12-Aug-99	24-Sep	11-Dec	09-Mar	15-Aug
Cantara	CNP	High	1-4	Spring '99	12-Aug-99		11-Dec	09-Mar	16-Aug
Mount Billy	FRNP	Low	1-4	Spring '99	16-Aug-99	05-Oct	05-Dec	11-Mar	24-Aug
Jones Camp	FRNP	Low	1-4	Spring '99	17-Aug-99	05-Oct	06-Dec	12-Mar	26-Aug
Bunyeroo	FRNP	Low	1-4	Spring '99	17-Aug-99	05-Oct	06-Dec	12-Mar	26-Aug
Yanyanna	FRNP	Low	1-4	Spring '99	17-Aug-99	05-Oct	07-Dec	12-Mar	26-Aug
Brachina	FRNP	Low	1-4	Spring '99	17-Aug-99	05-Oct	05-Dec	12-Mar	24-Aug
Dingely Dell	FRNP	Low	1-4	Spring '99	17-Aug-99	05-Oct	05-Dec	13-Mar	24-Aug

Horehound plume moth populations with 12-months of data were used to investigate the effect of founder size. The seasonal data consisted of a subset of the releases made in high plant quality areas ("Pitlochry"). This was done in order to minimize effects from changes in plant quality or percentage cover between release points, and the data used were from the censuses closest to day 167 after release. Day 167 was used because this was the maximum length of time the latest autumn release had been in the field.

Statistical analysis was undertaken using the software package SAS JMPIN 3.2.1. The untransformed data was checked for heterogeneity of variances using Brown-Forsythe test for unequal variances. Depending on the results, further analysis was applied to transformed or untransformed data to determine the level of significance for the main effects in the experiment. Microsoft Excel 9.0 was used to calculate Pearson's *r* values to investigate any association between independent and dependant variables. In this test *r* is a measure of correlation whose value lies between –1 and +1. Values of *r* near zero indicate a low level of correlation, negative values of *r* indicate low values of X are associated with high values of Y and positive values of *r* indicate high values of X are associated with high values of Y (Meddis 1975). The statistical significance of *r* can be estimated by computing *t:*

$$t_{N-2} = \frac{r}{\sqrt{\left[\left(1-r^2\right)/\left(N-2\right)\right]}} \qquad \text{(equation 1)}$$

where *t* has N-2 degrees of freedom and may be assessed using the critical value for the Student's *t* distribution (Meddis 1975). This test assumed the samples were drawn at random from a normally distributed population and the variables were not correlated (Meddis 1975).

Microsoft Excel 9.0 was used to plot the total population numbers for high and low plants releases, season of release and probability of establishment versus the founder size, to insert the linear trend lines. Microsoft Excel 9.0 spreadsheets were used to fit a sigmoidal curve, the general definition of which was described by Shea and Possingham (2000), to high and low plant quality releases with 12 months of data.

2.3 Results

2.3.1 Effect of size of release on probability of establishment for high and low plant quality releases

The results presented in this section are for the replicates with census data over the full 12-month period for both the high and low plant quality areas. These consisted of 5 and 6 replicates for each of the founder sizes (10, 30, 90 and 270) released in high and low plant quality regions respectively.

Brown-Forsythe testing for homogeneity of variances around the final recorded populations at 12-months, over the release sizes, indicated they were not equal ($F_{3,39} = 3.06$, $p < 0.04$). Consequently, both release size and the final recorded population at 12-months were log transformed in order to stabilize the variances before investigating the statistical significance of location and release size and any interaction between them in the model. The results show that both location and release size influence the outcome, although release size was the most significant factor (Table 2.4) with the ANOVA for the whole model having an $F_{3,39} = 39.98$, $p< 0.0001$. The Pearson's *r* test indicated that there was a positive relationship between size of the release and the population number after 12 months, irrespective of plant quality (Pearson's R for high plant quality areas = 0.70, $p<0.0004$, 18 df, Pearson's R for low plant quality releases = 0.40, $p<0.06$, 22 df).

Table 2.4 Effect test of the main factors effecting the population sizes at 12 months using log transformed data for original release size and recorded population at 12 months (JMPIN 3.2.1).

Source	Nparm	DF	F ratio	Probability of F
Location	1	1	3.376	0.074
log(Release size)	1	1	14.188	0.001
Location * log(release size)	1	1	1.585	0.201

The equation defining the general relationship between the expected number of insects recovered and the number of insects released 1 year earlier (Memmott *et al.* 1998) is

$$E(r) = kRN \qquad \text{(equation 4)}$$

where $E(r)$ = the expected number of insects recorded, k = probability of finding an insect on the plant, R = net individual rate of increase and N = original number of founders. Both k and R may depend on N, for example, if an Allee effect existed. To estimate k as a separate variable would have required releases of different numbers of a known number of individuals in high and low plant quality areas, followed by a census to determine the probability of detecting the larvae at known densities. This was not undertaken. Consequently, k and R were defined as a field estimate of the individual rate of increase (Memmott *et al.* 1998).

The data for the total population sizes and the release sizes for the different plant quality releases indicate that the high plant quality releases appear to have a consistently higher net replacement rate than those released where plant quality was low (Figure 2.4). Based on the relationship described by equation 4, the field estimates of the individual rate of increase, kR, were 161 and 2 for high and low plant quality releases, respectively.

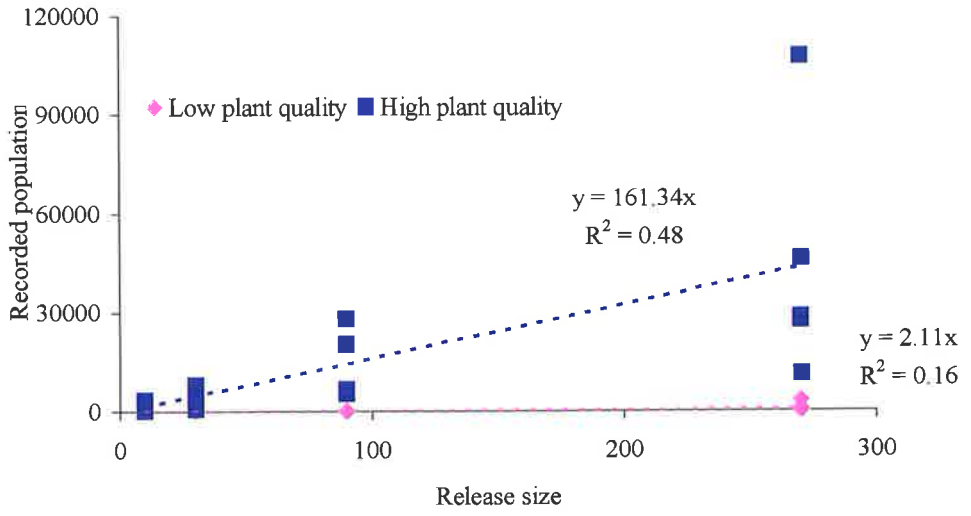

Figure 2.4. Estimated total population sizes for established horehound plume moth colonies after 12 months, for high and low host plant quality release sites showing linear trendline for each.

A sigmoidal curve was used to describe the relationship between the proportion of populations establishing and the release size, with the maximum probability of establishing set at 1. The likelihood of establishment where plant quality was low was more variable, with a lower probability of successful establishment observed for all release sizes, when compared to establishment in high plant quality areas (Figure 2.5). The likelihood of the maximum probability of establishing being less than 1 is discussed in chapter 8, but may explain the poor fit for the data from low plant quality areas. For the different levels of host plant quality, the relationship took the following form

High plant quality $$P_{est} = \left(1 - e^{-0.0082 * x^2}\right)^2$$ (equation 5)

Low plant quality $$P_{est} = \left(1 - e^{-0.000005 * x^2}\right)^2$$ (equation 6)

where P_{est} = proportion of populations establishing and x = release size. Two tailed T-test comparison between the percentage of successful colonisations in the different plant quality locations indicated significance differences in the ability to establish depending on whether the release was made in a high or low plant quality area (t_6=6.38, $p<0.0007$).

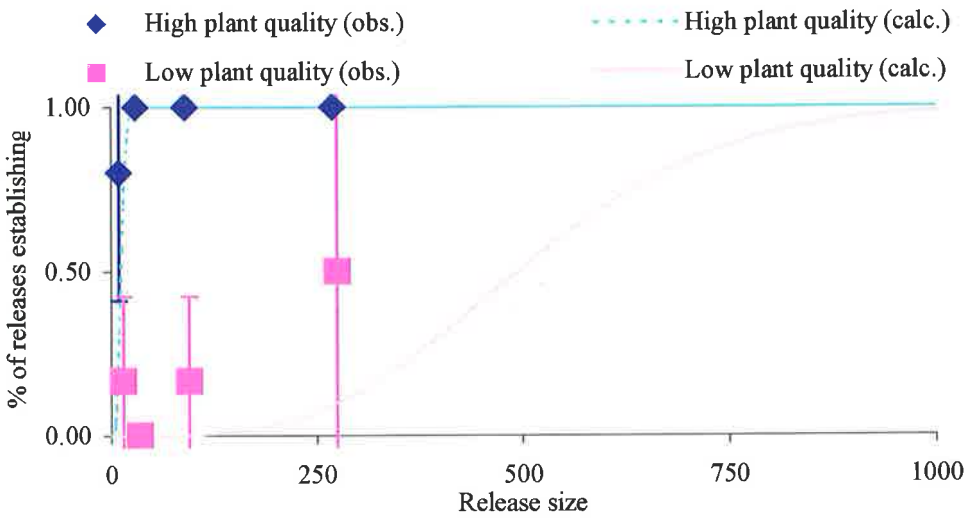

Figure 2.5. The proportion of horehound plume moth populations that successfully established over a 12-month time period in both high and low plant quality locations, based on 5 and 6 replicates for each release size of 10, 30, 90 and 270 for high and low plant quality areas, respectively. Error bars represent the 95% confidence intervals, high and low plant quality results are offset to allow the error bars to be clearly visualized.

One issue highlighted in this study was that, despite attempts to ensure a high number of replicates for final analysis, the logistics of breeding adequate numbers of insects for experimental use was constrained by space and time. This was exacerbated by extinction events, particularly in the FRNP. Low numbers of replicates impact on statistical significance by reducing the power of the statistical analysis.

2.3.2 Effect of size and season of release and probability of establishment in high rainfall areas

The data used to investigate seasonal effects came from releases made on the grazing property in the high plant quality area. Trends suggested that spring releases were more successful than releases made at any other time of the year (Figure 2.6).

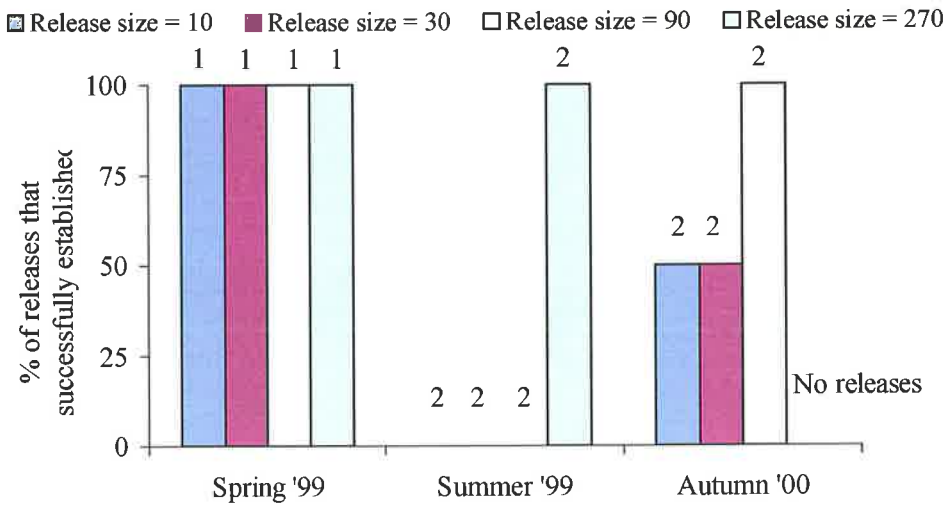

Figure 2.6. The percentage of horehound plume moth populations that successfully established over 4-7 months for high plant quality release areas by season. The number shown above each bar is the number of replicates initially released for each release size. There was no 270 size release made in autumn '00.

More extinctions occurred for summer releases than any other time of the year, but exact comparisons were not difficult because of differences in census dates and low sample sizes. The Brown-Forsythe test on the recorded population at 12-months for each release size indicated that the variances were unequal and both variables were log transformed before further statistical analysis ($F_{2,15} = 6.407$, $p = 0.01$). Analysis of the effect of season and release size indicated that the release size was statistically significant and that season and release size were interacting, although this interaction was only significant at $p = 0.08$ (Table 2.5). Pearons r values for the recorded population versus the release size were 0.99 ($p<0.01$, df = 2), 0.91 ($p<0.002$, df = 6) and 0.97 ($p<0.001$, df = 4) for spring, summer and autumn releases respectively.

Table 2.5 Effect test of the main factors effecting the population sizes at 12 months using log transformed data for original release size and recorded population at 12 months (JMPIN 3.2.1).

Source	Nparm	DF	F ratio	Probability of F
Season	2	2	0.196	0.824
log(Release size)	1	1	30.331	0.0001
Season * log(release size)	2	2	3.201	0.077

Plotting the recorded population size against the size of the original release indicated that the net rate of individual increase in the field the kR values for spring, summer and autumn releases were 6, 0 and 2 respectively (Figure 2.7). This suggested that both spring and autumn releases would increase in size as the net rate of individual increase is greater than 1, but that the summer releases with a net rate of increase of much less than one would rapidly become extinct.

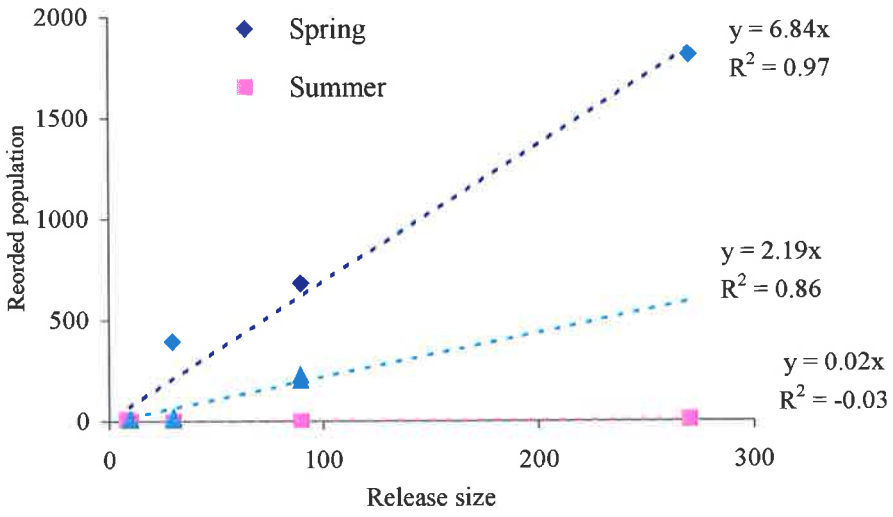

Figure 2.7. Estimated total population sizes for horehound plume moth colonies that successfully established by 4-7 months, in high plant quality locations, by season of release.

2.3.3 Effect of plant density on horehound plume moth population growth rates

Control areas, where no insects had been released, showed changes in weed cover similar to that observed at release sites over the 12-month study period (Figure 2.8). Based on the 95% CI, the high plant quality sites showed no significant change between spring of 1999 and spring of 2000. Percentage plant cover in the low plant quality areas differed between spring 1999 and spring 2000, but this also occurred in the control site and it was concluded that the decrease in cover was a seasonal effect and not the result of any damage caused by the horehound plume moth. The percentage plant cover recorded between the initial release and final census indicated that the insect densities recorded after 4 generations had no effect on the target weed in either host plant quality area.

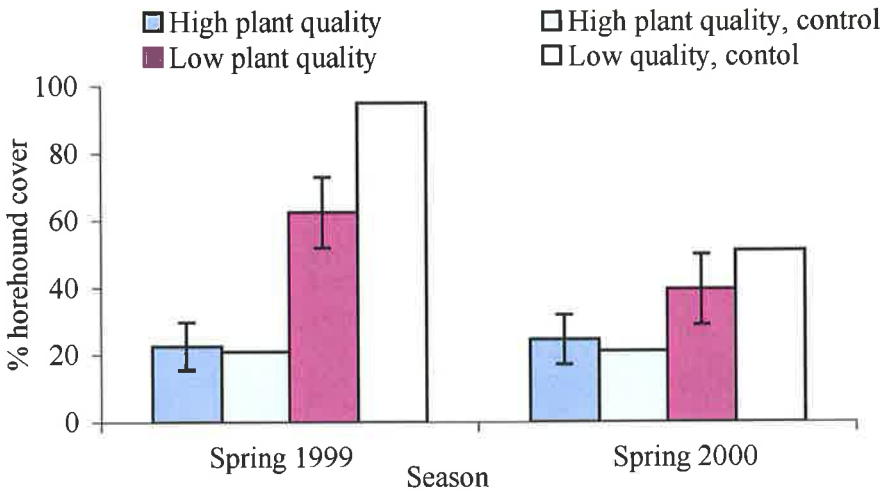

Figure 2.8. Percentage of horehound cover determined by Levy point quadrant method in high and low plant quality areas. Error bars represent 95% confidence limits for % horehound cover at the 270 horehound plume moth release sites. Sample size = 1, 5 and 6 for control, high and low plant quality areas respectively.

In the spring of 2000, percentage plant cover at sites where plant quality was consistently high (measured at the 270 release points), fell into 2 distinct groups with 2 release sites having 35 and 32% plant cover and the remaining 3 sites having 18, 18 and 19% plant cover respectively. For the

same season, the successful colonies at the low plant quality sites had percentage plant cover of 43, 42, 48, 38, 45, and 47% respectively.

These arbitrary groups, 2 in the high plant quality area and one in the low plant quality area, were used to investigate the effect of plant density and plant quality on successful establishment (Figure 2.9). Brown-Forsythe test for homogeneity of variances again indicated that log transforming both release size and the final population at 12 months was the best option to stabilize the variances before testing the model further. Analysis of the main effects (plant densities and release size) suggested that plant density and release size effect the final population at 12 months and that these to factors do not interact (Table 2.6). The Pearson's *r* values for high plant quality areas were: 18% plant cover = 0.88 ($p<0.0001$, 10 d.f.), 33% plant cover = 0.88 ($p<0.004$, 6 d.f) and the low plant quality areas, 44% plant cover = 0.42 ($p<0.42$, d.f. = 3).

Table 2.6 Effect test of the main factors effecting the population sizes at 12 months using log transformed data for plant density, original release size and recorded population at 12 months (JMPIN 3.2.1).

Source	Nparm	DF	F ratio	Probability of F
log(Plant density)	1	1	7.338	0.01
log(Release size)	3	3	8.367	<0.0001
log(Plant density)*log(release size)	3	31	0.715	0.55

Again, using the relationship outlined in equation 4, the net individual rate of increase in the field were calculated. The kR values were 280 (n=8) and 82 (n=12) for the 33 and 18% plant cover in the high plant quality areas. The low plant cover area only had a net reproduction rate of 2 (n=24).

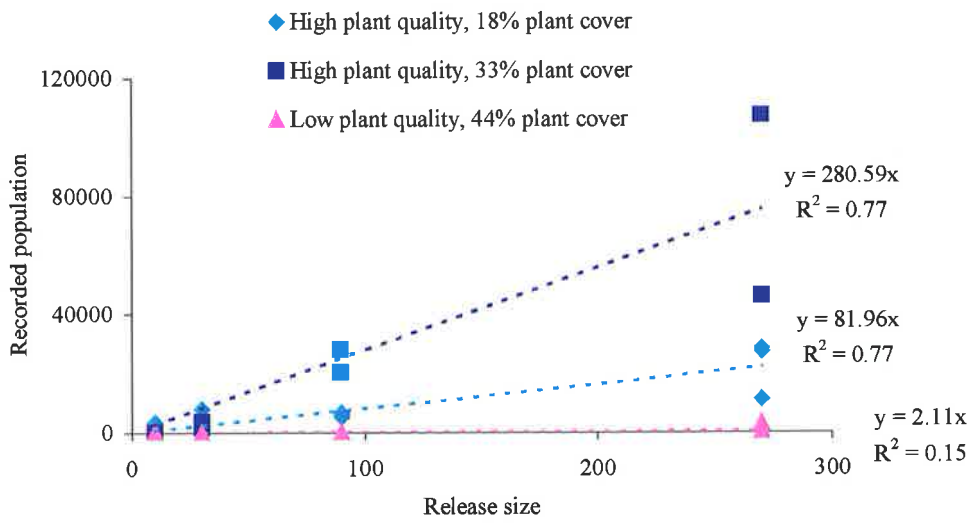

Figure 2.9. Estimated total population sizes for different release sizes for horehound plume moth colonies with 12-month data, by percentage plant cover and plant quality.

2.4 Discussion

2.4.1 Determining optimum release strategies for horehound plume moth in South Australia

The probability of establishment can be used to optimise release strategies when the goal is defined as the release size that returns the highest number of successful establishments (Memmott *et al.* 1998). These results indicate that releases made in spring established at a greater rate than any other season. No separation on basis of percentage plant cover was attempted when determining the following optimal strategy using the definition provided by Memmott *et al.* (1998). The observed percentage establishment for each size class was used to estimate the probability of establishment for the number of possible releases that could be made using a set number of insects for each release size used in this experiment. The 95% confidence intervals for these probabilities were calculated from a positive binomial distribution. For this example, it was assumed that a total of 2,200 horehound larvae were available for the release program and assigned to a given plant quality area. The figure of 2,200 available horehound plume moth larva was arbitrarily assigned based on the total number of insects reared for releases in this experiment. The observed probability of establishments for high and low plant quality locations was used to derive the expected number of establishments after 12 months from the maximum number of possible releases (Figure 2.10).

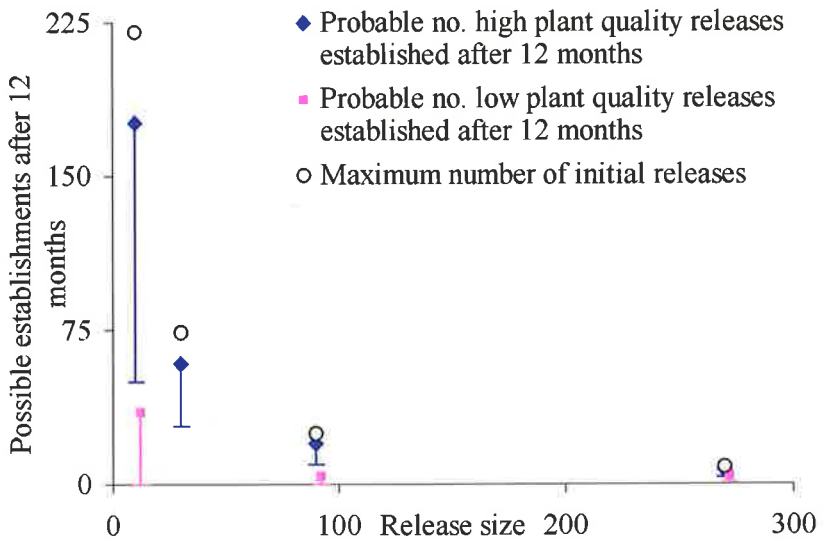

Figure 2.10. The maximum number of initial releases possible, assuming a total of 2 2000 individuals available for a spring release, and the number of likely establishments after 12 months for horehound plume moth released as 10, 30, 90 or 270 release sizes in high and low plant quality areas based on the observed number of successful establishments, with 95% confidence limits.

If the above definition described by Memmott *et al.* (1998) was used to qualify the optimum release size for the horehound plume moth, then the probability of establishment observed after 12 months would provide enough information for managers to determine the best release strategy for each plant quality location. Furthermore, if the 95% confidence limits were indicative of the level of risk associated with each of the strategies and the manager wished to ensure at least one release led to establishment then, irrespective of the plant quality, the largest release sizes would be advisable. This should ensure at least one secure site. However, this study suggests that releases sizes of about 10 - 30 horehound plume moth larvae would be an acceptable balance between risk and maximum distribution in high plant quality areas. In the low plant quality location, it appears that that managers should opt for release sizes of > 90 to achieve any establishments. This would limit possible distribution. While the above approach provides a more formalised decision making process for determining optimal strategies, it is still very generalised. Addressing the interactions of a cost-benefit analysis in conjunction with the risks and probability of establishment for a

biological control agent may more clearly identify the best option for managers. This issue will be expanded further in the final discussion (chapter 9).

2.4.2 Developing release strategies for horehound plume moth

The advantage of developing effective release strategies for a natural enemy is that the effort in rearing and producing the agent can complemented by a focus on increasing the probability of establishment in the initial stages and so improving the chances for eventual control of the host species. The controversy surrounding some of these reasons for such failures is still unresolved and includes questions about the founder effect, genetic impoverishment, integration into the new environment and host incompatibility due to the host having been subject to strong selective forces that have not influenced the natural enemy in its home range (Elton 1958; Goeden and Louda 1976; Peters 1977; Mackauer 1980; Lattin and Oman 1983; Kim 1983; Crawley 1986; Roush 1990; Hopper and Roush 1993; Ehler 1998).

The results from this field experiment provided some important empirical evidence regarding the establishment of horehound plume moth in the field. In summary these were:

1. releases where plant quality was low were less successful that those made where plant quality was high,
2. timing of the releases influenced the probability of successful establishment,
3. percentage cover of the host plant affected the size of the net population replacement rate over the initial 12-months,
4. smaller founder sizes had a higher probability of not establishing compared to larger founder sizes.

The decreased likelihood of small release sizes of horehound plume moth establishing support current theory that larger release sizes raise the probability that successful establishment will be achieved (Hall and Ehler 1979; Pimm 1991; Cameron *et al.* 1993; Hopper and Roush 1993; Memmott *et al.* 1998). Individual releases must still succeed or fail independently of other releases, unless there is a large-scale catastrophe. In this regard, there is an argument that release

size must be balanced with number of releases. The supposition that resource quality affects the success of establishment and population growth rates (Wilson 1960; Campbell 1976; Room and Thomas 1985; Center *et al.* 2000) was borne out by the conclusions of this study because colonisation was influenced by host plant quality and timing of release. The importance of host quality and abundance are highlighted as important issues, but as yet there have been few studies which investigate how these can be manipulated to create conditions that may improve the establishment of the natural enemy (Wilson 1960; Room and Thomas 1985; Center *et al.* 2000).

It is commonsense to acknowledge that environmental conditions are important factors for plant quality. Environmental effects are reflected in the response of the horehound plume moth to the season of release as well as plant qualities. This is indicated by the variable values for the horehound plume moth population growth rates and the probability of establishment for various release sizes at different seasons and plant quality. While long term temperature and rainfall averages smooth out any extreme fluctuations, the Bureau of Metrology statistics on conditions for both the Coorong (high plant quality) and Flinders Ranges (low plant quality) National Parks highlighted the variation encountered by the horehound plume moth after release (Figures 2.2 and 2.3).

When conditions were poor, such as in summer or in arid conditions, the probability of establishment was lowered. The survival of horehound plume moth depends heavily on temperature and humidity (Clarke 2001). The flowers of the horehound provide nectar for the adult, which extends their life span, providing greater opportunity to find mates and lay eggs. In spring the plants are in good condition, which provides excellent resources for the growth of the larvae. By summer, flowers are rare and the leaves becoming water stressed if rainfall is low and temperatures high. Clarke (2001) has shown that not only do excessive temperatures lead to higher adult mortality, but also that growth rates of the larvae are slowed on water stressed plants. Slower growth rates can impact in two ways. The first is that the larvae are exposed to predators for longer time periods, possibly increasing the mortality rates. The second is that the fecundity levels of the females are decreased by poor nutrition during the earlier life stages. By autumn, if the plants are

not water stressed, the leaves are in good condition, even if there are no flowers, and the larvae may have faster growth rates and do not face the same level of predation or stress.

Smaller colonies may also encounter larger Allee affects. Hopper and Roush (1993) believed the Allee effect could explain the lack of success of colonies founded by very few individuals. The decreased likelihood of establishment for the smaller release sizes observed in this experiment may suggest there is an Allee effect, although it was assumed none existed. The characteristics of the larvae themselves are such that if they are released with an adequate food supply, they do not tend to disperse over a large area (Clarke 2001). Thus, providing adults all emerge within a short time of each other Allee or inbreeding effects, even with small release sizes, may not become an issue for the horehound plume moth. If a species has low reproductive rates and/or rapid dispersal rates, Allee and inbreeding effects could be important. But the plume moth is polygamous, has many offspring, and disperses slowly (Leyson 1999; Menéndez and Thomas 2000), so it is unlikely these factors are of major importance unless conditions impose high mortality rates on the juvenile stages.

A population growth response to increased host plant density was unexpected and may have implications on how available resources, such as refuges, oviposition and food supply, affect the probability of establishment for biological control agents. It was not a parameter that was designed into the experiment and so any conclusions about the importance of host plant density, or why such a response should occur, are purely speculative. At first glance, it appeared that the higher the percentage cover of host plants presented a higher chance of successful establishment, but the results from the low plant quality area did not support this. If host plant density was the only reason for increased growth rates, then it would have been assumed that where the cover was 44%, populations would have established and expanded rapidly. This did not occur. It was unfortunate that percent plant cover in the low plant quality areas did not fall into distinctly different groups, such as they did in the high plant quality areas but, even if they had, the number of successful establishments in this region would have made any interpretation about releases difficult. It is possible that when host plant quality is very poor, plant density will have little influence on the

potential for survival. Factors influencing carrying capacity have not been investigated for horehound plume moth. There is the possibility that the increased density of host plants provided a more abundant resources and correspondingly higher survival rates. Even though plant density was high in the low plant quality areas, the plants were woody and carried less foliage.

The effects of plant density on population establishment and growth may provide some support for Goeden and Loudas' (1976) observation that generalist predators caused the failure of some releases. These predators could include invertebrates such as ants, true bugs, mecopterans and spiders as well as insectorivorous birds and reptiles. All of these are active in the dry warm conditions of the low plant quality area. Lower plant densities and/or poor plant quality may result in less refuges being available for the larvae, particularly as first instars. The first instar larvae burrow into leave tips until they increase in size. Fewer plants, or poor quality plants, suggest fewer available leave tips, with leaves being small and hirsute. This could leave the first instars exposed, not only to extremes of environmental fluctuation, but also to any predators.

Grevstad's (1999) conclusion that where environmental variability was high, or conditions permanently poor, a large number of very small releases would maximise the chance of overall establishment is based purely on probabilities that at least one release will establish. However, it should still be recognised that each release is an independent event and the probability of extinction remains the same for each population. In the case of the horehound plume moth, long term persistence, or control of the targeted host, could not be assumed in low plant quality locations because, not only was the probability of establishment for individual horehound plume moth colonies low, but the resulting population growth rates indicated that continued population survival would remain tenuous and susceptible to catastrophic events. Consequently, if risk assessment is included in the decision-making, then considering releases purely in spatial terms, as suggested by Grevstad (1999), could be detrimental to the success of a biological control program. Temporal issues, such as timing of release and plant quality over the first few generations should also be taken into account.

The investigation into optimal release strategies outlined in section 2.4.1 of this chapter did not include whether mixed release sizes would be a useful option in high or low plant quality areas. It merely suggested that releases as small as 10 were possible when plant quality was high and that releases in excess of 90 were required when plant quality was very low. Shea and Possingham (2000) suggested that various release strategies existed, depending on whether or not existing successful sites were present and the relationship that defined the probability of establishment for different release numbers. Their model for determining optimum release strategies and the possible benefits of mixed release strategies will be examined more closely in chapter 8.

Long-term persistence may be influenced by other factors, such as genetic paucity and adaptation, but it is clear that for species such as the horehound plume moth, with short life spans and high reproduction rates, small release sizes can be made if the host quality is high. This would allow a more rapid distribution of the agent and provide several established colonies, which would assist in avoiding localised catastrophic events that would otherwise devastate planned introductions.

Establishment of newly introduced biological control agents is a prerequisite to control of targeted pests. While there is no magic formula that would guarantee the choice, introduction and integration of a successful biological control agent, the interaction between quality of the host and its carrying capacity for the natural enemy have been highlighted as possible areas that must be evaluated if desired levels of control are to be achieved. Although all introductions must be evaluated on a case-by-case basis, along with the parameters intrinsic to the species being investigated, these results indicate that there is significant variation in establishment by geographic region. This variation appeared to be driven by the quality of the host resource. Consequently, it could be assumed that the efficacy of the horehound plume moth as a biological agent would vary across the target region, based on measurable and predictable local geographical, physical and climatic factors.

3. Microsatellite isolation and detection

3.1 Introduction

Microsatellites have been embraced as 'the right marker for the right job' (Scribner and Pearce 2000). The ability to define allelic states at the nucleotide level allows opportunities to investigate rates of mutation, evolutionary changes at the molecular level, kinship, random mating, population sub-division and differences between populations (Schlotterer 1998). Microsatellites are found in rapidly evolving non-coding DNA. They are highly polymorphic DNA markers with discrete loci and co-dominant expression. Microsatellites are uniformly distributed over the entire genome and are believed to be neutral, or nearly so, with regard to selection (Hughes and Deloach 1997). Despite their utility, marker development may be time consuming. For example, developing microsatellite markers requires the isolation of short repeat microsatellite sequences and then developing primers for use in polymerase chain reaction (PCR) using the DNA from the conserved flanking regions surrounding microsatellite repeat sequences is used to design specific primers to amplify a specific locus (Schlotterer 1998).

The traditional process of finding microsatellites involves the construction of a partial genomic DNA library and identifying and sequencing clones that contain microsatellites (Schlotterer 1998; Scribner and Pearce 2000). Once primers are designed, and the PCR conditions optimised, microsatellites are easily amplified from individuals collected from the populations under investigation (Schlotterer 1998; Scribner and Pearce 2000). Enrichment techniques, that do not involve the necessity for constructing partial libraries, have been developed that will decrease development time and make isolation of microsatellites easier (Kijas *et al.* 1994; Refseth *et al.* 1997). These techniques make use of streptavidin coated magnetic beads and biotinylated DNA sequences that anneal fragments containing repeat sequences. This allows them to be 'captured' and put through a preliminary PCR step to increase the concentration of microsatellite containing

fragments prior to ligation into a suitable vector. These techniques were not pursued here because of restraints on time, money and expertise.

One of the major advantages of microsatellite PCR techniques is that very small amounts of DNA are required for population analysis. Theoretically, 5 µL of haemolymph would be sufficient to run several PCR assays using different primer pairs. Such an approach would allow individuals forming founder stock for experiments to be analysed without sacrificing them. In essence, data would be available on all individuals contributing to any field releases. The feasibility of using microsatellite DNA markers to analyse the genetic structure of horehound plume moth populations following release was investigated in the following study.

3.2 Materials and methods

3.2.1 Individual DNA sample extraction

DNA from 20 individuals, 10 from the Spanish and 10 from the New French horehound plume moth cultures, was extracted as follows: individuals, as late instar larvae, pupae or virgin adults, were harvested from the relevant laboratory cultures. All individuals were killed by immersion in liquid nitrogen and then stored in separate tubes at –20°C until DNA extraction could commence. The wings and head were removed from the adults to ensure that pigments found in these body parts were not carried over by the extraction method, because there have been accounts of these pigments interfering with subsequent PCR (A. Reineke pers. comm.). The gut tube was removed cleanly, and without perforation, from the larvae in order to eliminate the possibility of DNA contamination by horehound leaves eaten by the larva and still retained in the tube. A modified cetyltrimethylammonium bromide (CTAB) protocol with an additional polyethylene glycol (PEG) precipitation, as described by Reineke *et al.* (1998), was used to extract total nucleic acids. This method is rapid, taking around 3 hours per sample and removed some of the inhibitors that may decrease yields obtained during amplification of PCR products. Following RNAse treatment, the DNA was stored at -20°C for up to one week, or at -80°C for longer storage.

3.2.2 Pooled DNA sample for microsatellite isolation

Twenty individual adult moths, minus wings and heads, were frozen in liquid nitrogen for 5 minutes. Using a chilled mortar and pestle the moths were finely ground into a powder and added to 1 mL of sarkosyl extraction buffer in a 2 mL microfuge tube (Appendix: Table A.2). The contents were mixed gently but thoroughly and a further 1 mL of extraction buffer added. The tubes were then placed in a 60°C water bath for 15 minutes and gently inverted at least 4 times during the incubation.

The tubes were then allowed to cool slightly and 10 µL of a 20 mg/mL solution of proteinase K added. The tubes were then gently mixed and placed in a 50°C waterbath for 36-48 hours, with periodic gentle mixing. After cooling to 37°C, 20 µL of a 10 mg/mL solution of Rnase A was added to each tube. The tubes were mixed gently and incubated at 37°C for 2 hours before cooling briefly on ice and splitting the contents into 2 clean tubes using a wide bore pipette tip. Equilibrated phenol was made on the week it was used by melting frozen phenol at 65°C, transferring it to a polypropylene tube containing an equal volume of 0.2 M Tris HCl pH 8.4. This was shaken for 30 minutes to mix the phases. The phases were allowed to separate and the top aqueous phase discarded. This mixing with 0.2 M Tris HCl pH 8.4 was often repeated several times and the equilibrated phenol was stored at 4°C when not being used. One volume of the equilibrated phenol was added to each tube and they were gently mixed by inversion for 2 minutes, left at room temperature for 1 minute before being mixed again for two more minutes. The tubes were then spun at 6 500 rpm for 10 minutes and the upper aqueous layer transferred to clean tubes using a wide bore pipette tip. One volume of chloroform:isoamylalcohol (24:1) was added and the tubes gently mixed by inversion before spinning again at 6 500 rpm for 5 minutes.

The upper aqueous layers were transferred to a 15 mL disposable centrifuge tube and chilled on ice for 5 minutes. A volume of 3 M sodium acetate (pH 5.2), equivalent to 1/10 the total volume of the aqueous layer, was added and the tube contents mixed gently by slow inversions (using a slow count of 1 to 14 for the time to make one complete inversion). A volume 2.2 times that of the tube contents of ice cold 100% ethanol was then added very slowly by running it down the side of the

tube and the tube again inverted as described previously. A layer of DNA appeared as a tangled mass around 3-4 mm below the surface. This DNA was transferred to a 2 mL microfuge tube using a wide bore pipette and the inversion steps repeated until no more DNA was recovered.

The tubes were then spun at 6 500 rpm for 10 minutes and the supernatant removed. The DNA pellets were rinsed with ice cold 80% ethanol, spun again and the supernatant removed. The DNA was allowed to air dry for several hours and then resuspended in 50 µL of Tris-EDTA buffer (TE), pH 8.0, at 4°C overnight before spot testing on agarose with ethidium bromide and visualization under UV light against various concentrations of a known quantity of DNA (Stratagene Kb DNA Ladder #201115) to determine approximate quantity of DNA in the extracted sample.

3.2.4 Size selection of DNA fragments

Twenty five µg of DNA from the pooled sample was incubated at 37°C with 20 Units of the restriction enzyme *Mbo*1 for 2 hours before heat inactivating the enzyme by placing the tube and contents in a 65°C water bath for 20 minutes. The DNA fragments formed after restriction enzyme digestion with *Mbo*1 were then separated on a 0.6% low melting point agarose Tris-acetic acid-EDTA (TAE) buffer, pH 7.7 – 8.0, gel at 4°C and 40V for 2 hours along with a suitable molecular weight marker before being visualized under a UV light (Marker IV, Boehringer, Mannheim) following ethidium bromide staining. Fragments in the range 400 to 1400 base pairs were excised from the gel and the low melting point agarose removed from the sample using the Jetsorb™ (Genomed, Bad Oeynhausen) 'cleanup' kit, according to the manufacturer's instructions. The concentration of the size selected DNA fragments was then estimated using the spot test described above.

3.2.5 Ligation and transformation and identification of positive clones

Fifty ng of size selected DNA fragments were ligated into Pharmacia Biotech 'Ready to Go' pUC18 *Bam*H (Product number 27-5260-01) as outlined in the manufacturer's instructions using a molar vector to insert ratio of 2:1. Prior to transformation of competent *E. coli* cells (JM109, Promega), the 'Ready to Go' stabilizers were removed as recommended by the manufacturer. *E.*

coli competent cells were used in the transformation of both sample and control DNA. The 'control DNA' plate was used to determine the transformation efficiency, and was prepared in parallel with the sample DNA ligation. Heat transformation was used as outlined in the technical notes supplied with the JM109 competent cells. Incubation in SOC medium and plating onto LB plates containing ampicillin, IPTG and X-gal also followed the procedure outlined by the technical notes (see Appendix: Table A.3 for recipes).

Control DNA supplied with the *E. coli* cells was used to calculate the transformation efficiency, or colony-forming units (cfu) per μg of the cells as described by the manufacturer. The use of X-gal and IPTG allow the selection of *E. coli* colonies containing plasmid inserts. White colonies were restreaked onto fresh ampicillin LB plates and grown overnight at 37°C. Initially, colony lifts directly onto Hybond^{TM} N+ positively charged membranes were attempted for the hybridisation and probing steps. The procedure used was that outlined in section 1.98 of Sambrook *et al.* (1989). Despite attempts to increase the stringency of washes in the hybridisation steps, followed by lysis of the bacterial colonies and remove cellular debris from the membrane (Sambrook *et al.* 1989), the occurrence of false positives, based on the blue/white screening, remained too high for this to be an efficient method for identifying plasmids containing microsatellites. Finally, in order to overcome this problem, crude plasmid purification from the white colonies was performed as outlined by Sellick (1997). After incubation each lysate was loaded into a well of a non-submerged 1% agarose gel, and separated by electrophoresis for approximately 1 hour at 30 V using a 1 x TBE buffer. The gels were then stained with ethidium bromide and the DNA bands visualised under UV light. A marker run alongside made it possible to estimate plasmids containing inserts and roughly what size the inserts were, even when allowing for the complications of plasmid circularisation and how this affected migration through the gel.

The gel was then rinsed in distilled water and set up for an alkali capillary transfer (Southern 1975) using 0.4 M NaOH as described in the Amersham Life Science Hybond^{TM}-N+ positively charged membrane booklet. Fixation after alkali blotting was not required and the hybridisation procedure followed the method described for the Hybond^{TM}-N+ membrane with 150 ng of the combined

biotinylated microsatellite probes $(GA)_8$, $(CA)_8$, $(AAT)_6$, $(CCT)_6$, $(CAG)_6$, and $(GACA)_4$ used in the probe mixture. The prehybridization and hybridisation steps used the blocking reagent recommended by Boehringer Mannheim (cat # 1096 176) and solutions were prepared according to the manufacturers instructions. The prehybridization conditions were 30 minutes at 42°C and hybridisation conditions were 30 minutes for 37°C. The first 4 stringency washes used 2 x SSC Appendix: Table A.5), 0.1% sodium dodecyl sulfate (pH7.2) at 37°C, for 15 minutes. The final stringency wash was 0.5 x SSC, 0.1% sodium dodecyl sulfate (pH7.2) at room temperature, for 15 minutes. Chemiluminscent detection utilised the CDPStar™ protocol (Boehringer Mannheim, cat # 1685 627) for the detection of plasmids that had hybridised with labelled microsatellite probe.

Once plasmids containing microsatellite inserts had been identified, the original colony was grown overnight, with gentle shaking, at 37°C, in 5 mL of LB broth with ampicillin (5 µL of a 10 mg/mL stock). The bacterial culture was then pelleted using gentle centrifugation of approximately 1000 rpm and the supernatant discarded into a container for autoclaving. A QIAprep Spin Miniprep kit (cat # 27104) was used to purify the plasmid from the *E. coli* culture. The DNA was then dissolved overnight at 4°C in 25 µL of nanopure sterile water before sequencing using an ABI 373XL automated sequencer (Perkin Elmer/Applied Biosystems).

3.2.6 Primer design and PCR optimisation

Oligo 4.0 software (Molecular Biology Insights) was used to design primers for the PCR detection of identified microsatellite regions. Based on the results from the Oligo 4.0 software, the following conditions were used to optimise PCR analysis. The annealing temperature was 58.6°C; primer concentration was 200 nM; and Mg^{++} concentration was 0.7 mM. The expected PCR product was 153 base pairs in length and the PCR analysis was run in a total volume of 20 µL, using 2.5 µL of sample, 5 µL each of 2 mM dNTPs and 1 Unit of Taq DNA polymerase. The thermocycle used to amplify the target DNA was:

1. 95°C 5 minutes
2. 95°C 30 seconds
3. 58°C 1 minute

4. 72°C 1 minute
5. repeat from step 2 a further 25 times
6. 72°C 7 minutes
7. stop and hold at 4°C

DNA from 20 individuals, 10 from the Spanish and 10 from the New French horehound plume moth collections were then diluted 1/5, 1/10, 1/100 and used to amplify microsatellites with the PCR cycle outlined above.

Visualisation of PCR products was undertaken by labelling one of the primer pairs with radioactive [γ^{32}P]ATP and using this in the PCR mix. Following PCR 5μL of loading dye (containing bromophenol blue and xylene cyanole) was added to each sample, the samples were denatured at 94°C for 4 minutes, then immediately transfered to an ice water-bath. Two μL of the PCR solution was then loaded and run out on a 40 cm 6% polyacrylamide gel at 40 W for 2.5 hours. The buffer solution used was 1x TBE buffer, at 1000 V and the gel was run until the bromophenol blue reached the bottom. The gel was then dried and transferred to an X-ray cassette with medical X-ray film (Fuji RX, 35 x 43 cm) laid over them. The cassettes were then stored for 24-48 hours before the X-ray film was developed.

3.3 Results

The DNA extraction from 20 adult moths yielded approximately 40 μg of DNA and half of this was used to size-select DNA fragments. The recovery of size-selected fragments was poor with only 600 ng of DNA fragments between 400 and 1400 base pairs being purified from the low melting point agarose gel. The control DNA plate returned a cfu of 8.92×10^8 and all colonies were blue indicating that the ligation and transformation procedure was working. The total number of colonies obtained from all the sample plates was around 500 and all colonies that appeared white were re-streaked onto fresh LB agar, containing penicillin/X-gal and IPTG, and re-incubated. Of these, only 60 colonies were actually white and processed through the crude plasmid purification process outlined in the method section to overcome the problem of false positives. This equated to

an approximate recovery of 12% of plasmids containing inserts from the plates. After Southern blotting onto N+ membrane and probing, only 4 plasmids appeared to be positive for microsatellite repeat sequences. This was a total return of 0.8% colonies being positive for microsatellites.

Each of the 4 'positive' colonies was grown in LB broth; the plasmids purified and sent for sequencing in both forward and reverse directions. Three microsatellite repeat sequences were confirmed. Two were imperfect di-nucleotide repeat sequences, $(CA)_9TG(CA)_8$ and $(CA)_{19}C(CA)_3C(CA)_4CC(CA)_5$, and the third was a tetra-nucleotide repeat $(CACT)_{12}$. Attempts to design primers for the $(CA)_{19}C(CA)_3C(CA)_4CC(CA)_5$ imperfect repeat and the tetra-nucleotide repeat $(CACT)_{12}$ failed as all potential primers were unsuitable when problems with annealing temperature, duplexes, hairpins and false priming sites were considered. Primers designed for the remaining imperfect repeat $(CA)_9TG(CA)_8$ were a 12-mer upper primer and a 17-mer lower primer (Table 3.1).

Table 3.1. Details of the primers designed by Oligo 4.0 for $(CA)_9TG(CA)_8$ for salt and DNA concentrations fixed at 50 mM and 250 pM respectively.

Primer	Tm(°C)	Td (°C)	ΔG(25°C) (kcal/mol)	Mr
Upper: 5' GCG GGG GAC CTG 3'	49.5	52.4	-28.6	3807
Lower: 5' GAG TGC GAG CGT ACA AG 3'	55.0	56.3	-32.0	5365

Visualisation of the PCR products indicated many stutter bands and null alleles (7/20 sampled) despite several attempts to optimise the PCR conditions. However, banding patterns obtained from those individuals where PCR products were amplified indicated a highly polymorphic microsatellite region. Dinucleotide repeat sequences are prone to PCR stutter and this phenomenon is a PCR artefact believed to be the result of slipped-strand mispairing during the Taq-mediated DNA replication (MacHugh 1996). These bands can make scoring gels difficult, but are reported to

form a characteristic signature to the practiced eye (Scribner and Pearce 2000). The presence of null alleles is potentially more of a problem. Null alleles are believed to be the result of further mutations occurring in the conserved DNA of the primer regions (Callen *et al.* 1993; Pemberton *et al.* 1995). The only way to confirm if this was the case, or if the mutations occurred in at the same point in all samples, would be to sequence every individual.

3.4 Discussion

The ability to determine allelic variation at the nucleotide level for a co-dominant marker would have provided an ideal opportunity to investigate the genetic influences on the successful establishment of biological control agents. Microsatellite loci can differentiate the individual and population-level demographic processes and, because they are co-dominant, also indicate homozygous and heterozygous states. However, 30% (6/20) of individuals tested resulted in no PCR product, i.e., a null allele. The presence of so many null alleles, the complex pattern of stutter bands and the low level of isolation of microsatellite regions were disappointing. Only one of the positive clones actually contained microsatellite repeat sequences that could be developed further. Even here, two of the repeats occurred within 2 bases of each other, so that designing primers to isolate each region was impossible in one case and limiting in the other. Only one of the primer pairs designed for the $(CA)_9TG(CA)_8$ amplified microsatellites in individuals and produced many null alleles, despite several attempts to optimise conditions by varying the magnesium concentrations in the PCR analysis. The DNA banding patterns for those individuals that did amplify microsatellite regions were also hard to interpret, with several stutter bands occurring. It was on this basis that analysis of microsatellite DNA was abandoned and AFLP analysis was developed for further studies.

Scribner and Pearce (2000) stated that genomic libraries used to develop microsatellites markers needed to be large. They suggested that such libraries need to be in the region of 50 000 to 100 000 recombinants as the recovery rate for positive clones is typically low (<0.5 – 2.0%). The recovery rate of 0.8% observed here would support the premise that large numbers of positive microsatellites

are necessary if loci are to be informative. Another problem faced when developing microsatellites are that recombinants may lack suitable flanking regions for primer development. This was also observed for 67% (2/3) of the microsatellites isolated here, with the remaining region being an imperfect dinucleotide repeat sequence.

To be useful for population studies many loci should be studied, as bias or the presence of null alleles at a single locus or few loci undermine the accuracy and precision of the analysis (Blouin *et al.* 1996). Given the time and effort involved in microsatellite isolation, primer design and optimising the PCR step, it is suggested that enrichment techniques should be utilized to isolate microsatellites (Refseth *et al.* 1997). This would result in the time invested in the isolation step being shortened considerably. Results reported in the literature indicate that enrichment techniques result in around 20% of clones being positive for micosatellites (Kijas *et al.* 1994; Refseth *et al.* 1997), as compared to the 0.8% recovered here. Recovering a larger number of microsatellite regions would allow the development several loci, which would overcome the problems encountered here.

4. Pilot study to investigate genetic divergence for horehound plume moth populations after 4 generations in the field

4.1 Introduction

Geographical and environmental differences between isolated colonies of the same species can produce variation in gene frequencies as well as in morphology and behaviour (Templeton 1980; Slatkin 1987). This variation is affected by systematic factors such as migration, mutation, drift and selection (Falconer 1982). There is certainly evidence that increased genetic divergence exists among populations that have become fragmented or undergone severe reductions in size (Leberg 1991 1993; Miyamoto *et al.* 1994). This may be due, in part, to the fact that restricted populations have fewer breeding individuals and inbreeding and genetic drift become driving forces.

This was a pilot study to investigate the usefulness of AFLPs for investigating the variation and genetic diversity within and among populations of horehound plume moth. The results also provide reference data on the genetic profile of the laboratory populations. These reference data provided useful baseline information for comparing changes that occurred in the genetic diversity for the founder experiment described in the next chapter. It was also undertaken to provide an insight to how rapidly small isolated populations showed evidence of divergence and to estimate the magnitude of the with-in population variation.

4.2 Materials and methods

4.2.1 Source of harvested individuals

Individuals used to found the New French laboratory population originated from a collection made at Cape d'Agde, France while the Spanish laboratory population was originally collected at Zaragoza, Spain. The climatic differences between these two regions were reported by Clarke (2001) and were quite different in regard to rainfall, temperatures and extremes experienced. The

Spanish collection was made 12 months earlier than the French collection (Table 4.1). Craig Clarke founded two field populations at Narrung, South Australia (New French) and Wilpenna, South Australia (Spanish) in 1998, using 200 founders from the original laboratory collections. Individuals were harvested for DNA from the two laboratory colonies in February 1999 as late instar larvae, pupae or virgin adults. In contrast, individuals from the Narrung and Wilpenna release sites were collected, haphazardly over the release area as larvae in February 1999. DNA was extracted as outlined in chapter 3.2.1.

Table 4.1. Summary of details for horehound plume moth collections from Cape d'Agde, France and Zaragoza, Spain (adapted from Clarke, 2001).

Collection	Region of origin	Climate	Importation date	No. founders originally imported	Generations in laboratory at time of analysis
New French	Cape d'Adge	Mediterranean, extremes rare	05/06/97	59	7
Spanish	Zaragoza	Arid, extremes common	17/07/96	50	10

4.2.2 Sample sizes

The question of sample size and optimal number of dominant loci (recorded as band present in an AFLP system) required to derive meaningful estimates for genetic parameters is influenced by the probability of detecting a given locus when it is present at low frequencies. The effect of sample size and probability of detection was aptly illustrated by Roush and Miller (1986) in their discussion on resistance monitoring. Using a formula derived by Roush and Miller (1986), which allowed one to find the necessary sample size for any given probability of detection, a simple plot of the frequency of a given loci, the probability of detection and the sample size was created

(Figure 4.1). Using the Roush and Miller (1986) formula, 28 individuals would need to be analysed to detect bands present at a frequency of 0.1 with 95% confidence, but if 80% confidence was acceptable then 15 individuals were sufficient. Once the frequency of the detectable band dropped below 0.1, the number of samples required to provide any level of confidence increased rapidly, with band frequencies of 0.05 and 0.01 requiring 58 and 298 individuals for a 95% probability of detection, respectively. Because AFLPs provided the opportunity for analysing a large number of dominant loci, it was assumed that this would offset biases produced by the presence of low frequency bands. As a result of examining the interaction of the sample size and probability of detection at low frequencies, the aim was to harvest a minimum of 16 individuals from each population for AFLP analysis.

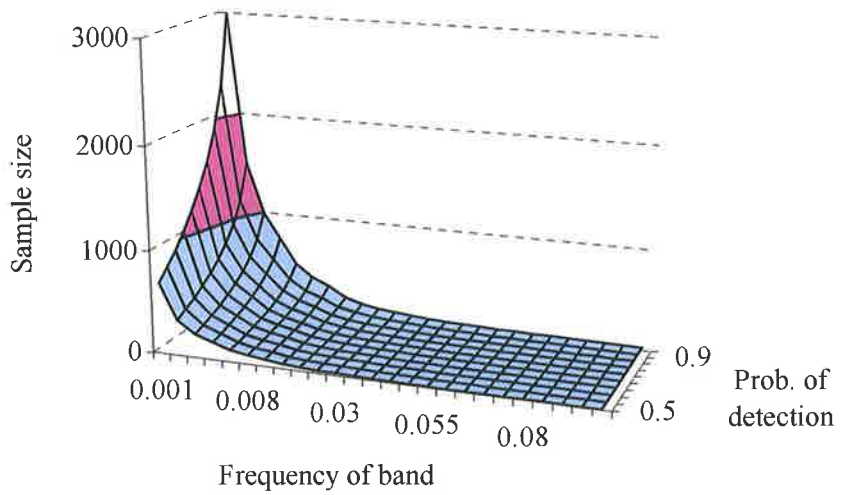

Figure 4.1. Sample size required for a given probability of detection for a given band frequency.

4.2.3 Preparation of AFLP samples

DNA finger-printing is the profile of a set of DNA fragments obtained from a specific DNA sample (Baker 2000). AFLP methodology allows the development of profiles without the requirement for sequence knowledge, using a limited set of generic primers (Vos 1995; Reineke *et al.* 1998). The amplified fragment length polymorphism (AFLP) technique is based on PCR amplification of a subset of genomic DNA fragments in which DNA is first cut with restriction enzymes ("cutters"), then double stranded adapters are ligated onto the ends of the fragments to generate template DNA

(Vos 1995; Reineke *et al.* 1998). The adapter sequence and the adjacent bases on the fragment of sample DNA serve as primer binding sites for further amplification of the restriction fragments where the PCR primers used have additional selective bases at the 3' end. This results in only a predetermined subset of the fragments being amplified during subsequent PCR steps (Vos 1995; Reineke *et al. 199*8). The restriction fragments for amplification result from cutting with a "rare cutter" and a "frequent cutter". The frequent cutter generates small fragments that amplify well and are within the optimum size for separation on a sequencing gel. The rare cutter ensures that the selective amplification is limited (Vos 1995; Reineke *et al.* 1998). Thus, using two different restriction enzymes provides flexibility in producing a final profile with the optimum number of fragments for scoring, while various combinations of a low number of primers provide the opportunity for producing many different profiles (Vos 1995; Reineke *et al.* 1998). Several technical problems noticed in other DNA techniques have not been observed using AFLPs. These included the advantage that repeatable AFLP profiles could be obtained from different concentrations of template DNA, which still resulted in fingerprints of equal intensity despite such variations in template concentration (Vos 1995; Jones *et al.* 1997). Although large numbers of variable markers are generated, most AFLP markers are dominant (Jones *et al.* 1997; Witsenboer *et al.* 1997).

The AFLP method used was derived from Vos *et al.* (1995), with modifications by Greg Penner (Adelaide University, Molecular Plant Breeding Laboratory). An additional variation was that approximately 0.5 μg of genomic DNA was used instead of the recommended 1.0 μg with no subsequent reduction in final yield after the PCR amplifications. This was because plume moths return smaller amounts of extracted genomic DNA than is possible from larger plant samples. Pst1, a six base pair rare cutter, and Mse 1, a four base pair cutter, where used to cut extracted DNA and the double stranded adapters chosen for this study matched the Pst1 and Mse1 sequences. The pre-amplification step used primers compatible with the ligated sequences with one additional base pair consisting of A on the Pst1 and C on the Mse1. The optimum annealing temperature for the PCR was set at 56°C and repeated for 20 cycles.

For the selective amplification step, eleven primer pairs were used to screen the DNA from 10 horehound plume moth individuals. Selective amplification was performed on the pre-amplified fragments with each primer having 3 additional nucleotides (Table 4.2). The exception was one test run using Pst1A/Mse1CAG. One μCi ^{32}PγATP was used to label the Pst1 primer in all cases. Of the 11 primer pairs tested, the 4 most informative primer pairs were chosen for further analysis. A step-down PCR procedure was used to amplify the sub-set of the DNA fragments (Table 4.3). The step-down procedure was used to ensure enrichment of the target sequences during the earlier higher temperature steps and decrease the formation of secondary amplification products in subsequent cycles. The final volume of the PCR reaction was 20 μL, and once the cycle was complete, 20 μL of loading dye was added to each tube.

Table 4.2. Pst 1 or Mse 1 primer pairs used in preliminary screening of horehound plume moth DNA in order to select the most informative primer pairs for genetic analysis.

Primer pair	Conclusion
Pst1A / Mse1CAG	Not selected
Pst1AAT / Mse1CAA	Selected
Pst1AAT / Mse1CAT	Not selected
Pst1AAT/ Mse1CCC	Not selected
Pst1AGA / Mse1CAA	Selected
Pst1AGA / Mse1CAT	Selected
Pst1AGA / Mse1CCC	Not selected
Pst1AAC / Mse1CAA	Not selected
Pst1AAC / Mse1CAT	Not selected
Pst1AAC / Mse1CCC	Not selected
Pst1AAC / Mse1CGT	Selected

Table 4.3. Selective step-down PCR program used to amplify the sub-set of DNA fragments following the pre-amplification PCR step.

	Temperature (°C)	Time (seconds)
Step 1	94	30
Step 2	65	30
Step 3	72	60
Step 4	Followed by 9 cycles of steps 1 to 3, with the annealing temperature in step 2 decreasing by 1°C per cycle until it reaches 57°C	
Step 5	94	30
Step 6	56	30
Step 7	72	60
Step 8	Followed by 25 cycles of steps 5 to 7, then stop	

4.2.4 Visualisation of AFLP products

Polyacrylamide gel electrophoresis was used to separate the PCR products. Denaturing Sequagel® 6 (National Diagnostics, cat # EC-836) was prepared and used according to the manufacturers' instructions. Sequagel® 6 is a monomer solution containing urea as well as acrylamide and bis-acrylamide (19:1 w/w) in distilled deionized water. Sequalgel® 6 was accompanied by Sequagel Complete Buffer Solution, which is 5X TBE (89 mM Tris Base, 89 mM boric acid, 2 mM EDTA) and a catalyst in distilled and deionized water. When the two are combined in the appropriate ratio they form a 6%, 6 M urea, pH 8.3 denaturing gel. Two µL of the selective PCR amplification products, after dilution with loading dye (containing bromophenol blue and xylene cyanole), were denatured at 94°C for 4 minutes followed by transfer to an ice water-bath, then loaded and run out on the gel at 40 W for 2.5 hours. The gels were transferred to Whatman's paper and dried at 80°C for 45 minutes. Gels were transferred to X-ray cassettes and medical X-ray film (Fuji RX, 35 x 43

cm) was laid over them. The cassettes were then stored for 48 hours before the X-ray film was developed. Bands were scored visually and recorded in binary format as either a 0 or 1 (band absent or present). Scoring, and subsequent analysis, also assumes that fragment sizes have been accurately assessed, populations sampled at random and the same primers used among all individuals (Baker 2000).

A lambda marker was labelled and run at regular intervals during the development stages of the technique. Subsequent runs used a selected sample as a standard marker. This was run at regular intervals on the same gel to assist scoring.

4.2.5 Genetic analysis

AFLPs generate dominant markers and this means that incomplete information is available because heterozygotes cannot be distinguished. For dominant markers, each band is assumed to correspond to a single locus and the presence of the band is assumed to represent the dominant genotype while a blank represents the homozygous recessive genotype. When analysis of AFLP bands is undertaken, it is assumed that there are only 2 alleles at each locus, represented by present or absence of a band.

Determination of heterozygosity is based on co-dominant expression of alleles and AFLP data do not readily provide this information within a population. Band absence (null allele) represents (aa), but band presence can be (Aa) or (AA). To determine which bands of a given subset are co-dominant the exact paternity of individuals would have to be known and their prepared AFLP fragments analysed. Such detailed analysis requires specific breeding programs and identification of possible co-dominant bands was not undertaken here. However, heterozygosity can be estimated.

As the presence of a band dominates over the null allele, which cannot be amplified with PCR, estimating allele frequencies causes some difficulties in statistical analysis. The frequency of null alleles can be estimated by taking the square root of the observed proportion of individuals that are homozygous for the null alleles (Stewart and Excoffier 1996). This transformation may lead to

statistically biased estimates and, in order to reduce the bias, Lynch and Milligan (1994) introduced the recommendation that those samples with fewer than 3 null homozygous individuals be ignored. However, this favours loci with a high frequency of null-alleles and underestimates the frequency of bands (Zhivotovsky 1999). This means that information is lost as loci are omitted from analysis and also causes biases of genetic variation and genetic distances. For this reason, estimates of genetic diversity at the nucleotide level were also examined along with estimates of genetic diversity using the Lynch and Milligan (1994) Taylor expansion approach.

Genetic diversity at the nucleotide level represented a measure of the frequency of different alleles, measured as presence or absence of a band, and hence estimated the variation within a population. The measure of genetic diversity at the nucleotide level was subsequently referred to throughout the study as an estimate of genetic diversity. The equation used to calculate the average genetic diversity across the loci was defined as (Tajima 1983; Nei 1987; Schneider *et al.* 2000):

$$\hat{\pi} = \frac{\sum_{i=1}^{k}\sum_{j<i} p_i p_j \hat{d}_{ij}}{L} \qquad \text{(equation 1)}$$

where $\hat{d}_{ij}$ is an estimate of the number of mutations having occurred since the divergence of haplotypes i and j, k is the number of haplotypes, p_i is the frequency of the haplotype i and L is the number of loci. The total variance (over the stochastic and sampling process), assuming no recombination between sites and selective neutrality, was calculated as (Schneider *et al.* 2000):

$$V(\hat{\pi}_n) = \frac{n+1}{3(n-1)L}\hat{\pi}_n + \frac{2(n^2+n+3)}{9n(n-1)}\hat{\pi}_n^2 \qquad \text{(equation 2)}$$

where n is the number of gene copies in the sample.

The value of genetic diversity at the nucleotide level will provide some indication of if, and how, genetic diversity differed between those populations harvested from the field after 4 generations versus individuals collected from the laboratory colonies. Concern has also been expressed that the loss of rare alleles from populations may limit the potential for future adaptation (reviewed in Roush 1990). Although measuring a dominant system, the number of polymorphic loci for each

population was also determined to investigate if the number of polymorphisms between populations differed markedly. Essentially, the questions being examined using these two parameters were; could any difference be detected between the populations, and if there was, was there any pattern in differences between the field populations and the laboratory populations?

Total genetic diversity in a species is the function of two components, the average gene diversity within a population and the average variance in allelic frequency between populations (Nei 1975). This relationship is used to calculate the F_{ST} statistic and is the ratio of within and among population components expressed as a proportion of the total. Slatkin (1995) considered the probability that populations diverged and remained isolated with no gene flow occurring between them and defined an estimate of Slatkin's linearized F_{ST}. Average genetic diversity over the loci, decrease in number of bands recorded and Slatkin's linearized F_{ST} (Slatkin 1995) were used to investigate the genetic differences among the four populations.

The neutral mutation hypothesis was examined using Tajima's D and Fu's Fs (Tajima 1989; Fu 1997). These two tests are based on the infinite site model without recombination and both rely on the assumption that polymorphisms are maintained at intermediate frequencies under the model of neutral selection. The theory of neutral selection suggests that genetic diversity should be low in small populations and high in large populations. But significant deviation from the assumption of selective neutrality can be attributed to factors other than selective effects, for example, factors such as population expansion, bottlenecks or heterogeneity of mutation rates, so interpreting results can be difficult. If a mutation has a selective advantage, then the chance of its surviving is increased. It can then spread through the population as a 'selective sweep', increase in frequency and perhaps become fixed. Both Tajima's D and Fu's Fs were calculated to determine how useful they were in detecting whether changes were due to selection and/or if bottlenecks occurring in the field populations could be detected because they test somewhat different predictions and complement each other.

Tajima's D examines whether the average number of pairwise nucleotide differences is larger or smaller than expected from the observed number of polymorphic loci. For a population at equilibrium, with random mating, the Tajima's D statistic would be expected to have a value of zero. Statistically significant positive values would imply balancing selection or population sub-division, which generate an excess of heterozygotes compared to those expected from Hardy-Weinburg principles. But a statistically significant negative value would suggest directional selection or a bottleneck and excess homozygotes. Large population expansions will also shift Tajima's D to a significantly negative value (Bonatto and Salzano 1997; Schierup and Hein 2000). Simonsen *et al.* (1995) examined Tajimas' D as a method of determining selective neutrality and concluded that Tajima's test was powerful against the alternative hypotheses of selective sweep, bottlenecks and sub-division, but that Tajima's D could only detect selective sweeps or bottlenecks within a specific time interval in the recent past, or if population sub-division had occurred for a long period of time.

Fu (1997) argued that his F_S test was more powerful, particularly when detecting population expansion. Fu's test is based on the hypothesis that background selection gives rise to different polymorphic patterns than does logistic population growth, although both show excesses of rare alleles or recent mutations (Fu 1997). Fu's F_S tends to be negative when there is an excess of rare alleles (or recent mutations). A large negative value is taken as evidence against the neutrality of mutations and is indicative of rapid population growth, selection or genetic hitchhiking (Fu 1997). Fu (1997) described genetic hitchhiking as the situation that occurs when a neutral locus is linked to a locus experiencing natural selection. The outcome of this linkage is that when the favourable locus is selected, the neutral locus is 'dragged' along and increases in frequency within the populations as well. Fu (1997) suggested that the F_S value was particularly sensitive to detecting population expansion and genetic hitchhiking and, if used in conjunction with other tests, such as Tajima's D, could be used to differentiate between selection and population expansion. For example, if only the F_S value was significantly negative, then the cause is more likely to be population growth or hitchhiking and not selection or bottlenecks.

Tools For Population Genetic Analysis Version 1.3 (Miller 1997) and Arlequin 2.0 (Schneider *et al.* 2000) population genetics software was used to calculate the genetic population parameters. These software packages examine conventional and molecular polymorphism, either within or between populations, and provides average genetic diversity across the loci as defined by the equation 1, the number of polymorphic loci, genetic distances (Nei 1987), Slatkins linearized F_{ST} for pairwise comparison between populations (Slatkin 1995), AMOVA (Excoffier *et al.* 1992), Tajima's D (Tajima 1989) and Fu's Fs (Fu 1997).

4.3 Results

4.3.1 Screening for primer pairs

In an initial survey, the modified AFLP technique was used to screen 11 AFLP primer pairs using 10 Spanish and 10 New French individuals. The amplification using Pst1A/Mse1CAG produced a pattern that was too complex for hand scoring. The remaining primers pairs all showed varying degrees of polymorphism and complexity. Of the primer pairs tested, 4 primer pairs were selected for screening of the New French and Spanish populations. These pairs were chosen for reproducibility, simplicity in scoring and the number of observable bands, but provided differentiation to the level of the individual. A total of 93 bands were used in a screening of plume moth individuals from 4 different sub-populations.

The number of bands present and genetic diversity in the Spanish and New French laboratory and field populations were calculated using the Lynch and Milligan (1994) Taylor expansion and at the genetic diversity at the nucleotide level (Arlequin 2.0). The results indicated that the field populations had fewer observable bands and lower genetic diversity than observed in the corresponding laboratory cultures (Table 4.4). This is consistent with the assumption that the field colonies would have been founded from a subset of the genetic variation available from the source and may have experienced further bottlenecks or adaptation in the field. Despite the original laboratory cultures being founded with a similar number of individuals (50 for the Spanish and 59 for the New French) and the extra generations the Spanish population had been maintained in the laboratory, the Spanish laboratory culture had more genetic variation than that observed in the New

French laboratory culture. This cannot be readily explained and may be due to the composition of the original collection, subsequent bottlenecks and/or unidentified differences during rearing.

Table 4.4. Comparison of horehound plume moth AFLP results for the number of bands present, the average heterozygosity using the Lynch and Milligan (1994) Taylor expansion estimate (L&M) and the genetic diversity at the nucleotide level as calculated by Arlequin 2.0 (A2.0).

Population	Source	Number of individuals sampled	Number of bands present	Average heterozygosity (L&M)	Genetic diversity (A2.0)
Spanish	Laboratory	22	86	0.335	0.342
Spanish	Wilpenna	16	65	0.277	0.247
New French	Laboratory	21	62	0.223	0.202
New French	Narrung	18	48	0.166	0.153

A graphical representation of genetic distance data was made using the clustering method defined by the UPGMA algorithm using the Lynch and Millgan (1994) Taylor expansion estimates (Swofford and Olsen 1990) (Figure 4.2).

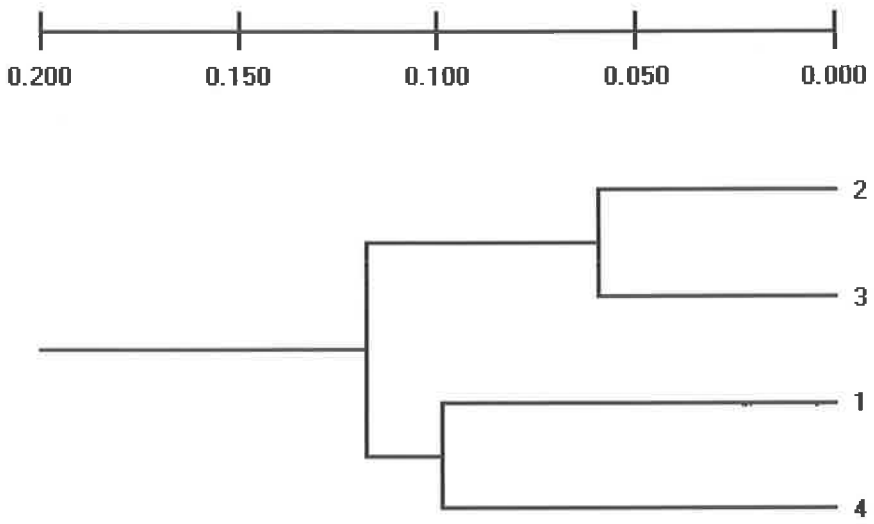

Figure 4.2. Genetic distance based on UPGMA clustering method. 1 = Spanish, laboratory; 2 = New French, laboratory; 3 = New French, Narrung; 4 = Spanish, Wilpenna

Slatkin's (1995) linearized F_{ST} values calculated using Arlequin 2.0 were used to produce pairwise comparisons between the populations and defined the differences between the populations (Table 4.5). All estimates made using Arlequin 2.0 software reflected those calculated based on the Lynch and Milligan (1994) Taylor expansion approach to estimating population statistics. Because no difference between the two approaches to estimating genetic population data could be determined, subsequent chapters use the parameters calculated at the nucleotide level in preference to the Lynch and Milligan (1994) estimates. This was done purely for the sake of simplicity.

Table 4.5. Slatkin's pairwise linearized F_{ST} values for the Spanish and New French laboratory and field populations, calculated by Arlequin 2.0.

	Spanish Lab	Spanish Wilpenna	New French Lab	New French Narrung
Spanish Lab	0			
Spanish Wilpenna	0.139	0		
New French Lab	0.206	0.302	0	
New French Narrung	0.289	0.443	0.185	0

The differences between population structure reflected what was expected from the known population history, i.e., because the original Spanish laboratory culture provided the founders for the Wilpenna release, they were expected to be more similar than to the New French populations and vice versa for the New French populations when compared to the Spanish populations. The pairwise comparisons suggested that there was some differentiation among the populations, particularly between the Wilpenna (Spanish) and Narrung (New French) releases. This may provide evidence that, purely by chance, the founders were very different in their genetic profile, or that there has been some adaptation occurring in the two different geographical locations.

Tajimas' D values were not statistically significant, but Fu's Fs were negative for all populations and were statistically significant at the 5% level (Table 4.6). This is indicative of rapid population growth, selection or genetic hitchhiking (Fu 1997). Clarke (2001) reported that the expected population increase per year for horehound plume moth releases varied from 39.1 to over 532.8 for the Spanish culture and from 21.7 to over 548.5 for the New French culture. Rainfall following the releases influenced the expected population increase. The recorded population increases observed by Clarke (2001) together with Tajimas' D and Fu's Fs statistics suggested that rapid population growth, not selection or genetic hitchhiking, was occurring within the 4 populations examined here.

Table 4.6. Tajimas' D and Fu's Fs values for the Spanish, New French, Narrung and Wilpenna field populations based on AFLP data, calculated by Arlequin 2.0.

Population	Tajimas' D	P(D simul < D obs)	Fu's Fs	Prob(sim Fs <=obs Fs)
Spanish Lab	1.402	0.914	-6.018	0.015
Spanish Wilpenna	0.738	0.795	-4.320	0.032
New French Lab	0.370	0.701	-8.466	0.003
New French Narrung	0.067	0.578	-7.957	0.005

4.4 Discussion

Population genetics is often based on a single sample in time, which means there is no absolute reference on which to base interpretations for any change (Wright 1965; Rousset and Raymond 1997; Woolliams *et al.* 1999). The potential for genetic drift and selection to occur immediately after sampling is not well understood in nature. Therefore, attributing the actual cause of genetic variation between populations is very difficult. Multi-locus data, such as AFLPs, are analysed as pairwise comparisons of complex patterns, scored only as a presence/absence of a band. Consequently, they have meaning only in relation to other patterns analysed in exactly the same way, or within the same study. But this is precisely what may be required when investigating biological control introductions, when the generation of large numbers of bands and complex profiles can be compared easily across individuals within the species and sub-populations.

Studies using AFLPs demonstrate that this method is a sensitive technique for distinguishing genotypes from different geographic origins, as well as detecting differences between local populations (Beismann *et al.* 1997; Arens *et al.* 1998; Reineke *et al.* 1998). These authors attributed a certain degree of the observed genetic variability between populations to the accumulation of polymorphisms resulting from both historical population bottlenecks and the adaptation to different environmental conditions. The study undertaken here established that molecular analysis using

AFLPs could be used to estimate the decrease in the frequency of bands present and genetic diversity. It also provided the opportunity to compare the molecular population genetic estimates produced using the Lynch and Milligan (1994) and the Arlequin 2.0 analytical methods. As both approaches produced similar results, the use of Arelquin 2.0 in subsequent chapters was considered adequate for the genetic population analysis. The data also provided information about the differentiation and variation possible between and within geographically separated releases made in Australia over a short time period.

Many of the statistical tests traditionally used for interpretation of genetic data are of little use when assumptions of equilibrium, such as Hardy-Weinburg, are violated (Davies *et al.* 1999). For example, it is frequently assumed that any statistical significance in genetic differentiation is the result of isolation (van Oppen *et al.* 1997), but Hedrick (1999) pointed out that if any of the populations under examination had undergone substantial reduction in population size, then calculated genetic distances may increase very quickly as the populations expand. This means that any measure of differentiation may reflect differences in effective population sizes and expansion rates, not genetic distance. Factors such as populations' equilibrium, rapid expansion, random mating or bottlenecks must be taken into account when interpreting results. However, if statistical differences are detected between populations, then it becomes possible to investigate the reasons for the differences based on all known information about particular cases.

Analysis of population structure is based on the probability of genes occurring within and between populations (Slatkin 1987; Rousset 1997). Estimators such as F_{ST} are used to define patterns of sub-divisions between populations (Rousset 1997). For example, the Slatkin's linearized F_{ST} table and the Lynch and Milligan's (1994) analytical methods used to determine genetic distance based on UPGMA clustering method defined the expected differences among the sub-populations of horehound plume moth released in the field and their origin. They also highlighted that, even over 4 generations, differentiation may actually be the result of random variation caused by rapidly expanding populations and founder variation, because the results of Tajima's D and Fu's F_s were indicative of rapid population growth, selection or genetic hitchhiking. Although no investigation

of genetic hitchhiking was made, there was no suggestion that individual loci were becoming fixed across populations released in similar conditions. The results provided further support for the hypothesis that isolated populations, although they maybe less genetically diverse within the population, were more genetically variable between populations (Futuyma 1979; Slatkin 1987).

The importance of differentiation between populations can be further explained in light of the results observed by Gaudel *et al.* (2000) and Clarke (2001). In a study on the endangered alpine plant, *Eryngium alpinum* L. (Apiaceae), using AFLP markers Gaudel *et al.* (2000) found the F_{ST} values revealed high differentiation among populations (mean pairwise $F_{ST} = 0.40$). This appeared to be independent of geographical distance. The authors proposed that founder events during postglacial colonisations and/or bottlenecks explained this high but random genetic differentiation. Clarke (2001) investigated the genetic distance between a French and a Spanish laboratory horehound plume moth culture. Using 13 polymorphic allozyme loci, Clarke (2001) concluded that differentiation between the Spanish and New French laboratory cultures was minimal. Assuming that the New French and Spanish cultures have not undergone strong genetic drift, or inbreeding, since the original releases made by Clarke (2001) and the individuals harvested for the AFLP analysis presented here, then the importance of biological versus statistical significance for the interpretation of population hierarchy using pairwise comparisons becomes more apparent. The results of my AFLP analysis consisted of 93 bands, which permitted the identification down to the level of an individual. This produced more variation than could be expected within or between populations that would be observed for allozyme data using 13 polymorphic loci. This indicated that the differences between the laboratory cultures were far higher than shown by Clarke's (2001) allozyme study. The field populations were also assumed to be rapidly expanding. Consequently, it was concluded that the high genetic differentiation observed between the horehound plume moth populations examined in this pilot study was likely to be the result of random founder effects or subsequent bottlenecks experienced in the field and not from any pattern of isolation resulting from the distance or environmental differences between the release sites.

All field populations appeared to have a lower frequency of bands present and average genetic diversity than the laboratory populations. It could be noted that such loss of diversity in populations founded using a sub-set of individuals from a larger source population has been observed for smaller, fragmented populations when compared with larger populations (Korn 1994; Berger and Cunningham 1995; Frankham 1995b; Young *et al.* 1999). Additionally, analysing an even smaller subset of individuals could mean that rare alleles are not detected at all. Assuming the laboratory cultures have maintained the maximum number of possible bands present, there were 11(12%) bands present at a frequency of less than 0.10. All of these were detected in the Wilpenna (Spanish) population and 7 where detected in the Narrung population, so it can be assumed that failure to detect rare bands was not an issue.

One complication with the founder experiment was that the sample sizes of harvested individuals might not be equal in these experiments. Consequently, a simple bootstrapping simulation was undertaken to investigate the effect sample size had on Fu's Fs to determine if it would be a useful parameter to investigate selection, drift and/or rapid population expansion when sample sizes were different. The profiles obtained from the AFLP data in this experiment were used to create simulated populations of varying size, and the Fs statistic calculated for each and plotted against the number of polymorphic loci in each simulated populations (Figure 4.3). The results showed that Fu's Fs value was affected by the number of individuals analysed, while the number of recorded bands had little effect. This raised concerns about the usefulness of the statistic unless sample sizes were similar. As a result, it was decided that the frequency of individual bands would be compared between field releases in subsequent experiments to determine if they showed evidence for selective sweeps or fixation for any locus.

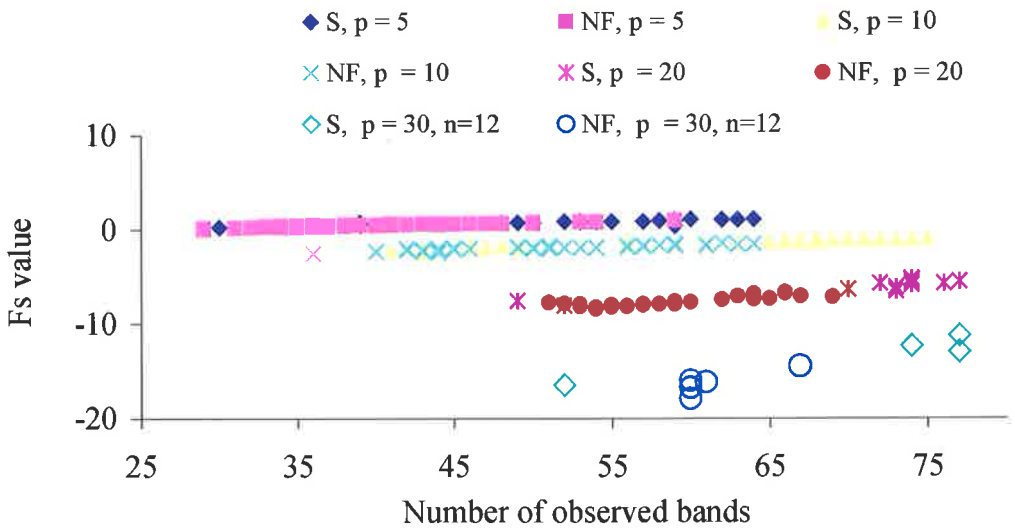

Figure 4.3. Estimates of Fu's Fs, 50 iterations for simulated populations containing 'p' individuals for 'n' iterations. 12 iterations for simulated populations containing 30 individuals. Populations simulated by bootstrapping AFLP data. S=Spanish, NF=New French.

In summary, it can be said that the populations appeared to show differentiation in the field over relatively few generations. But determination of whether this was due to the original geographical separation of the two laboratory colonies and/or historical bottlenecks, selection or random genetic drift was not possible, although the Tajima's D and Fu's F_S statistics suggested that the differentiation was the result of random chance and population expansion and not selection. However, the results of the bootstrapping analysis suggested that the use of these statistics were of limited value unless sample sizes were similar. Consequently, it was decided that future studies would use Bonferroni Chi squared tests (Rice 1986; Rice 1990), and pairwise tests of the band frequencies order to investigate the possibility of fixation for a particular band was occurring across all populations in a given location. If this occurred it may indicate selection in that loci or one closely linked.

5. The effect of release size on genetic diversity of horehound plume moth populations established in high and low rainfall areas of South Australia

5.1 Introduction

Loss of genetic diversity and inbreeding are expected consequences of poorly managed introductions of a classical biological control agent. This loss of genetic diversity in very small populations has been associated with decreased population fitness and/or a reduced potential for responding to changes in the environment (Ewens *et al.* 1987; Soulé 1987; Ryan and Seigfried 1994; Ouberg and Vantreuren 1994; Hedrick and Gilpin 1997; Newman and Pilson 1997; Fischer and Matthies 1998; Vrijenhoek 1998). But the literature also contains examples of colonies founded by a few as two individuals or populations with low genetic diversity that have persisted for long time periods (Newsome and Noble 1986; Ralls *et al.* 1986; Cameron *et al.* 1993; Caro and Laurenson 1994; Quemman 1996).

If the existence of genetic variability is regarded as a prerequisite for successful establishment of biological control agents, what level of losses will limit the colony's ability to adapt to the new conditions via natural selection processes? Several studies have investigated associations among population size, fitness traits and genetic variability but the conclusions are contradictory, with some authors finding no association of fitness traits, genetic diversity and population size (Ouberg and Vantreuren 1995; Young *et al.* 1999), while others reported a positive correlation for some fitness traits, such as growth rates (Newman and Pilson 1997; Fischer and Matthies 1998; Vrienhoek 1998; Rebordinos *et al.* 1999). Unfortunately, none of these studies were able to determine if loss of variation was important to long-term viability, although Newman and Pilson (1997) concluded that small effective population size significantly increased the probability of population extinction, over and above that due to random demographic factors. Given that some

authors reported decreased genetic diversity together with decreased population fitness in some populations, it is possible that small populations are losing genetic diversity, but the quantitative genetic variation underlying fitness traits has not yet been affected. If such genetic erosion is a major cause of viability problems for long-term survival of a population, then genetic diversity is necessary to preserve overall population fitness, and allow for post-release adaptation. Thus, genetic diversity would have to be considered when planning releases of a biological control agent. The purpose of this study was to investigate the effect of release size on genetic diversity of horehound plume moth populations established in high and low plant quality areas of South Australia and to determine if there was a correlation between the richness of either polymorphisms or genetic diversity and population replacement rates.

5.2 Materials and methods

5.2.1 Preparation of harvested individuals and AFLP analysis

The experimental design and releases were those outlined in chapter 2.3. In spring of 1999 late instar larvae were collected at random from the Spanish laboratory colony, frozen in liquid nitrogen and then stored at –20°C until DNA analysis could be undertaken. During the final spring census in 2000, late instar larvae were harvested haphazardly from established release sites, stored separately with a supply of fresh horehound leaves until returned to the laboratory. Once in the laboratory, they were also frozen in liquid nitrogen and stored at –20°C for DNA analysis. Collection of at least 20 individuals was attempted, but this was not always possible at sites where population establishment was low.

The method for DNA extraction from individuals was the same as that used in chapter 3.2.1 while the methods for AFLP analysis, band visualisation and data analysis was outlined in chapter 4.2.3, 4.2.4 and 4.2.5. Because of the time constraints on the project and the number of samples that were processed, one primer pair was selected. This primer pair (AAT/CAA) resulted in a pattern providing a total of 30 repeatable bands for scoring, with the Spanish laboratory population (source for the releases) showing 28 polymorphic sites.

5.2.2 Data analysis

Arlequin 2.0 was used to calculate the number of polymorphic loci and the average genetic diversity across the loci as outlined in chapter 4.2.5. It was also used to calculate the pair wise Slatkin's linearised F_{ST} values between the populations. Tools for population genetic analysis (Version 1.3) were used to develop the tree using the UPGMA algorithm with bootstrapping (Miller 1997).

To investigate differences between the frequencies of the bands present at each locus in each field population, each locus was compared with the laboratory source population in a two by two contingency table containing the frequency of band present versus band absent in each population. A Chi-squared test was then applied. The approximation of the Chi-square applies when the samples are drawn at random and the sample size is adequate (Meddis 1975). Expected frequencies should reflect the frequencies found in the source Spanish population and a significantly large value of Chi-squared indicates one of the assumptions is false. If one of the cells in the two by two table is less than 5, it presents a problem because this implies that the observed frequencies do not reflect the population distribution from which the sample was drawn (Meddis 1975). When this occurred, the Fisher exact test was applied. Epi6.0 (Harvard University) was used to calculate the Chi-square values (alpha = 0.05), or the Fisher exact test when it was necessary. To address the problem of a Chi-squared test being significant purely by chance, a sequential Bonferroni correction was applied to the resulting probabilities for each comparison (Rice 1989; Rice 1990).

Population replacement rates were estimated for each replicate with population data recorded 12 months after releases were made. These results were used to calculate the population replacement rate, which was determined as the recorded population number at 12 months divided by the original release size. These figures were used to determine if there was any correlation between population replacement rates and the number of observed bands present, or the average genetic diversity across the loci, in either the high or low rainfall areas. Pearson's r-values were used to determine the measure of correlation between the population replacement rates and the number of bands

recorded or the average genetic diversity for each population in either high or low plant quality locations.

5.3 Results

Field populations were sampled after 12 months, or an assumed 4 generations. All field populations sampled showed fewer bands present and lower average gene diversity at the nucleotide level across the loci when compared to the source population (Table 5.1).

Table 5.1. Number recorded bands present and average genetic diversity for field populations in high and low plant quality locations after 4 generations compared to the source Spanish population.

Founder size	Plant quality	No. of successful releases	No. of individuals analysed mean (sd)	No. of recorded bands present mean (sd)	Average genetic diversity mean (sd)
Source	Laboratory	NA	22 (NA)	28 (NA)	0.323 (NA)
10	High	5	11.6 (4.0)	18.6 (5.3)	0.219 (0.047)
30	High	5	12.8 (3.3)	19.6 (3.9)	0.227 (0.036)
90	High	5	14.6 (0.5)	20.6 (1.8)	0.235 (0.024)
270	High	5	14.0 (1.0)	17.4 (2.3)	0.200 (0.029)
10	Low	1	9.0 (NA)	19.0 (NA)	0.267 (NA)
90	Low	1	14.0 (NA)	19.0 (NA)	0.227 (NA)
270	Low	3	11.3 (2.5)	19.0 (3.5)	0.245 (0.047)

The pairwise Slatkin's linearised F_{ST} values did not suggest any pattern in differentiation between the high or low plant quality locations, with variation apparently being dispersed at random between locations and founder sizes (Appendix: Table A.4). To visualise the differentiation

between populations estimates of genetic distances (Wright 1978) were used to prepare a tree using Tools for Population Genetic Analysis (Miller 1997). There was no apparent pattern of grouping between the low and high plant quality releases (Figure 5.1). The results of the Bonferroni corrected Chi-squared tests (p = 0.05) indicated the band present frequencies at 3 loci were significantly different from the source laboratory population ($p < 0.0001$). All 3 of these bands were from different populations and the frequencies were unique to each population. Molecular markers appear to be poor indicators of heritable variation in adaptive traits (McKay and Latta 2002) but these results supported the notion that the differentiation between the populations was due to founder effects and/or genetic drift and not to selection processes.

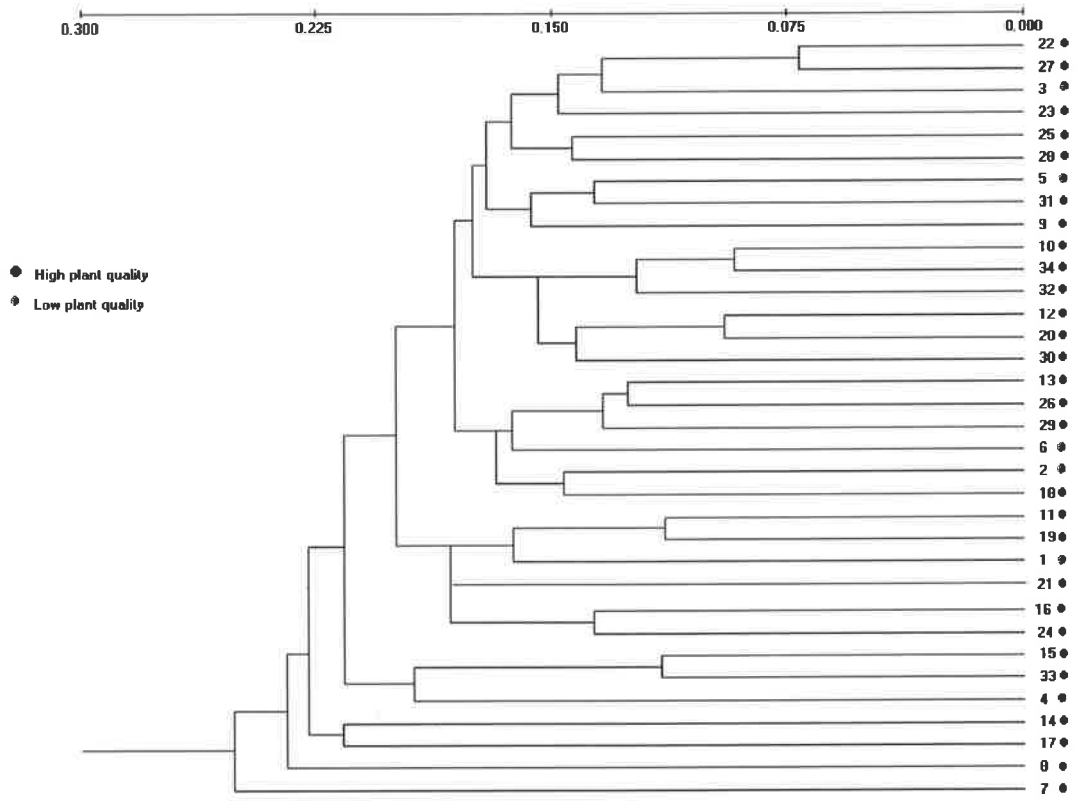

Figure 5.1. Genetic distance based on UPGMA clustering method. Releases made in the low plant quality locations represented by 1 through 6; releases made in the high quality locations represented by 7 through 34.

The number of bands present and the average genetic diversity, both within and between sites, showed a high level of variation, but did not appear to be different at any of the founder sizes

(Figure 5.2, Figure 5.3). Higher within and among population genetic variation was observed at lower founder numbers, although this would not be unexpected because the chance that rare alleles may be omitted by sub-sampling is more likely.

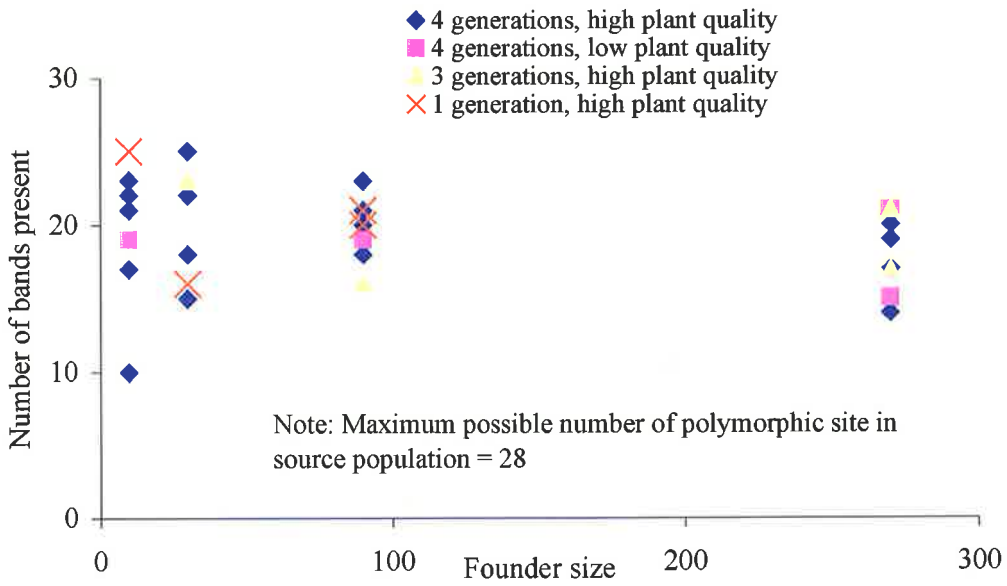

Figure 5.2. Number of bands present remaining in successful horehound plume moth populations as detected by AFLP analysis using Pst 1 AAT/ Mse 1 CAA primer pairs for selective amplification.

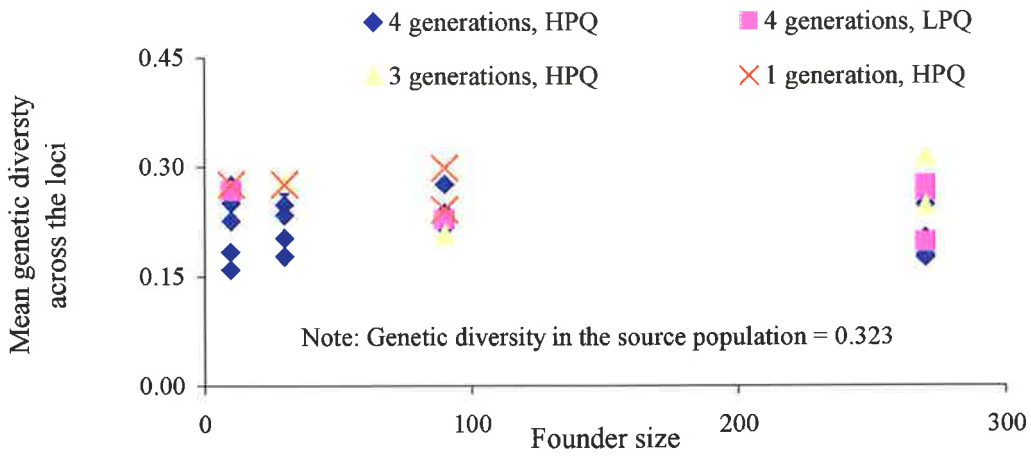

Figure 5.3. Average genetic diversity in successful horehound plume moth populations as detected by AFLP analysis using Pst 1 AAT/ Mse 1 CAA primer pairs for selective amplification. HPQ = high plant quality, LPQ = low plant quality.

To test the predictions of Nei *et al.* (1975) that genetic variation would be maintained by rapid population growth because the impacts of genetic drift would be minimised by rapid population growth, the number of observed bands or genetic diversity were correlated with population replacement rates (Table 5.2). There was no clear association between population replacement rates over 4 generations, bands present or average genetic diversity. However, the possibility of any correlation was tested with Pearson's *r*. For all but 2 of the populations with an assumed 4 generations of growth the population replacement rates were high, therefore the assumption was made that genetic variation would be maintained when populations were undergoing rapid expansion. Consequently a 1-tailed *t*-test for the number of bands present versus the population replacement rates was calculated for all sampled populations with 4 generations data (Table 5.3).

Table 5.2 Population replacement rates, number of bands present and average genetic diversity by plant quality and generations in the field for horehound plume moth.

Plant quality	Generations in the field	No. founders	Population replacement rates	No. bands present	Ave gene diversity
High	4	10	0.5	10	0.16
		10	22.2	17	0.184
		10	203.3	23	0.275
		10	347.4	22	0.25
		10	87.3	21	0.226
		30	127.8	25	0.27
		30	58.1	15	0.203
		30	80.0	22	0.234
		30	265.6	18	0.248
		30	22.7	18	0.178
		90	12.2	21	0.227
		90	228.4	23	0.276

		90	59.5	18	0.218
		90	74.5	21	0.237
		90	68.2	20	0.217
		270	170.4	17	0.178
		270	378.0	19	0.202
		270	42.4	20	0.248
		270	105.0	14	0.198
		270	101.3	17	0.175
High	3	30	0.7	23	0.279
		90	4.7	16	0.206
		270	2.9	17	0.311
		270	11.6	21	0.245
High	1	10	0.9	25	0.276
		30	0.7	16	0.276
		90	2.6	21	0.298
		90	2.3	20	0.241
Low	4	10	34.2	19	0.267
		90	14.9	19	0.227
		270	6.8	21	0.266
		270	0.7	15	0.196
		270	1.9	21	0.274

Table 5.3. Pearson's r and t-values (alpha = 0.10) for the number of bands present and average genetic diversity across the loci against population replacement rate for horehound plume moth populations released in high plant quality locations over an assumed 4 generations.

Plant quality	No. of populations	d.f.	No. of bands present			Average genetic diversity across the loci		
			r-value	t_{obs}	p	*r*-value	t_{obs}	p
High	20	18	0.329	1.671	p=0.08	0.385	2.001	p=0.03

The results indicated a significant positive correlation for the average genetic diversity and the population replacement rates, but R^2 value for the linear trend line only explained 15% f the variation. The paucity of data due to population extinctions in low plant quality areas restricted definitive conclusions regarding the possibility of an association between the population replacement rates and the number of polymorphic loci or the average genetic diversity.

5.4 Discussion

The few laboratory and field studies that have been undertaken indicated that a decrease in genetic heterogeneity and loss of rare alleles would occur when sub-samples were taken from their source population (e.g. Korn 1994; Berger and Cunningham 1995; Frankham 1995b). These results agreed with these findings and the observations made in chapter 4 that sub-populations tend to have more null allele polymorphic loci and lower average genetic diversity than the source population. However, the probability that the genetic diversity or number of polymorphic sites can be related to successful establishment of a biological control agent remains moot. These results suggested that there was a positive association between population replacement rate and the number bands present or the average genetic diversity. In this respect, they supported the finding (Rebordinos *et al.* 1999) that there was a positive association between multilocus heterozygosity and growth rates and provided some theoretical correlation with the finding (Gaudeul *et al.* 2000) that there was a

positive correlation between genetic diversity measurements and population size for the endangered alpine plant, *Eryngium alpinum* L. (Apiaceae). Additionally, the results also provided support for the conclusion that reduced population growth caused genetic losses (Fischer and Matthias 1998), but did not in themselves support the premise that reduced genetic variability affected population viability because population growth rates remained high for almost all the released populations.

Genetic diversity within populations has been proposed as an indirect indicator of extinction risk because it affects the evolutionary potential of a species (Allendorf and Ryman 2000). Genetic diversity has also been proposed as an indicator of inbreeding depression (Hedrick and Miller 1992). There was a positive correlation between genetic diversity and population replacement rate, which suggested that slower population replacement rates would indicate lower levels of heterogeneity for the horehound plume moth. However, it still seems unlikely that extinction risk could be based on levels of genetic diversity alone. However, the amount of genetic differentiation among populations or observed decreases in genetic diversity could provide useful data for planning actions such as re-introduction or augmentation with further individuals. For example, if large decreases in genetic diversity are recorded for releases then further individuals from other locations with different genetic profiles can be added to the population to increase genetic diversity.

On a locus-by-locus basis, the bands that returned significant statistical differences from their source population were not the same across the populations. Neither the Slatkin's pairwise linearised F_{ST} nor the tree calculated from Wright's genetic distances indicated the presence of selection. Consequently, it could be concluded that any differences observed between the populations were due to founder effects and/or genetic drift and not due to selective forces.

It should be acknowledged that selection might take a long time to become evident in a genotype. Eccleston (1996) tested the hypothesis that a parasitic wasp, *Brachymeria intermedia* (Nees),

introduced into the USA as a biological control agent against the gypsy moth, *Lymantria dispar*, had undergone adaptation. This wasp was originally introduced on several occasions from 1908 to 1963 but establishment was considered unsuccessful until 1965. Offspring from different crosses between Corsican and Massachusetts individuals differed significantly in weight and in the proportion surviving freezing. These observations suggested that adaptation had occurred, although it was unclear which introduction(s) were responsible for the final establishment and the author assumed that the wasp was present for a relatively long time before establishment was confirmed. However, it is possible that the adaptation occurred over the time period between 1908 and 1965. In a study on grayling, *Thymallus thymallus*, populations Haugen and Vollestad (2000) found that differences in early life-history traits suggested that natural selection resulted in local adaptation over a time period of 13-18 generations. The observations made on the horehound plume moth covered a maximum of 4 generations, so the possibility of future selection cannot be dismissed. But more generally, the data support the supposition that for small, slow growing populations the founder effect and random genetic drift, not selection, are the driving forces of genetic differentiation.

The number of extant populations available in the low plant quality area at the end of the project made it impossible to draw any conclusion on the possibility of a relationship between population growth rates and decrease in the number of recorded bands present or average genetic diversity. The lack of replicates for seasonal releases also prevented investigation of possible correlation between population replacement rates and other factors in those cases.

Recorded population numbers over 4 generations for populations released where host plant quality was high indicated that populations were rapidly expanding despite observed losses genetic diversity. Those populations that managed to establish in the low plant quality release areas had low intrinsic growth rates. Over subsequent generations this restriction on population growth could exacerbate any inbreeding depression and result in increasing homozygosity. Later experiments on inbreeding (Chapter 7) indicated that inbreeding could severely affect the viability and potential for populations to persist if numbers remained low and growth rates slow. The effect this may have

had on attempts to establish horehound plume moth in low plant quality locations will be discussed further in chapter 8.

The use of AFLP techniques permitted a large number of polymorphisms to be identified and used for analyzing the population structure and differentiation in this study. But AFLP markers are thought to be dominant with many alleles per locus (Hoelzel 1998; Kraus and Peakall 1998; Baker 2000) and dominant markers are not as efficient at detecting heterozygosity as co-dominant markers (Lewis and Snow 1992; Lynch and Milligan 1994). Estimates of heterozygosity from populations released on high and low quality plants and their relationship to release sizes would have been useful, as such information would have helped examine the hypothesis that inbreeding levels in populations founded with fewer individuals or at low plant quality locations would have been higher than corresponding levels with greater numbers or at high plant quality locations. Therefore, the use of co-dominant marker techniques such as microsatellites would be a useful option in further studies.

Measuring the effect of decreased genetic diversity on long-term viability was not possible given the time frame of this project, but low population replacement rates appeared to be associated with lower average genetic diversity and fewer bands present in the populations. Low population replacement rates would have the potential for increasing the likelihood that a population would be driven to extinction if a local catastrophe or extreme environmental fluctuations were encountered. If this were the case, management decisions for biological control programs may need to be altered. Not only would it be necessary to ensure maximum genetic diversity, and the inclusion of rare alleles in releases, as recommended by Roush (1990), but monitoring the genetic profile of a population over successive generations to ensure genetic diversity was maintained would prove to be a useful strategy until sites were well established if decreased genetic diversity was known to be associated with long-term viability.

The rate of gene frequency change caused by genetic drift depends on the effective size of the population (Futuyma 1979). The effective population number is defined as the size of a uniformly

reproducing population, with equal contribution from all adults, that would have the same homozygosity as that of the observed population (Crow and Kimura 1979). However, little is known about the effective size of natural populations (Nei and Tajima 1981). In a study investigating the effective population size of olive fly (*Dacus oleae*), Nei and Tajima (1981) noted that their estimate of the effective population size, calculated from changes in allele frequency, was very small when compared to the actual size of the population under investigation. The authors presented several explanations for this discrepancy. The first was that not all individuals participate in mating, and the second was that the sampling of the alleles was inadequate. Nei and Tajima (1981) also noted that estimating effective population size was subject to a large sampling error unless a large number of generations and a large number of genes are sampled.

This study provided the opportunity to examine the effective population size for the horehound plume moth to determine if the effective population was low compared to the census size, or if it differed between the release sizes. However, using frequencies estimated for markers that are dominant would result in biased estimates (Jorde *et al.* 1999). Jorde *et al.* (1999) investigated the quantification of biases for allele frequency estimators and developed an estimator for the effective population size when dealing with dominant gene markers, such as AFLPs. They noted that twice as many loci or sampled individuals were required when using dominant markers to achieve the same precision as was observed for co-dominant markers. Using the formula developed by Jorde *et al.* (1999), the harmonic mean of the genetically effective population size from populations established for 4 generations in the field was calculated (Table 5.4). The following assumptions were made in the estimation of the effective population size: generations were discrete, no selection was occurring and no migration occurred from outside populations (Nei and Tajima 1981). The following equation (Jorde *et al.* 1997) was used to calculate the effective population size

$$\hat{N}e = \frac{1}{2\left[F_k - \frac{\left(1-\hat{q}^2{}_z\right)}{2\hat{n}\hat{q}_z\left(1-\hat{q}_z\right)}\right]} \qquad \text{(equation 1)}$$

where $\hat{n}$ is the harmonic means of the 2 sample sizes, $\hat{q}_z$ is the mean frequency of the recessive alleles ($\hat{q}_x$ and $\hat{q}_y$) from both samples, calculated using the Lynch and Milligan (1994) estimator,

and F_k is the measure of the temporal allele frequency, originally defined by Pollack (1983) and written by Jorde *et al.* (1999) as

$$F_k = \frac{(\hat{q}_x - \hat{q}_y)^2}{\hat{q}_z(1-\hat{q}_z)} \qquad \text{(equation 2)}$$

Table 5.4. Initial founder size and harmonic mean of genetically effective population size for horehound plume moth colonies after 4 generations of field release, estimated according to Jorde et al. (1999).

Plant quality	No. populations extant	Founder size	Effective population size mean (sd)
High	5	10	35 (22)
High	5	30	24 (5)
High	5	90	57 (66)
High	5	270	30 (16)
Low	1	10	32 (NA)
Low	1	90	27 (NA)
Low	3	270	43 (16)

The results calculated were of the same magnitude as the value calculated by Clarke (2001) for the horehound plume moth laboratory culture after 25 generations. Clarke (2001) used Wright's (1931) equation to calculate an average effective population size of 23. No statistically significant variation in effective population size on the basis of founder size or plant quality could be detected and the overall mean value of the genetically effective population size for the field populations in this study was 35. This suggested that, provided the assumptions applying to the genetic effective population size are met, a horehound plume moth population with at least 35 adults could maintain the homozygosity of the observed populations. A review by Frankham (1995c) suggested that the ratio of effective population size to census size is in the order of 0.1, based on estimating changes

in homozygosity over time. Thus, the census population size required to maintain the homozygosity of an observed horehound plume moth population should be greater than 350.

Chapter 2 provided evidence that, as long as host plant quality was high, small releases could be made and populations would establish and grow rapidly. But no prediction on how long-term genetic effects would influence long-term persistence could be made at that time. Observation taken in the laboratory indicated that random mating produced around 14 adult offspring per female (results shown in chapter 7). Hence, it could be assumed that each female has the potential to increase population numbers (as determined by the number of females present) by at least 10-fold per generation. Thus, the finding that the effective population size was quite small has implications when planning releases of horehound plume moth. Together with the observations from chapter 2 that successful establishment using very small release sizes could occur, the finding that the effective population size was quite low suggested that initial release numbers could be quite small, without managers becoming concerned that inbreeding would become a problem in small colonies.

The standard method for estimating allele frequencies for dominant markers assumes Hardy-Weinburg proportions in order to estimate allele frequencies at dominant loci (Jorde *et al.* 1999). Unfortunately, dominant markers do not allow the testing of Hardy-Weinburg assumptions. However, because the results of the Chi-squared tests indicated that no selection was occurring in any of the field populations, it could be assumed that each release was a single, randomly mating population with no other evolutionary force acting on it other than genetic drift. In this case, large deviations from Hardy-Weinburg proportions would be unlikely to occur and the temporal method of calculating changes in gene frequency could be applied (Jorde *et al.* 1999). The horehound plume moth exhibited high mortality rates among the juveniles but showed a high fecundity rate in the survivors, even if all adults did not mate (21% of randomly paired moths failed to mate or produce offspring in the laboratory, cf: chapter 7.3.1). This meant that the total population, observed in the field, may well be very much larger than the effective population size in each generation, even though the survey data consisted primarily of juvenile life stages.

The hypothesis that selection is a driving force when populations establish in novel environments was not supported by these data. Because the Spanish horehound plume moth was specifically selected on a climate-matched basis, it is possible that there was enough plasticity in the source population for strong selection or adaptation not to emerge as detectable factors for releases made in high and low plant quality areas of South Australia. When insect numbers are low, some individuals contribute more to the next generation, purely by chance. Thus, genetic drift can lead to the decay of genetic diversity in a population (Hopper et al 1993), but as the population increases, the effect of genetic drift is minimized (Nei 1975; Roush 1990). The results observed in this experiment suggested environmental conditions and random founder effects would have more of an effect on the probability of successful establishment than any of the other hypothesised genetic causes of extinction.

Theory predicts that genetic drift in small populations and inbreeding both cause a decrease in mean population fitness (Newman and Pilson 1997; Frankham 2000). The 3 successful populations investigated from the low plant quality locations did not show statistically significant differences in the magnitude of decrease in the recorded number of bands present(or genetic diversity) compared to populations from high plant quality locations. Harvesting individuals from field populations in both locations and determining fitness traits such as fecundity and egg to adult survivorship could elucidate if there has been a loss of viability when population growth rates are lower or if they are due to environmental factors. Fewer colonies established in the low plant quality areas, and no measurements of genetic diversity could be made for extinct populations. It could be possible that inbreeding and genetic drift, exacerbated by slower population growth rates in these low plant quality areas, contributed to an increased probability of extinction where the environmental conditions were more extreme.

The increased application of AFLPs to population genetics could stimulate the development of new statistical tests such as those attempted by Tajima (1989), Fu (1997) and Jorde *et al.* (1999). Restriction fragments generated by AFLP reactions and selected for use can number in the 1000s if many primer pairs are used. For example, Seki *et al.* (1999) generated 745 DNA bands using 19

arbitrarily chosen primer combinations. Hand scoring multiple samples becomes the limiting factor, but software packages can be utilized that allows the easy scoring of this number of bands. This means that the ease of AFLP methodology can be utilized to full advantage for studies involving genetic variability and divergence between populations. If this technique is coupled with even a few allozyme or microsatellite markers, then the discriminatory power of AFLPs for detecting variability, divergence and selection and the power of co-dominant markers could be combined to investigate the interaction of environment and genetics in population studies.

6. Effect of outcrossing between Spanish and French horehound plume moth populations on establishment and population growth rate of horehound plume moth colonies

6.1 Introduction

A physical barrier, such as the geographical separation of individuals of the same species, can preclude gene flow and cause diversification between populations as local selection and adaptation occurs. As a result a particular trait may become depleted or lost from a population (Slatkin 1987; Connor and Ferguson-Smith 1993). Differences between strains that have evolved in separate locations can be expressed in performance that is the result of selection for advantageous alleles and not merely the result of genetic plasticity. Hopper *et al.* (1993) reviewed several case studies for various species of biological control agents where there was a recorded between-population variation in several traits. These traits included the acceptance of hosts, developmental time, fecundity, host range, insecticide resistance, mortality, searching behaviour, sex ratio, host suitability and temperature tolerances, all of which could affect the establishment of a biological control agent.

There are many documented examples of situations where outcrossing vigour does occur, especially in plants where selfing can deplete genetic variation (Allard 1965; Ouberg and Vantreuren 1994; Ruckelshaus 1995; Smitherson *et al.* 1996; Carr and Dudash 1997). But the assumption that outcrossing will restore genetic variation, improve individual fitness and the flexibility for coping with environmental challenges is not always valid (Wilson 1965; Berger and Cunningham 1995; Nagy 1997). Wilson (1965) hypothesised that when one species invaded the range of a closely related second species, the two could compete or hybridise in such a way that their average genotype fitness would be lowered. One hypothesis for decreases in average genotype fitness is that outcrossing may disrupt gene complexes that have evolved in populations as a result

of co-adaptation (Dobzhansky 1955; Futuyma 1979). Co-adaptation occurs when alleles at different loci have evolved to be advantageous together, but not separately (Futuyma 1979). Futuyma (1979) suggested that the breakdown of co-adaptation causes deleterious interactions to be expressed in hybrid populations because formerly advantageous complexes are disrupted and the alleles may show unpredictable, non-additive effects when acting in a new genetic background. The risk of outcrossing depression is rarely acknowledged (van den Bosch 1975; Berger and Cunningham 1995; Nagy 1997) but resultant depressions in reproductive rate, fecundity or survival may be large enough to place the survival of a small population in jeopardy.

Published literature investigating genetic profiles for outcrossed populations and fitness traits is virtually non-existent. The persistence of differences between strains, when reared in a common environment, can suggest a genetic basis, but it does not exclude maternal effects like nutrition and vertically transmitted diseases (Hopper *et al.* 1993). Collections of the horehound plume moth from Spain (Spanish) and France (New French and Old French) were imported into Australia. Clarke (2001) analysed insects from these Spanish and French sources using 13 polymorphic allozyme loci and noted there were no observable fixed differences between the two populations and Neis' genetic distances were small (Nei 1978). Clarke (2001) reported that egg number and viability of reciprocal crosses between French and Spanish individuals produced fewer eggs and had lower egg viability for some crosses when compared to crosses within each culture. Clarke (2001) concluded that the cause for this observation was unclear but may be genetic in origin.

Whatever the cause for the observed decrease in fecundity and viability of Spanish and New French horehound plume moth crosses made by Clarke (2001), such losses in fecundity and viability could lead to a slowing of the population growth rate. This could have negative impacts for horehound plume moth establishment and/or long-term persistence of colonies if outcrossing occurred. It is important that the consequences of possible outcrossing depressions be investigated or inappropriate release strategies could endanger the release program of a biological control agent.

Outcrossing depression could result in either of the following: the establishment rate of releases may be unacceptably low or a prolonged low population growth rate could increase the likelihood of extinction, the agent itself may not be detected for many generations or the density of the agent may remain too low to cause any detectable effect on the pest species assuming an outcrossing depression persists. Unless the cause for any of these outcomes can be clearly identified, it is possible that an agent could be declared a failure because of an outcrossing depression, not because it was an unsuitable choice for the environment it was released into.

The question addressed in this study was whether outcrossing of two horehound plume moth populations, collected from different regions and maintained separately in the laboratory, would affect the descendants of the crosses between the horehound plume moth populations in a detrimental manner and subsequently reduce the probability of establishment. Additionally, the experiment was used to investigate if genetic differences could be identified as the cause of decreased fecundity for crosses between the Spanish and New French cultures. AFLP analysis was undertaken to investigate the genetic profiles of individuals harvested from field populations and resultant population profiles were compared with the recorded growth rates of extant field populations to determine if outcrossing depression could be correlated with results from the AFLP analysis.

6.2 Materials and methods

6.2.1 Foundation stock and field releases

Reciprocal outcrossing of the two populations (Spanish and New French) was undertaken in the laboratory. All but one of the laboratory crosses were founded with 8 adult virgins of each sex. This was to ensure each of the hybrid and 'pure' populations experienced similar sized bottlenecks prior to release. The exception was the New French female by New French male, which consisted of 8 virgin females and 6 virgin males. It was assumed that random mating occurred and no correction for family size was made when late instar larvae were collected for field release. The initial Spanish female by Spanish male (SxS), New French female by New French male (NFxNF), Spanish female by New French male (SFxNFM) and New Frecnh female by Spanish male

(NFFxSM) crosses produced 56, 68, 101 and 61 offspring, respectively. Minimums of 20 larvae were harvested for genetic evaluation. The remaining late instar larvae formed the founders for the field releases. The shortage of larvae limited the number of founders for final field releases and replication, particularly as some of the individuals from each cage had been harvested for molecular analysis. The final numbers allowed only one replicate each of the SxS and NFxNF parental lines. Numbers of hybrid offspring allowed two replicates of NFFxSM and three replicates of the SFxNFM crosses. Late larval instars within each line were randomly allocated to replicates for field release.

Field releases were all made on a single farming property on the same day, 3/03/2000, and differed only in the composition of the crosses and the numbers of F1 late instar larvea released (Appendix: Table A.1). The farming property was approximately 15 km from Corny Point, on the Yorke Peninsula, South Australia (Figure 6.1., Table A.1). The SxS, NFxNF, 2 SFxNFM and one NFFxSM field releases all consisted of 30 founders (see Chapter 2 for release methodology). There were two additional releases were made, a SFxNFM release of 21 founders and a NFFxSM release of 11 founders. Subsequent total population numbers were estimated using the same technique as outlined in chapter 2.2. The autumn release ensured there was no chance to survey the field sites prior to the spring of 2000 because the insects went into diapause over winter. This provided one census of horehound plume moth numbers in the field in Spring 2000. Thus, only one generation, the second generation following outcrossing, was surveyed to provide data to determine the population establishment and growth rate for each release. The establishment levels and growth rates for each cross were examined to determine if an outcrossing depression could be detected.

Figure 6.1. Map of release location for the outcrossing populations at the Yorke Peninsula, South Australia, 1=area of release.

6.2.2 Data and analysis

Individuals for molecular analysis were collected from the laboratory populations prior to field release and from each surviving field population and stored at –20°C until molecular analysis could be undertaken.

DNA and AFLP analysis was undertaken as described in the Material and Methods section of chapter 5. The molecular data, from individuals from the Spanish, New French and laboratory outcrossed populations, were used as a basis for comparison with those obtained from individuals harvested from the field populations in spring 2000.

The sign test (Meddis 1975) was used to determine if there was any significance in differences observed between laboratory foundation and the corresponding field populations for the number of bands present or average genetic diversity across the loci. The prediction was that the field populations would have fewer bands and lower genetic diversity than their corresponding source population. Consequently, a 1-tailed test was used to determine statistical significance. If significance is found it leaves the implication that the distribution characteristics differ, i.e., the medians and possibly the means of the two populations are different.

6.3 Results

6.3.1 Census data and population replacement rates

After the winter diapause, first generation offspring were found at 5 release sites, but insects could not be located at either the NFxNF or one of the SFxNFM releases and it was assumed these populations did not survive. The census data, collected one generation after field release, indicated that there was no significant difference in the population numbers observed among any of the outcrossed populations, but they were greater than the SxS release (Figure 6.1). The hybrid populations increased approximately 50 times the original release size compared with an increase of 1.5 times the original release size for the SxS release. This suggested that outcrossing vigour was occurring.

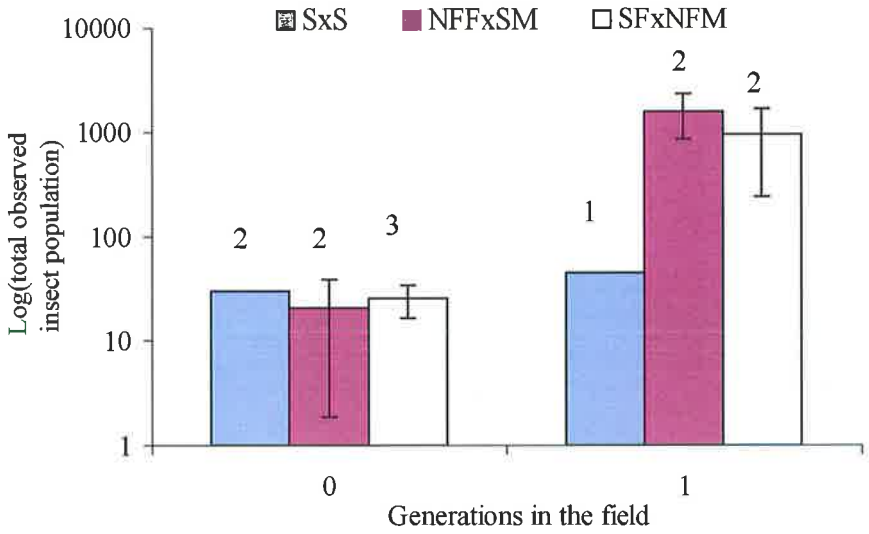

Figure 6.1. Total observed horehound plume moth numbers (log scale) in each surviving population after one generation. Founder stock indicated as follows: SXS = Spanish female by Spanish male; NFFxSM = New French female by Spanish male; SFxNFM = Spanish female by New French male; no New French female by New French males (NFxNF) observed in the field. Number over the bars equals sample size. Error bars show 95% confidence intervals.

6.3.2 AFLP analysis

In all cases the extant field populations had fewer bands recorded and lower genetic diversity (Figures 6.2 and 6.3). The sign test comparing the laboratory source populations and the corresponding field populations indicated that both the number of bands present and the average genetic diversity were lower in all field populations ($A=0$, $n=5$, $p<0.05$, 1-tail test). This mirrors those trends observed in chapters 4 and 5. When the sign test was applied to the combined data from chapters 4, 5 and 6, it was significant at the 0.1% level ($A=0$, $n=36$, $p<0.01$, 1-tailed test). This provided further support for the hypothesis that smaller populations have lower genetic diversity and fewer bands present when compared to their source.

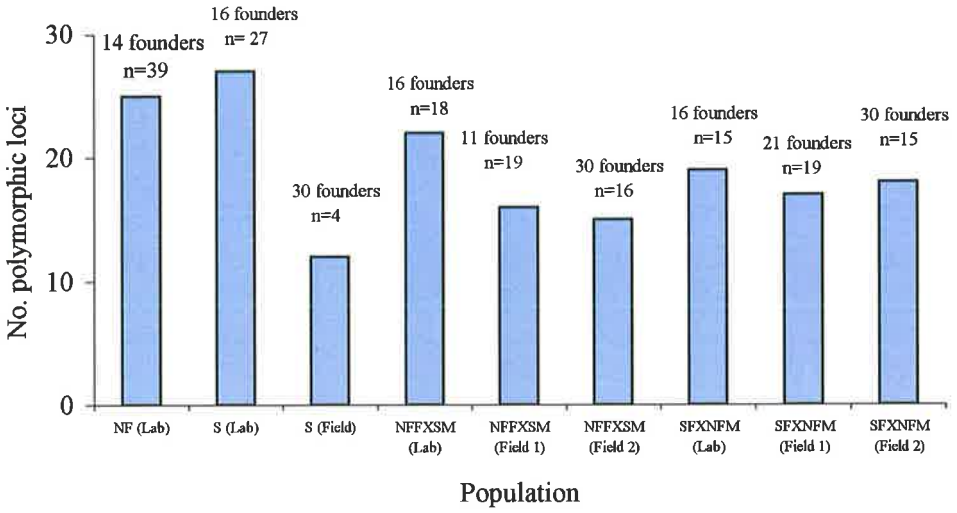

Figure 6.2. Number of bands present observed for horehound plume moth laboratory foundation stock and first generation field populations. The numbers over bars represent the original founders released in the field and n = no. samples analysed after 1 generation.

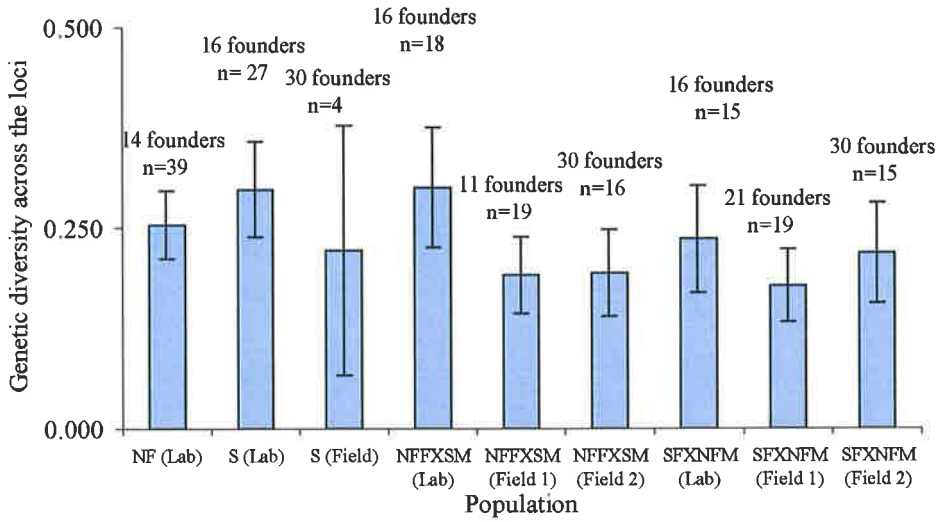

Figure 6.3. Genetic diversity across the loci for laboratory foundation stock and first generation field releases. Error bars show 95% confidence limits; numbers over bars represent original founders in the field; n = no. samples analysed after 1 generation.

The number of bands present and population replacement rates were higher for all outcrossed populations than for the single pure cross that established in the field (Figure 6.4). The Pearson's *r* value was 0.34, and the calculated *t* value was not statistically significant therefore no weight could

be given to any relationship between the number of bands present and the population replacement rates.

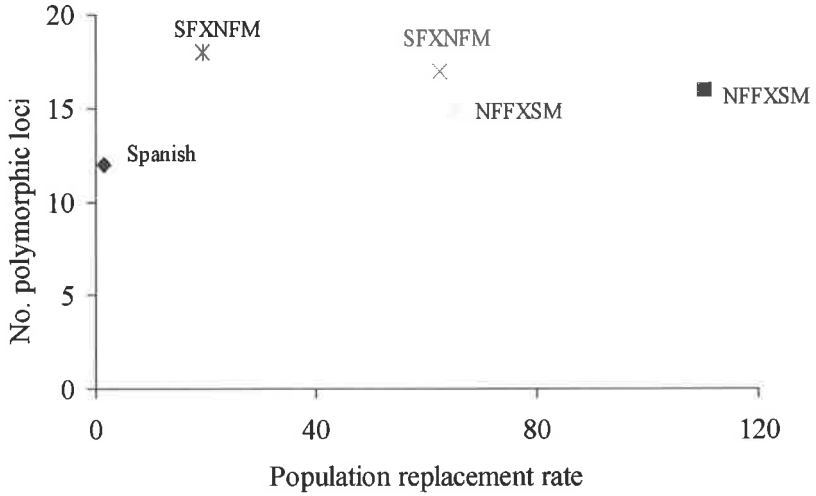

Figure 6.4. Number of bands present versus population replacement rates for horehound plume moth field populations for the different crosses after one generation.

The results for average genetic diversity across the loci versus the population replacement rates suggested a slightly negative correlation between average genetic diversity and population replacement rates (Figure 6.5). This negative association was contrary to that observed in chapter 5 for average genetic diversity and population replacement rate. The Pearson's r value was –0.74, but the calculated *t* value was not statistically significant. Additionally, the sample sizes were low, with only 2 values for each outcrossed line and one for the single pure line that persisted in the field. This meant that no relationship between average genetic diversity and population replacement rates could be determined.

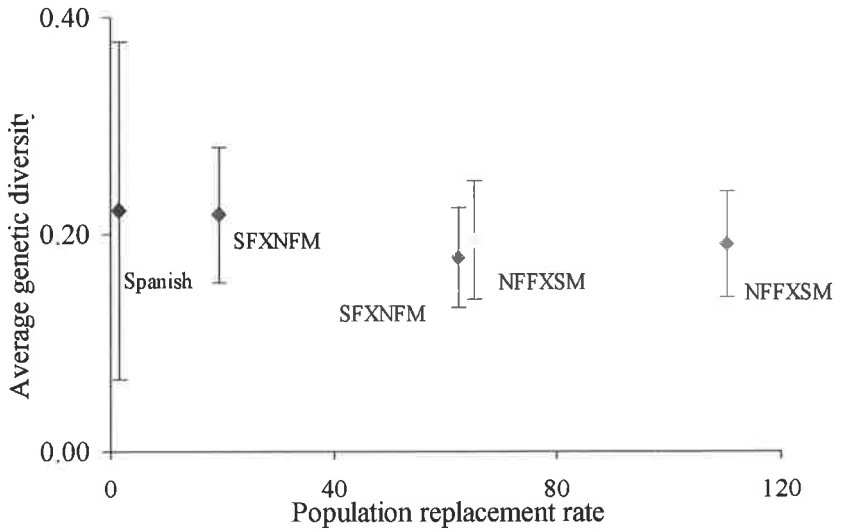

Figure 6.5. Average genetic diversity across the loci versus horehound plume moth population replacement rates for field populations after one generation. Error bars represent 95% confidence limits.

The frequencies of the band present for individual loci for the laboratory populations, after the forcing the laboratory populations through similar sized bottlenecks, were compared with each other to determine if there were statistically significant differences among populations in the frequency of bands present for selected loci prior to field release. After comparing the New French and Spanish laboratory populations and applying sequential Bonferroni correction, 4 loci were found to be significantly different in the frequency of band present. These were bands identified as 3, 4, 22 and 24. If a bias for these bands in the outcrossed lines was observed, it could indicate a reversion to a parental line via selective mating. Consequently, the frequencies of the bands present for the loci observed in the field populations of Spanish, New French and reciprocal crosses were also examined, with reference to their original foundation population to clarify this question.

The outcrossed laboratory populations differed from both the New French and Spanish populations at 2 bands only, band identified as 3 for the SFxNFM and band 7 for the NFFxSM. In both cases, an increase in frequency of the band present in the outcrossed populations was observed, but neither was statistically different from their outcrossed laboratory source, so it was concluded that these differences were the likely result of random founder effects. The NFFxSM field populations

were both significantly different (alpha = 0.05) from the laboratory NFFxSM foundation stock for the frequencies of band present for bands identified as 6, 10 and 14. A further comparison of the frequencies of these 3 bands for NFFxSM field populations with the original Spanish and New French laboratory populations was undertaken. Again, after sequential Bonferroni correction was applied, the frequencies of only two bands remained significantly different from the original pure lines. The frequency of the band identified as 6 remained significantly different from the laboratory New French population but not the Spanish. The cause of this could not be identified and may be due to random founder effects or mating patterns. The only band that had a frequency that was significantly different from all laboratory populations was band 14, which was present at a lower frequency than the original pure lines (Figure 6.6). Again, the cause of this could not be specifically identified and may be due to random founder effects or genetic drift.

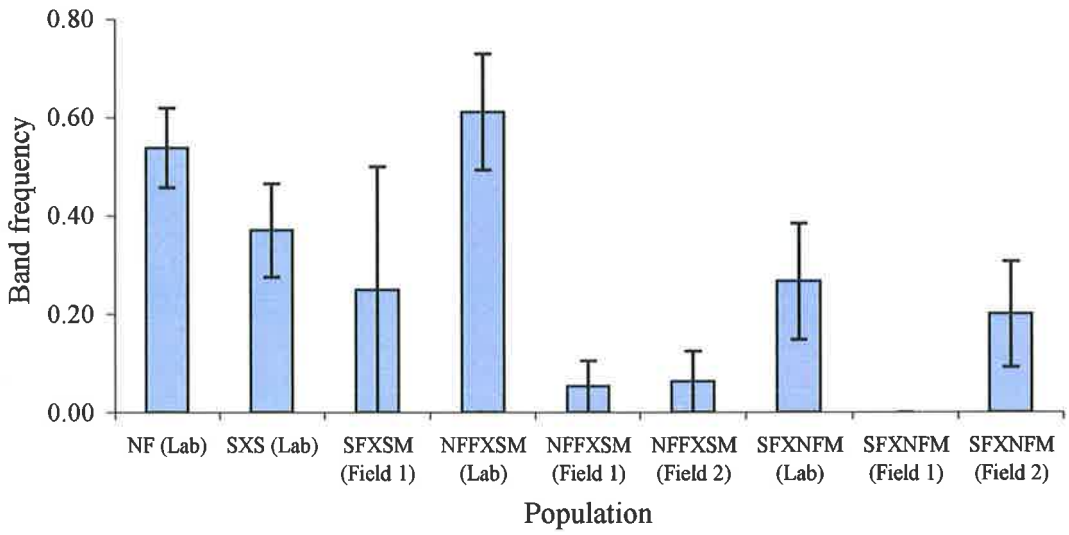

Figure 6.6. The observed frequency of band 14 for all populations. Error bars represent the 95% confidence limits.

6.4 Discussion

Decreases in fitness traits of the individuals within the population following outcrossing can mask the real cause for the failure of a biological control agent to establish (Hopper *et al.* 1993; Berger

and Cunningham 1995). In an earlier trial using horehound plume moth collected in France or Spain, Clarke (2001) noted an outcrossing depression in some family lines of reciprocally crossed horehound plume moth. The causes of the apparent outcrossing depression could not be readily identified. Factors other than genetics that could cause similar effects could result from differences in behaviour, such as subtle mating differences, that might develop between the two geographically separated populations of horehound plume moth originating in Spain and France. The results presented in this section suggested that the variation in fecundity and viability observed in the original crosses undertaken by Clarke (2001) was not caused by genetic differences between the populations, but was due to random founder effects or experimental artefact.

In this study, only one release of each of the pure Spanish or New French lines was possible and the New French population failed to establish. This failure to establish could have occurred by chance; just as the low population replacement rate for the SxS observed in the field could have been an anomaly. To investigate if this was the case, data recorded after one generation in the field for the releases discussed in chapter 2 were compared with the single observation for the SxS population in this experiment. It must be noted that variation in plant quality and the physical environment, both temporally and geographically, influence the population growth rate of the horehound plume moth, however, the recorded population increase for autumn releases made in chapter 2, using 30 late instar larvae, was 0.33. If all chapter 2 larval releases of size 30, made on high plant quality sites, irrespective of season of release, were included in a population growth estimate then the population increase averaged 0.70, which was still lower than the SxS increase for one generation recorded here. Consequently, the population increase of 1.5 the original release size could not be regarded as an anomaly. There were no corresponding data for either the pure NFxNF or the outcrossed populations to permit a similar comparison but, as there was no evidence to indicate otherwise, it could be concluded that the increases observed for the outcrossed populations demonstrated that outcrossing was unlikely to cause depression between the Spanish and French populations.

In contrast to the findings of Berger and Cunningham (1995), where the results of mixed lineages of Badlands bison suggested an outcrossing depression, the outcrossing of two populations of horehound plume moth that originated in different geographical locations did not suggest any depression. Instead, these results suggested that, because of increased population growth rates, the establishment of horehound plume moth populations would benefit from the outcrossing between the two colonies. The rapid increase in horehound plume moth numbers for the hybridised populations observed in this experiment would provide a buffer against small-scale localised catastrophe and provide increased opportunity for dispersal over available horehound plants.

Time limitations meant only one generation could be followed after field release. A further complication was that the field generation included the winter diapause, which Clarke (2001) reported was associated with increased mortality rates for the horehound plume moth. But it appeared there was no evidence for any selective or assortative mating and provided further support for the hypothesis that that smaller populations do lose genetic diversity when compared to their source. However, there was evidence that outcrossing between the New French and Spanish laboratory colonies resulted in increased probability of establishment in the field, which was accompanied by rapid population replacement rates. Unlike the cases cited by Ford (1971), there was no evidence that the direction of the cross affected population replacement rates for any of the populations. This may suggest that genetic diversity is not correlated with fitness in the horehound plume moth.

Comparison of the frequencies of individual bands, after sequential Bonferroni correction, was used to determine if competition between New French and Spanish populations or selective mating could be detected. There were significant differences in frequencies of bands present at 4 bands for the laboratory New French and Spanish colonies, while the laboratory outcrossed colonies were not significantly different from each other at any band. The bands that were significantly different in outcrossed populations were not the same as those between the pure colonies. This suggested a random founder effect rather than any bias due to selective mating. The observed decrease in frequency for both NFFxSM populations at band 14 may be purely by chance. However, in one of

the SFxNFM field populations band 14 was not detected and in other populations the frequency had decreased slightly. This may be indicative of active selection at this band. In order to determine whether this was a random effect or an adaptation, it would be necessary to harvest the field populations over subsequent generations for AFLP analysis.

Overall, when the population growth rates are considered in association with the AFLP results, it could be concluded that outcrossing between the horehound plume moth populations collected in Spain and France would result in a significant increase in population fitness, when measured as population replacement rates. Anecdotal evidence, based on the early releases of horehound plume moth in Victoria, Australia, indicated that the original importation from France might have experienced inbreeding or adaptation while being reared in the laboratory. The subsequent field releases in Victoria did establish, but when these field releases were augmented with a more recent importation from Spain it appeared that the populations increased rapidly and dispersed over larger areas. Clarke (2001) reported that the original French colony, which had undergone many generations in the laboratory, had lower heterozygosity and demonstrated lower fecundity and percent egg hatch than the later French or Spanish imports. Clarke (2001) undertook a series of crosses between these 3 colonies in the laboratory and noted that outcrossing restored vigour in the original French population. It appeared that, at the very least, inadvertent mixing of horehound populations resulted in little harm, but that there was more evidence that outcrossing improved fecundity and viability.

While practitioners of classical biological control have expressed concern that inbreeding can have deleterious effects on successful establishment the consequences of outcrossing are rarely considered. Early in the 1970s' Legner (1971) and Hoy (1975) investigated outcrossing in parasitoids. Legner (1971) studied outcrossing and heterosis of parasitoids of synanthropic flies and found expressions of hybrid vigour in both the F1 and F2 generations of crosses between strains of parasitoids from climatically similar but geographically isolated areas. However, there was an apparent lack of such vigour among progeny from crosses between tropical and temperate regions. On the other hand, Hoy (1975) hybridised 3 strains of *Apanteles melanoscelus* collected from

Connecticut, France and Yugoslavia and reported that the crossing did not appear to produce a more effective parasitoid. However, Hoy (1975) had no parallel releases of pure strains with which to directly compare hybrid results and used data from Weseloh and Anderson (1975) to conclude that outcrossing did not produce a more effective parasitoid.

Given the paucity of data on how outcrossing may directly affect the vigour of biological control agents, thus indirectly affecting successful establishment of releases, it may be time for this issue to be revisited. The results of this experiment and the literature indicating improved vigour after outcrossing, especially for plants (Allard 1965; Legner 1971; Ouberg and Vantreuren 1994; Ruckelshaus 1995; Smitherson *et al.* 1996; Carr and Dudash 1997) encourages the prospect that outcrossing would be helpful in some biological control programs. In the case of the horehound plume moth, for example, mixing horehound plume moth colonies collected from widely different locations may provide some benefit, by improving the probability of establishment and increasing population growth rates. Wang (2000), in a computer simulation, hypothesised that the survival of the inbred lines and the inbreeding level attained were generally highest when between-line selection was used as a breeding strategy. This suggested that line crossing could lower the risk of extinction substantially. Wright (1931) argued that the optimal population structure was a complex of small populations, among which there was a slight, but persistent gene flow. If a complex of small populations with slight gene flow is optimal and line crossing does reduce the risk of extinction, then the active management of artificially created populations, such as those resulting from the release of biocontrol agents, using the outcrossing of laboratory or field populations may improve the success rate for the establishment of biological control agents.

7. Effect of inbreeding on establishment of horehound plume moth colonies

7.1 Introduction

It has been argued for some time that the probability of extinction in small populations increases as heterozygosity decreases. The rationale behind this is that an increased homogeneity of the genes will result in reduced survival, fertility and individual fitness (Charlesworth and Charlesworth 1987; Simberloff 1988; Soulé and Mills 1998). This reasoning is based on the hypothesis that high levels of heterozygosity are the product of a positive feedback cycle that provides a buffer against the deleterious genetic load (Lesica and Allendorf 1992; Soulé 1994). In effect, maintaining heterozygosity is the 'cheapest' way of avoiding the costs of deleterious or lethal alleles becoming homozygous. Loss of fitness may also include an increase in susceptibility to diseases and random environmental and demographic events (Templeton and Read 1983; Simberloff 1988; Soulé and Mills 1998). Critics of this hypothesis have pointed out that an increase in the inbreeding co-efficient does not always express itself as an inbreeding depression because some small populations have persisted over an ecologically long time frame, despite having been founded by few individuals (Caro and Laurenson 1994; Caughly 1994; Quemmen 1996).

The major reason for the controversy is the difficulty in determining the actual cause of extinction in small populations. There is ample evidence that inbreeding can reduce fitness (Wright 1977; Charlesworth and Charlesworth 1987; Thornhill 1993; Jiménez *et al.* 1994; Frankham and Ralls 1998; Saccheri *et al.* 1998; Soulé and Mills 1998; Westemeier *et al.* 1998). Recent studies on fragmented populations, such as those of Westemier *et al.* (1998) and Saccheri *et al.* (1998), confirm that decreasing heterozygosity was negatively correlated with long-term colony persistence of isolated colonies. Jiménez *et al.* (1994) and Miller (1994) have attempted to investigate if inbreeding will decrease the likelihood of survival in a natural or artificially stressed environment.

These two studies reported that the effects of inbreeding were more severe under stressful, or widely fluctuating conditions, than in the relatively benign conditions of a laboratory or zoo. However, there have been no studies designed to observe how deliberate inbreeding, in an attempt to purge deleterious alleles, affected the establishment of biological control agents. The extent of inbreeding depression varies among species, among populations within that species and within family lines (Thornbill 1993; Pray and Goodnight 1995) and this also contributes to how inbreeding affects populations.

The success of any program to purge genetic load without increasing the probability of extinction is dependent on the genetic basis of the inbreeding depression. Is the genetic load composed of alleles that are lethal when made homozygous or is the genetic load due to many alleles that have an additive deleterious effect as inbreeding levels increase? Differences between inbred strains reared in a common environment or observed in the field can suggest a genetic basis when in fact the causes are maternal effects, such as nutrition or vertically transmitted diseases (Hopper *et al.* 1993). Maternal effects can be excluded by using half- or full-sib crosses and analysing observed differences in the offspring (Hopper *et al.* 1993).

Evaluating the interaction of attempted purging of inbreeding depression with demographic and environmental factors could assist in developing conditions that assist the successful establishment of horehound plume moth colonies. The following experiment was designed to evaluate the usefulness of purging any inbreeding depression from small populations, via half- or full-sib crosses and then to determine if the viability of the population would be compromised by loss of genetic variation. It was envisaged this could be achieved by breeding full- or half-sib crosses over successive generations until the inbreeding co-efficient for each mating strategy was approximately equivalent. Field releases of the resultant offspring, along with outcrossed lines as controls, would then be used to determine the population growth rates for the different mating strategies in an attempt to elucidate the effects of purging on population persistence.

7.2 Materials and methods

Randomly selected virgin adults from the Spanish horehound plume moth colony were mated as pairs to form foundation family lines. The offspring of these pairs were then used to establish inbred lines, either as full-sib crosses over two generations or as half-sib crosses over four generations. The first method results in an estimated inbreeding co-efficient of 0.375 and the second, an estimated inbreeding co-efficient of 0.381 (Falconer 1981). The number and sex of offspring from each of the lines was recorded for each generation. It was planned that, after the two full-sib or four half-sib matings, the surviving adults in each line be allowed to mate at random within their sib group with the aim of breeding up to 200 individuals to provide late larval instars for field release at habitat matched locations.

In December 1998, ten virgin males were paired with ten virgin females and caged on single plants in an environmentally controlled room. The temperature of these rooms was maintained at 21° C with 14 hours of artificial daylight. Plants were watered as necessary but usually every two to three days and fresh plants were introduced into the cages when the original plants became severely defoliated by the larvae. Once the larvae, neared maturity the plants were checked at each watering session and any pupae were harvested into separate labeled containers. Any adults that were present at the same time were also collected into labeled containers, with the sexes being kept separately. A weak horehound infusion was prepared by boiling fresh horehound plants in distilled water and allowing it to cool. Sucrose (10%) was added and this was available to all collected adults because no flowering horehound was available at the time. This solution was replaced every two days. The number and sex of all the emerged adults was recorded for each line. Offspring were mated as either a full- or a half-sib cross and the above pattern of recording and care continued for the duration of the experiment.

Statistical analysis was based on ANOVA and Students t-test was applied to detect differences among means obtained for each of the breeding strategies and each generation (Snedecor and

Cochran, 1967, p259-273). To describe the linear correlation between two variables, X and Y, Pearson's *r* was used.

An estimation of the number of deleterious mutants expressed as lethal equivalents carried by the Spanish horehound plume moth was calculated using the formula derived by Morton *et al.* (1956) to calculate the number of lethal equivalents per gamete. This formula is:

$$S = e^{-A-BF} \qquad \text{(equation 1)}$$

where S is the proportion of survivors in a population, A is the fraction of deaths expressed under random mating, B the fraction of deaths arising from the expression of recessive genes via inbreeding and F is the co-efficient of inbreeding. The formula assumes independence between genetic and environmental factors with respect to survivors (Morton *et al.* 1956). As the experiment was done under controlled laboratory conditions it was accepted that this assumption held for the observed data. The formula can be rewritten in order to estimate A and B using a linear relationship defined by Makov and Bittles (1986):

$$-\ln S = A + BF \qquad \text{equation 2}$$

If there were no survivors and the BF equaled zero, then the assumption could be made that A would also equal zero. This allowed the estimation of B using a linear regression forced through zero. It should also be noted that the number of lethal equivalents per zygote, or per individual, is twice the number of lethal equivalents per gamete, therefore the estimated value of lethal equivalents is twice the value of B obtained by the above formula (Crow and Kimura 1970; Ralls *et al.* 1988). Deleterious alleles, when homozygous, would result in the rapid loss of viability when inbreeding occurs. Crow and Kimura (1970) defined the genetic load as the fraction by which the population mean differed from a mean reference genotype, i.e., changes that produce increases as well as changes that cause decreases in fitness. This is usually measured by fitness or some component of fitness. In this study, the term genetic load referred only to the detrimental component of the genetic load.

7.3 Results

Eight out of 39 of the initial random matings failed to produce any offspring. This may just be due to the difficulties of replicating optimum mating conditions within a laboratory situation, but could also be caused by unidentified mating strategies employed by the horehound plume moth. If this is indicative of the percentage of males or females that fail to mate under normal conditions, then it could be assumed that 21% of pairs would fail to mate in the field. The decrease in the number of viable adults produced as the inbreeding co-efficient increased suggested the expression of an inbreeding depression (Table 7.1). Both F1 crosses showed fewer adult offspring, although the half-sib crosses appeared to be less affected. By the second generation of full and half-sib crosses the level of inbreeding depression was of a similar magnitude. The sex ratio and developmental time were not affected (Table 7.1).

Table 7.1. The effects of inbreeding method on production and development of offspring.

Generation	No. of family lines	Average no. males	Average no. females	Sex ratio	Average no. adult offspring	First adult (days)
Random crosses	38	7.32 ± 7.66	6.55 ± 8.43	1.12 ± 0.91	13.87 ±15.68	54.2 ± 13.45
Full sibs, F1	22	4.58 ± 2.31	3.58 ± 4.06	1.28 ± 1.54	8.16 ± 10.67	56.51 ± 15.21
Full sibs, F2	19	1.59 ±3.36	2.05 ±4.78	0.78 ± 0.74	3.64 ±8.02	55.00 ±18.14
Half sibs, F1	27	6.55 ±6.44	5.55 ±6.77	1.19 ± 0.95	12.05 ±12.66	56.68 ±15.44
Half sibs, F2	22	1.52 ±6.75	2.05 ±4.78	1.05 ± 0.63	2.96 ±6.1	55.06 ±18.59

ANOVA on number of emerging adults for each generation and breeding strategy indicated that the number of adult offspring decreased with each generation and as the inbreeding co-efficient increased (Table 7.2). Two-tailed t-test comparison of the adult emergence for each generation and breeding strategy (alpha = 0.01) indicated that the initial random mating and first generation crosses were different from both second-generation crosses. The first generation half-sib crosses were different to both second generation crosses but there was no difference between the second generation crosses. This suggested that there was a negative correlation between the inbreeding level and the number of adult offspring produced, but that the expression of the inbreeding depression would reach a level where the number of viable adult offspring produced would stabilize.

Table 7.2. ANOVA for the number of emerging adults by inbreeding level.

Source of variance	SS	df	MS	F	P-value	F-crit (alpha=0.01)
Between groups	2719.30	4	679.83	4.97	0.001	3.47
Within groups	16831.880	123	136.80			
Total	19551.18	127				

The survivorship data was standardized using the number of viable adults obtained from the randomly out-crossed pairs as the 100% value and scaling all other results to this (Table 7.3). Pearson's *r* for the proportion of adults emerging versus the inbreeding co-efficient was –0.37, indicating a negative correlation between inbreeding co-efficient and the proportion of adults emerging. To test the significance of the Pearson's r value a one-tailed T-test was applied ($t_{125} = 1.75$, $0.1 < p < 0.05$). Therefore, there was a negative correlation for inbreeding co-efficient versus viable adults obtained.

Table 7.3. Proportion of horehound plume moth pairs mating and the proportion of adults emerging standardized against initial number of viable adults obtained from randomly paired male and female horehound plume moths.

Inbreeding co-efficient	n	Proportion of pairs mating	Mean proportion of adults emerging
0	38	0.79	1.00
0.125	27	0.76	0.87
0.250	22	0.69	0.59
0.305	22	0.48	0.21
0.375	19	0.39	0.26

These proportions were used to estimate the average number of lethal equivalents per individual, which was 3.6 per gamete using the equation described by Markov and Bittles (1986) (Figure 7.1). Ralls *et al.* (1988) estimated the lethal equivalents in 40 captive mammalian populations, covering 38 species, and found the number of lethal equivalents ranged from –1.4 to 30.3, with a mean of 4.6 per gamete. Haag and Dearaujo (1994) calculated the genetic load of the lepidopteran species, *Dryas iulia* to be 3 lethal equivalents per gamete when egg to adult survival was considered. Roush (1987) reported on a case of inbreeding depression and laboratory adaptation for tobacco budworm, *Heliothis virescens*. Using the mean data from this study, the number of lethal equivalents per gamete for tobacco budworm was calculated to be 3.2. On this basis, the number of lethal equivalents carried by the horehound plume moth was found to be a similar magnitude to that found in other species.

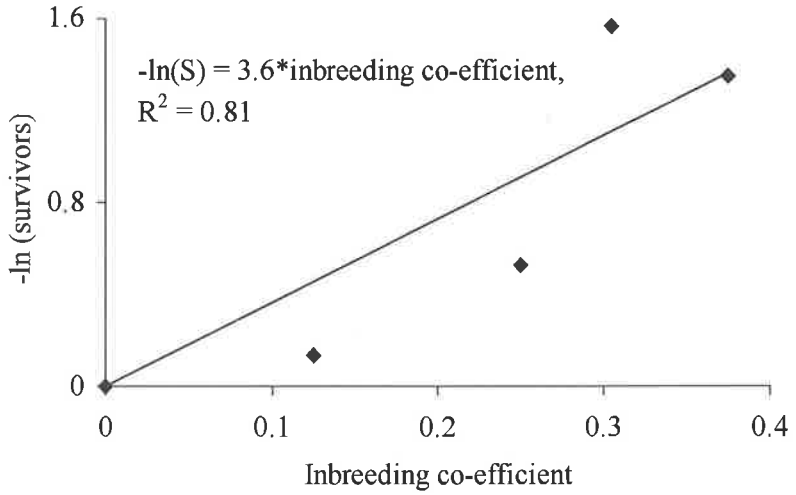

Figure 7.1. Estimation of the number of lethal equivalents per gamete (B) based on a linear regression forced through zero and values obtained for the proportion of adult horehound plume moths emerging at different inbreeding levels using the equation first described by Markov and Bittles (1986) and expressed as $-\ln S = BF$ ***, where F = the inbreeding co-efficient.***

7.4 Discussion

Several factors can cause decreased heterozygosity in biological control programs. These include the initial failure to harvest rare alleles or wide genetic variation from the original habitat, the possibility of bottlenecks, inbreeding and genetic drift that may occur purely by chance in a small population (Roush 1990). Purging deleterious alleles has been suggested as a breeding method for improving the likelihood of successful establishment based on the prediction that a population with a history of inbreeding will not show increased inbreeding depression with further inbreeding (Crow and Kimura 1970). The rationale behind this hypothesis is that a fraction, possibly a large one, of the lethal, or deleterious genetic load has already been eliminated by the loss of individuals homozygous for the deleterious alleles. In practical terms, intensive inbreeding may rapidly eliminate lethal and near lethal alleles but the population viability may be so severely affected that the probability of extinction becomes unacceptably high.

Although no specific field experiments have been undertaken to investigate purging as a management strategy, some computer simulations have been undertaken (Fu *et al.* 1998; Wang 2000). One such study examined 5 breeding schemes and concluded that mild inbreeding, such as half-sib mating, was the breeding system of choice, as it was effective in purging deleterious alleles and carried a low risk of extinction (Fu *et al.* 1998). A simulation study on full-sib crosses undertaken by Wang (2000) concluded that the survival of the inbred lines and the inbreeding level attained were generally highest with between-line selection and lowest with within-line selection. This suggested that, compared with no crossing, line crossing could lower the risk of extinction and the inbreeding coefficient of the purged population substantially with little loss of the effectiveness of purging. The observed results for the horehound plume moth in this study indicated that both full- and half-sib breeding strategies carried a similar risk of extinction and within-line selection was associated with decreased survival of the lines. However, these experiments did not test Wang's (2000) supposition that survival of inbred lines would be higher if between line selection was included as a breeding strategy. Wang (2000) concluded that purging was not justified because the risk of extinction associated with inbreeding was too great, unless the species had a reproductive fitness large enough to cope with the decrease in fitness resulting from inbreeding. Insect species are generally regarded to be highly fecund, but inbreeding can affect the individual fitness across all life stages. Because inbreeding can affect all life stages using the results of adult viability would be more realistic than using the number of eggs produced per female when investigating the effect of inbreeding.

One aspect not addressed here was the decrease in offspring that resulted from non-mating. Confirming matings is essential to separate non-viable from non-mating pairs. This would allow a more accurate estimate of the level of lethal alleles. In this study it was only possible to determine the impact of non-viable and non-matings together, as matings were not observed or recorded. However, this would not have altered the conclusions that the horehound plume moth experienced an inbreeding depression, because in a controlled laboratory situation the decrease in number of-matings could be caused by inbreeding.

The other factors examined in this study, time to emergence and the sex ratio, did not vary significantly among the inbreeding levels. The overall decrease in viable adults produced in the second generation clearly indicated the potential severity of inbreeding depression if population numbers remain low for more than one generation. The fecundity of the lines after two generations of deliberate inbreeding showed that it would have been impossible to continue the field release section of the experiment as first planned. This suggested that inbreeding would have a deleterious effect if a small number of horehound plume moth were released in the field.

Using the formula derived by Morton *et al.* (1956), the number of lethal equivalents per zygote that were carried by the horehound plume moth was estimated at 7.2. The magnitude of the lethal equivalents carried by the horehound plume moth observed here was similar to that found in other species (Roush 1986, Ralls *et al.* 1988; Haag and Dearaujo 1994).

In his study on the horehound plume moth, Clarke (2001) found evidence that one of the horehound plume moth laboratory cultures (Old French) suffered from inbreeding. Not only did this culture have lower levels of heterozygosity, but it also had lower fecundity (Clarke 2001). This finding provided further support for the hypothesis that the level of heterozygosity could be used to predict inbreeding depression (Hedrick and Miller 1992). However, Clarke (2001) reported the effect of inbreeding in the field and found that the field release for the inbred culture recovered after 3 generations although the population growth rate remained lower than that observed for the other two cultures. Saccheri *et al.* (1996) also recorded evidence of a fitness rebound in the butterfly, *Bicyclus anynana*. In the face of the possibility of fitness rebound and the genetic variation found between family lines (such as that observed for this experiment) accurately predicting the magnitude of an inbreeding effect would be difficult.

Still, beyond these tantalising anecdotes, there is little empirical evidence that purging is a useful management strategy (Templeton 1979; Templeton and Read 1983; Willis and Wiese 1997). The results described in this chapter indicated the difficulty in developing successful purging methods without many lines becoming extinct in the interim. The actual effects of inbreeding will depend on

the genetic basis of the depression (i.e., whether the deleterious effect is due to highly lethal or many additive loci) and this is not always obvious. The accompanying loss of genetic variation resulting from inbreeding remains a significant cause for concern for long-term colony persistence. Hedrick and Miller (1992) suggested that the level of heterozygosity could be used to predict inbreeding depression, but Prey and Goodnight (1995) concluded from their study that it was not possible to predict the level of inbreeding depressions based on an estimate of heterozygosity. These data on plume moth support the premise that there was a negative relationship between the number of adult offspring per female and the level of inbreeding, but that such a relationship would be difficult to define due to the amount of genetic variation found among family lines.

The major conclusion of this study was that in order to achieve a confident understanding of whether slow or rapid inbreeding could be used to purge deleterious alleles, then experimental analysis would have to involve a large number of lines, monitored over several generations, with parallel outbred controls. It is doubtful that the effort to carry out such experiments on the scale required to understand the genetic basis of any inbreeding depression, and then devise and execute a breeding method to purge deleterious alleles, would be worthwhile. The avoidance of potential inbreeding depression and the determination of the number of founders required for long-term persistence is likely to be more useful than purging strategies, given the time required to implement them.

8. Predicting the probability of establishment for the Spanish plume moth and planning optimal release strategies

8.1 Introduction

The major aim of any biological control program is successful control of the target species. Perhaps the most critical step in this process is the successful establishment of the selected biological control agent in the field (Shea and Possingham 2000). Establishment is influenced by factors that increase the risk of extinction for small and isolated populations and there are numerous well-written reviews of the major factors affecting persistence. These include assessments of the impact of environment, catastrophe, demography and genetics on the persistence of small populations (Roush 1990; Caughly 1994; Soulé 1986 1994; Barton and Whitlock 1997; Hedrick and Gilpin 1997, Hedrick *et al.* 1997; Lacy 1997). However, general theory on strategies required for successful establishment remains relatively untested, with few guidelines available (Memmott *et al.* 1996; Grevstad 1999; Shea and Possingham 2000).

Conservation biologists have been investigating the use of population viability analysis (PVA) to assess the 'risks' being faced by small or isolated populations for some time (Lacy 1992; Lindenmeyer *et al.* 1993, Mangel and Tier 1994; Brook *et al.* 1997a 1997b; Forys and Humphry 1999). Consequently, there are a number of generic software packages in existence, which allow managers to simulate the extinction processes acting on populations in order to predict population viability. Models can be written on a case-by-case basis, but there is some merit in using a generic package for PVA (Lindenmeyer *et al.* 1993), particularly in the early planning stages of a biological control program. Generic packages have the advantage that they have frequently been tested against empirical data even though they may require some modifications or refinements before they can be effectively utilised for the species under investigation (Brook *et al.* 1997b). VORTEX is one such generic package that has been used to model a number of endangered species

(Clark *et al.* 1991; Lindenmeyer *et al.* 1994; Lindenmeyer and Lacy 1995; Brook *et al.* 1997b; Forys and Humphrey 1999).

It should be pointed out that there are dangers inherent using such models for simulating real populations (Gotelli 1998). These dangers include the possibility that models are too complex and contain parameters that cannot be realistically measured or solutions that are biologically meaningless. As a result, the simplest models may actually be the most useful for predicting outcomes. Perhaps the most frequently overlooked danger is that models are merely representations of nature. Nature is not bound by human logic or assumptions and this can result in models departing from reality. The ultimate test of any model is whether it can accurately assess risks and likelihood of failure or success when challenged by empirical data, but models may help prioritise key factors affecting outcomes and/or indicate where further information is necessary. This allows a more focused direction for future research.

The primary aim of this study was to determine if generic PVA software, such as VORTEX, could be used in tandem with decision-making analysis to develop suitable release strategies for biological control agents. This was probably the first time VORTEX had been used to evaluate the probability of establishment for an invertebrate classical biological control agent and was achieved by using the empirical data from the horehound plume moth project to retrospectively test the outcome of the simulations. Comparing 'virtual strategies' with actual outcomes could highlight limitations in current programming techniques. Assumptions, parameters or factors that require experimental study prior to release could also be identified and clarified when selecting agents or planning release strategies. More importantly, this started evaluating if generic approaches, based on rules of thumb, could be realistically applied to specific species and situations and provide support for the notion that biological control programs could utilize predictive decision-making tools.

8.2 Materials and methods

8.2.1 Assumptions made when describing the lifecycle of horehound plume moth for PVA

The PVA program VORTEX (Version 8.0) used in this study has been employed in numerous studies of a wide array of taxa (Clark *et al.* 1991; Lindenmeyer *et al.* 1994; Lindenmeyer and Lacy 1995; Brook *et al.* 1997b; Forys and Humphrey 1999). A detailed description of the model is presented in Lacy (1993, 2000) and will not be repeated here.

The usual annual sequence of events occurring in VORTEX consisted of the following repeating cycle for each generation (Lacy 1992):

Census → Reproduction → Growth and Development → Mortality

Species with high fecundity, measured as the number of offspring per 'litter' per female,can be modelled by redefining what is meant by reproduction. For example, in this case study of horehound plume moth, the mortality rates of the egg, larval and pupal stages of the moth were condensed. This simplified the reproduction and mortality events because the "litter" sizes consisted of the number of adults produced per female.

The assumptions used for horehound plume moth population simulations were:

- scenarios assumed that all releases were made in spring using late instar larvae.
- one week equalled one VORTEX year. This was because VORTEX was developed for vertebrates where life cycles were calculated in years, but it was simpler to regard one week of the horehound plume life cycle as one year for this analysis.
- populations were simulated for 30 VORTEX years (4 generations of horehound plume moth), initially at 500 iterations, then 10 000 iterations per scenario,
- extinction was defined as no animals of one or both sexes,
- death was at 7 VORTEX years,
- sex ratio at birth (percent males) 50,
- 1 population only, with no immigration and no migration,

- no allowance was made for winter diapause because the 30 VORTEX years was in sequential timesteps,
- polygamous mating,
- percent mortality of adults was set at randomly applied 10% across the entire adult lifespan, with environmental variation (EV) providing the level of standard deviation. EV in reproduction and mortality was concordant across the population,
- frequency for the probability of a catastrophe event was set at 0.15%, i.e., potential for 2 catastrophes over the 4 generations. The multiplicative effect of reproduction and survival was 0.6+[0.4*RAND], where RAND is a function that generates a random number between 0 and 1; i.e., if a catastrophic event occurred reproduction was decreased to 60% of the expected, with a degree of randomness superimposed over the assumed mortality normally experienced. This was to simulate the effect of the unpredictability severity random catastrophes on populations,
- number of lethal equivalents was set at 5 (maximum possible in the program) with 100% assumed to be lethal when homozygous. VORTEX used an infinite alleles model, in which the number of distinct alleles at the hypothetical locus was twice the number of available animals. This hypothetical locus was used to simulate the transmission of alleles. At each step, an insect was assigned 2 unique alleles and each offspring randomly received 1 of these alleles from each parent. Where inbreeding was included it affected the percentage of females breeding and mean litter size and decreased the viability in the first stage of life,
- there was no harvesting or supplementation of individuals,
- carrying capacity set at 30 000. Decreasing carrying capacity with time, in order to represent deterioration of habitat at high population numbers, was not included,
- the calculation of the deterministic population growth rate was based on females, with assumptions of no limitation of mates and no density dependence,

8.2.2 Release Scenarios

The initial scenarios were based on combinations of 2 different host plant qualities, the effect of including the genetic component and 4 different founder sizes (Figure 8.7). The simulations were run for 500 iterations to determine the sensitivity of the program to the incorporation of inbreeding. Following this, a final set of simulations were run, with 10 000 iterations for each scenario to ensure statistically reliable predictions (Harris *et al.* 1987).

Life-cycle constraints that applied to the horehound plume moth, such as egg to adult life span, were derived from previous studies on the lifecycle of the horehound plume moth (Weiss and Lippai 1996; Clarke 2001). The EV was determined using the raw data from Clarke's (2001) study, in which he demonstrated that higher temperatures increased mortality rates at all life stages while concurrently decreasing fecundity. The percentage larval survival rates obtained for high quality plants versus low quality plants were approximately 81 versus 67%. Additionally, Clarke (2001) noted that the number of larvae observed on poor plant quality stems could be up to 80% lower than that observed on high plant quality stems. High or low plant quality categories used in this study were based on visual observations of the leaf size for plants receiving high or low water treatments (Clarke 2001), and average rainfall and variation in temperature per annum for the release location (Australian Bureau of Meteorology 2000, see Figure 2.2 and 2.3). The EV levels were determined based on the variation in population replacement rates recorded by Clarke (2001) (Figure 8.1). These observations led to initial EV levels for the high plant quality and low plant quality scenarios being set at 20% and 40% respectively, with a similar magnitude in variation used to affect randomly the number of females breeding when environmental conditions were poor.

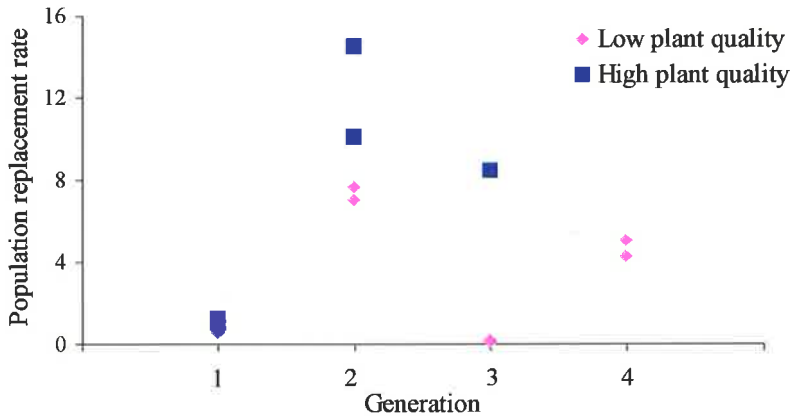

Figure 8.1. Population replacement rates observed for high and low plant quality locations for initial releases of 200 individuals made in early spring (data source: Clarke 2001).

The average population replacement rate across all generations was 14.11 (sd=11.67) and 5.97 (sd=6.13) for high and low plant quality areas. These values were used to estimate the replacement rate of a single female. To conform to constraints within the model system, each female was assumed to have two litters, one at 6 weeks and the other at 7 weeks (6 and 7 VORTEX years respectively), with the total of the litters equalling a mean replacement rate per female of 14.11 or 5.97 depending on plant quality.

It was assumed seasonal, rather than random effects, would be the major influence on reproduction. A pilot study, using 200 iterations per scenario, was run to investigate the effect of using random versus a relationship depicting sinusoidal variation, to define the relationship between the percentage of females breeding and the average litter size.

. On the basis of the observations made in chapter 7.3, which estimated the number of adults pairs actually breeding, only 79% of adult males were made available for mating to all females in the simulations, as the mating system was polygamous. The final VORTEX function defining the % adult females breeding was described as:

(79+(25*(SIN((PI*(Y+5))/7.5)*(RAND*2)/2))) (equation 1)

where Y was a VORTEX year, as constrained by the program.

This assumed an average of 79% of females in the breeding pool with sinusoidal variation that simulated annual variation in fecundity caused by the seasons (Figure 8.2). EV in % adult females breeding was set at 20% for high plant quality releases and 40% for low plant quality releases.

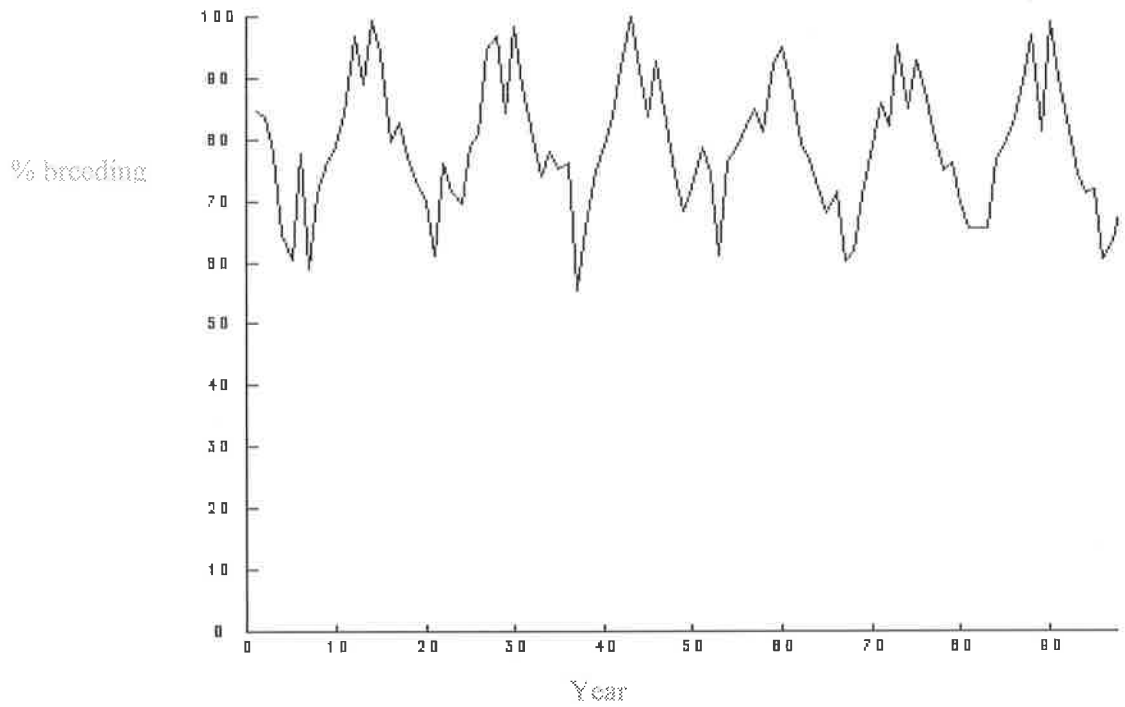

Figure 8.2. Example of % of adult females breeding, no inbreeding effect included, over 100 VORTEX years.

Inbreeding decreased the percentage of horehound plume moth adults mating (Figure 8.3) as well as the number of viable adults produced (Figure 8.4). When the genetic component was incorporated the number of lethal equivalents was set at 5, with an estimated 100% of these described as lethal when homozygous. The data were used to model inbreeding depression by decreasing both the number of females in the breeding pool and the number of viable adults produced per female as the inbreeding co-efficient increased. In a natural system inbreeding co-efficients would be unlikely to reach levels over 0.1. For example, the effect of the level of the inbreeding coefficient can be placed in context when considering Bulmers' (1973) estimation of 0.0036 for the inbreeding co-efficient for a natural population of greater tits This suggested that, unless founder numbers were small and population growth rates remained low for some time,

inbreeding would be unlikely to impact on the number of females breeding or the number of adult offspring produced per female

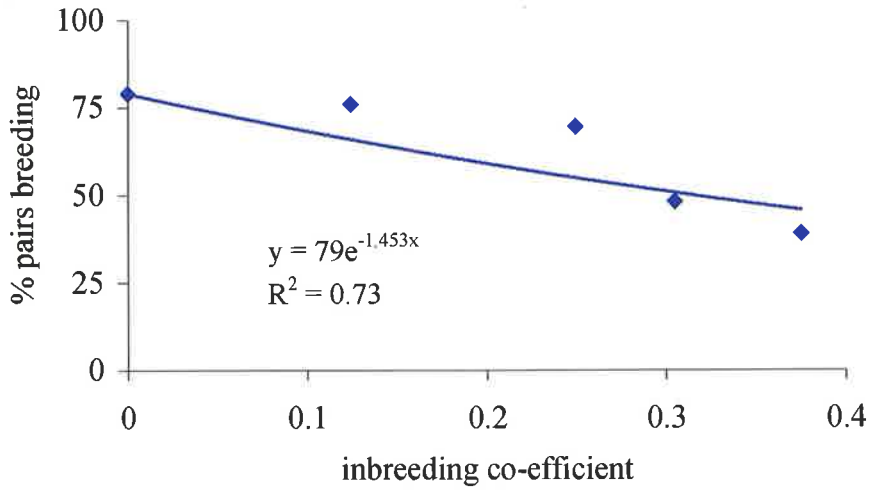

Figure 8.3. The observed combined percentage decrease in horehound plume moth pairs mating as the inbreeding co-efficient increased (data from chapter 7, Table 7.3).

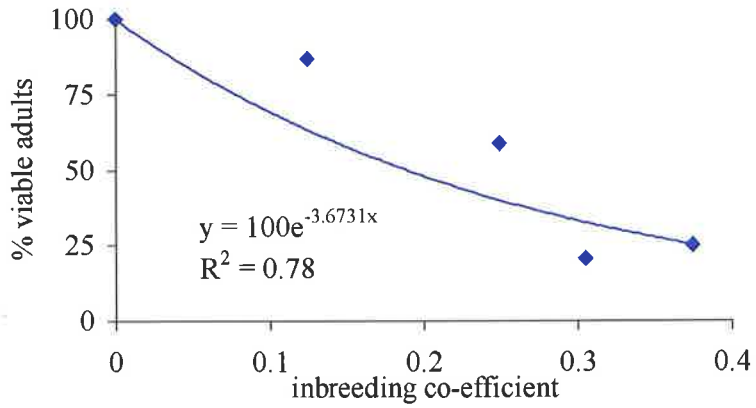

Figure 8.4. The observed combined percentage decrease in horehound plume moth offspring per female as inbreeding co-efficient increased (data from chapter 7, Table 7.3).

The equation describing the exponential trend line for the decrease in the percentage of pairs mating when inbreeding was included was:

$$\%pairs\_breeding = 79 * e^{-1.46I} \qquad \text{(equation 2)}$$

where I = inbreeding co-efficient and the R^2 = 0.73 (Figure 8.3). The final VORTEX function describing the percentage of females breeding, when seasonal and random effects were incorporated in the relationship was (Figure 8.5):

((79+(25*(SIN((PI*(Y+5))/7.5)*(RAND*2)/2))))*exp(-1.46*(I/100)) (equation 3)

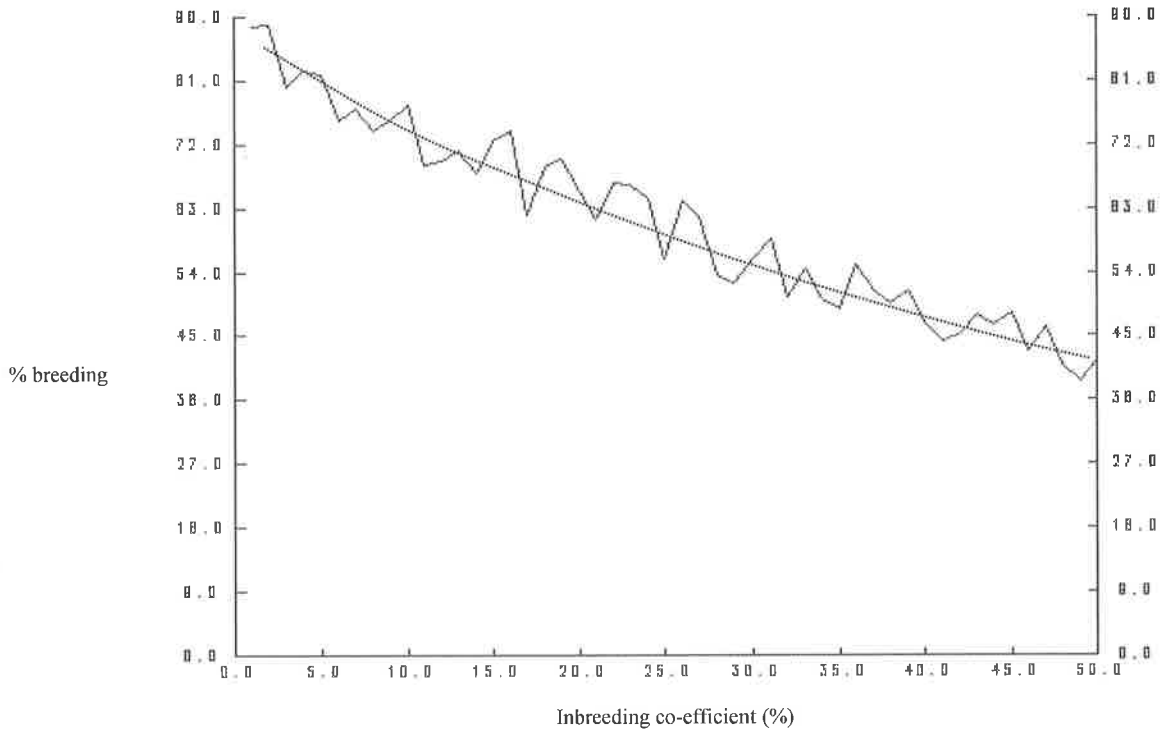

Figure 8.5. Example of how inbreeding (I) affected the percentage of females breeding, dotted line shows mean level of inbreeding.

Similarly the decrease in the number of viable adults emerging from pupae was estimated and used to modify the relationship describing the average litter size when inbreeding was included as a component. The exponential trend line describing the percent decrease in viable offspring emerging was:

$$\%viable\_adults = 100 * \exp^{-3.67I} \qquad \text{(equation 4)}$$

where I = inbreeding co-efficient and the R^2 =0.78 (Figure 8.4). The final VORTEX function describing the percentage of females breeding and the mean litter sizes as the inbreeding co-efficient increased and seasonal effects where incorporated was then set as (Figure 8.6):

(([2.18+(3*(SIN((PI*(Y+5))/7.5)))]*(RAND*3))/2)*EXP(-3.67*(I/100)) (equation 5)

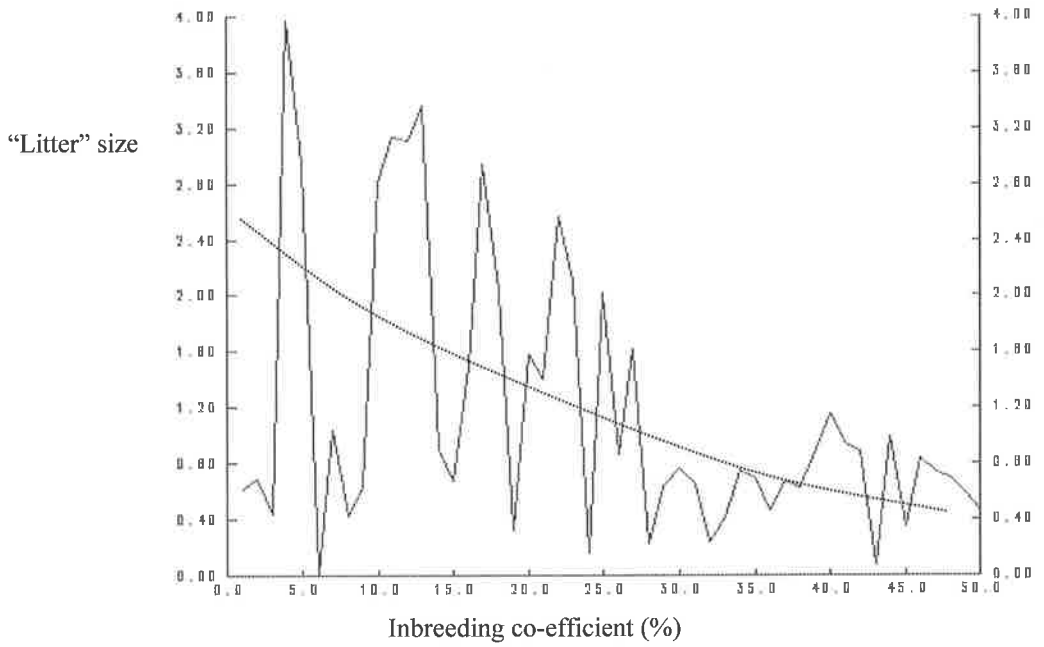

Figure 8.6. Example of how inbreeding (I) and EV affected the 'litter size' in low plant quality areas as the inbreeding co-efficient increased, dotted line represents mean litter size at different levels of inbreeding.

The VORTEX relationship defining the % of adult females breeding was defined as equation 1. The mean litter size for high and low plant quality scenarios respectively was defined for VORTEX as:

([7+(4*(SIN((PI*(Y+5))/7.5)))]*(RAND*3))/2 (equation 6)

and

([2.18+(3*(SIN((PI*(Y+5))/7.5)))]*(RAND*3))/2 (equation 7)

with a standard deviation in litter size set at 7.5 and 3.0 respectively. The final scenarios consisted of high or low host plant quality for founder sizes of 10, 30, 90 or 270, without inbreeding.

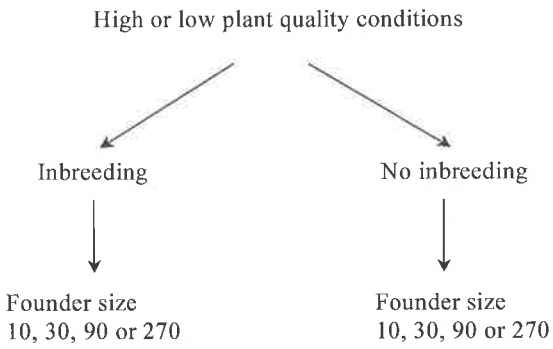

Figure 8.7. Key criteria for establishing the parameters for the populations simulated using VORTEX 8.0.

8.2.3 Decision making analysis

The estimates for the probability calculated by VORTEX for horehound plume moth colonies establishing for the simulated populations were compared with results observed for the field populations described in chapter 2. The equation described by Shea and Possingham (2000) for calculating the probability of establishment was used to determine the 'a' value for each founder size released in high and low plant quality areas for both observed and simulated populations as well as the optimal release strategy for each situation. This equation was represented as:

$$p(x) = p_m(1 - e^{-ax^2})^2 \qquad \text{(equation 8)}$$

where x is the release size, p_m is the maximum probability of establishment and 'a' is a constant that determines the shape of the establishment curve (Shea and Possingham 2000). The simulated values for probability of establishment, the shape of the establishment curves and the final decisions on optimal release strategy were compared with the observed results to determine if the PVA combined with the optimal release strategy model described by Shea and Possingham (2000) was supported by the empirical data.

8.3.1 Pilot study comparing random versus sinusoidal variation for population simulations

Equations using random or sinusoidal variation to determine the mean number of offspring and the proportion of females breeding were evaluated. Typical curves incorporated a seeded random variation or sinusoidal variation around the estimated mean for the number of offspring in released in different locations, defined as high or low plant quality areas (Figure 8.8). Each scenario was run using random or sine relationships for % of females breeding and mean litter size in order to determine if either relationship produced differences in the predicted outcomes. However, it was assumed that the sinusoidal variation was a more realistic representation of seasonal effects. This was because the horehound plume moth is capable of 4 cycles per year (Clarke 2001) and the sinusoidal relationship altered both the number of pairs mating and the final number of viable adult offspring produced in a cyclic fashion to mimic the 4 seasons observed in South Australia over the 30 VORTEX years simulated. So the final functions defining mating and number of adult offspring produced were represented using this approach.

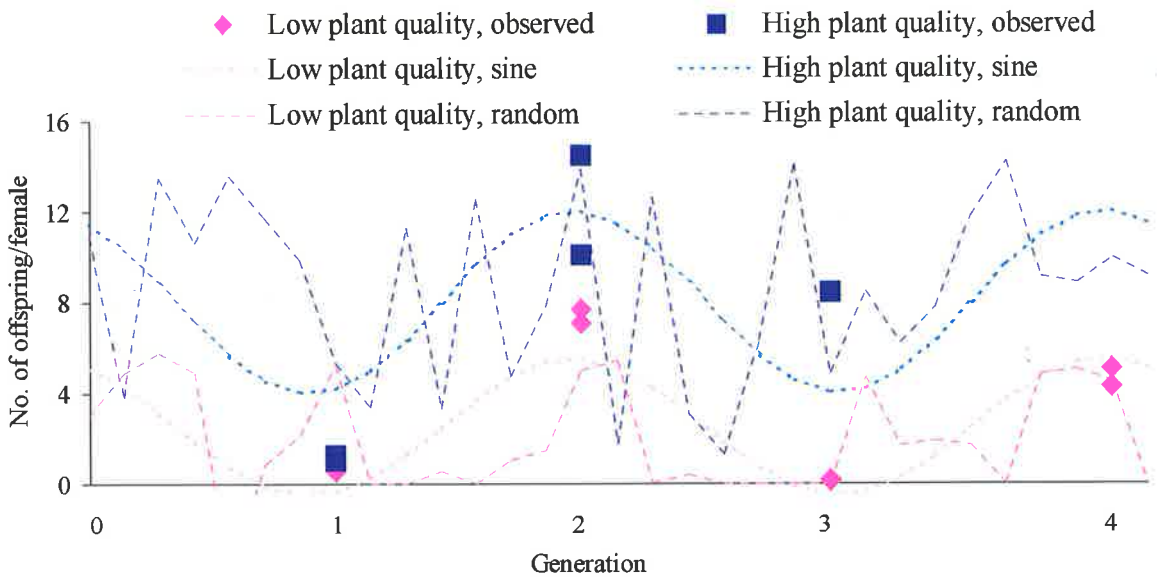

Figure 8.8. An example of the curves produced by random and sine function relationships describing the number of offspring for Spanish horehound plume moth compared with the observed results for high and low plant quality field releases (data source: Clarke 2001)

8.3.2 Testing the sensitivity of inbreeding as a parameter when simulating horehound plume moth populations

The relationships describing the inbreeding effect on mating and number of adult offspring produced were either included in the model or omitted while all other base parameters were kept constant Table 8.1).

Table 8.1 Base parameters used when simulating horehound plume moth populations

Parameter	High plant quality locations	Low plant quality locations
1 VORTEX year	Equals 1 week of horehound plume moth lifespan	
Maximum life span	7 weeks (7 VORTEX years)	7 weeks (7 VORTEX years)
Sex ratio	1:1	1:1
Environmental variation	20%	40%
Total offspring per female	14.11	5.97
Percentage of males mating	79	79
Percentage of females mating	79	79
Normal adult mortality (%)	10	10

The results indicated that incorporating inbreeding had little or no effect (Figure 8.9), except where release numbers were very low (Figure 8.9; founder size 10), but even here the effect of incorporating inbreeding was still small. Based on this result, the inclusion of the inbreeding component was not continued because of the increased computer time for each simulation to be completed, especially when population numbers were high.

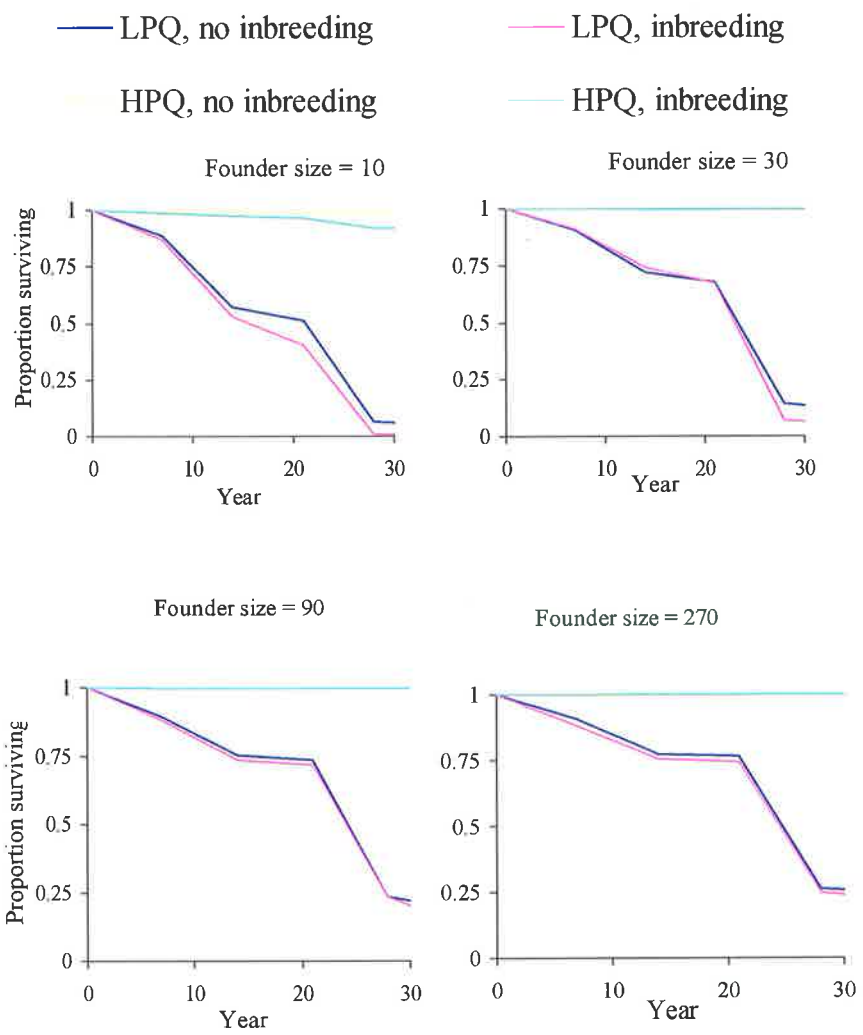

Figure 8.9. Proportion of populations surviving in the low plant quality areas (LPQ) or high plant quality areas (HPQ) after 4 generations for 4 founder sizes, with and without inbreeding (200 iterations per scenario).

8.3.3 Simulations for varying release sizes made in high or low plant quality locations

To test the effect of the variation in the simulated population growth, the quantiles at 5, 10, 25, 50, 75, 95 and 100% for population numbers over 4 generations of horehound plume moth were calculated for the 270 size releases in high and low plant quality areas (Figure 8.10). It should be pointed out that the vertical axes have different scales in order to clearly demonstrate how the simulated populations behaved. The pattern for release sizes of 10, 30 and 90 horehound plume moth individuals were similar to the patterns observed for release sizes of 270 (for both high and low plant quality scenarios and across the quantiles) and are not shown here. These results

indicated the detrimental effect that large variation in seasonal environmental conditions had on successful establishment. The poor record of population persistence supported this result where host plants were of continually low quality and population replacement rates halved (observations by Clarke 2001; and in chapter 2 of this project). Where host plant quality was high, the seasonal effects slowed population growth in winter and summer (when conditions were harsher) but did not cause extinction.

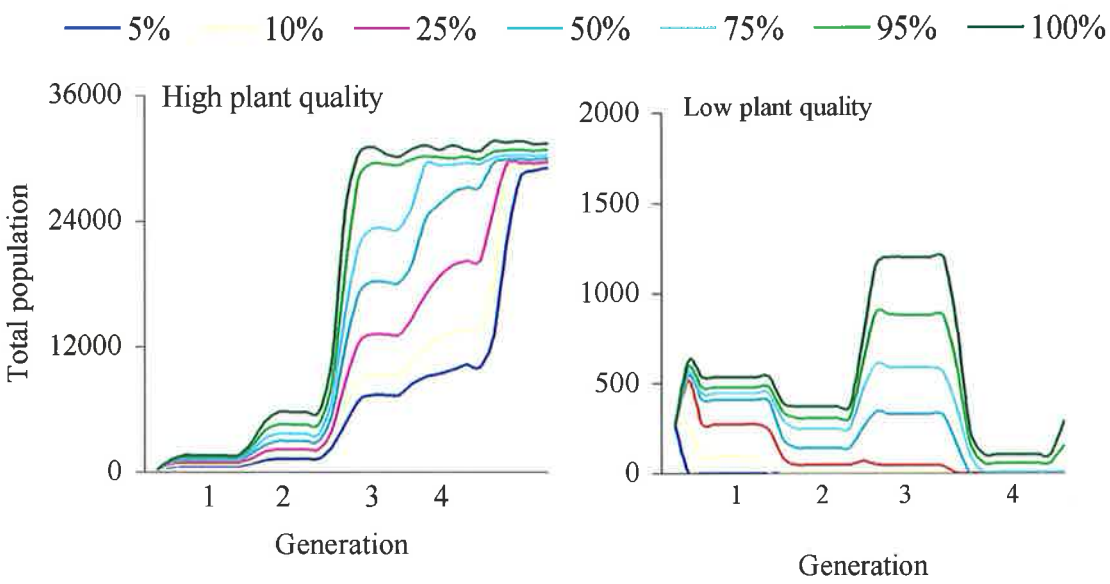

Figure 8.10. Total number of individuals calculated for 4 generations, for an original early spring release size of 270, for indicated quantiles in high and low plant quality scenarios, with maximum carrying capacity set at 30 000.

The proportion of populations predicted to successfully establish over 4 generations and the mean time to extinction suggested that if host plant quality was high successful establishment was likely (Table 8.2). The few populations simulated on high quality plants that were observed to go extinct all occurred at the time of release or first generation after release. This suggested that extinction was due to chance. The extinction pattern where host plant quality was low suggested that numbers steadily declined over the first 3 generations in the field and indicated the likely cause of extinction was due to low population replacement rates caused by the habitat and environment encountered by these insects. These observations provided an important 'rule of thumb' for practitioners distributing horehound plume moth in Australia. This was that if no offspring or plant damage

could be detected by the second generation where host plant quality was high a second re-introduction should be attempted. However, if horehound plume moth numbers were steadily declining over 3 generations and host plant quality was poor, then further management options or approaches should be considered. These decisions include the possibility of manipulating the host plant quality to improve conditions (Room and Thomas 1985) and/or augmenting the existing field population or abandoning the attempt until distribution and dispersal in other, more favourable, areas was complete.

Table 8.2. The proportion of simulated horehound plume moth populations extant at generation 4 and the mean number of generations before extinction (10 000 iterations).

	High Plant Quality		Low Plant Quality	
Release size	Mean no. generations to extinction	Proportion extant	Median no. generations to extinction	Proportion extant
10	>4	0.99	2.0	0.07
30	>4	1.00	2.0	0.14
90	>4	0.99	3.0	0.23
270	>4	1.00	3.0	0.26

The simulated probability of establishment after 4 generations corresponded to the observed probability of establishment after 12 months in the field (assumed 4 generations) for both the high and low plant quality areas. With P_{max} set at 1, the *a*-values describing the slope of the establishment curves (Shea and Possingham 2000) for the high plant quality releases were 0.082 and 0.08 for observed and simulated populations respectively. These two values were similar. However, if P_{max} was assumed to be 1 for the low plant quality releases the a-values were 0.000005

and 0.000008 (Chapter 2, equations 5 and 6, which follow the same form as equation 8 in this chapter), but both the observed and simulated probabilities of establishment indicated that the P_{max} for establishment where plant quality was low would be less than 1. Fitting the observed and simulated data to equation 8 results in P_{max} values of 0.5 and 0.23 for the observed and simulated results, while the *a*-values for the low plant quality releases were 0.00006 and 0.002 (Figure 8.11). These values did not concur with each other in the same way that the results for the observed and simulated probability of establishment in high plant locations did. This may reflect the uncertainty in achieving establishment where host plant quality was poor and environmental variation high, but both the empirical and simulated results suggest that P_{max} is less than 1 where host plant quality is continually poor.

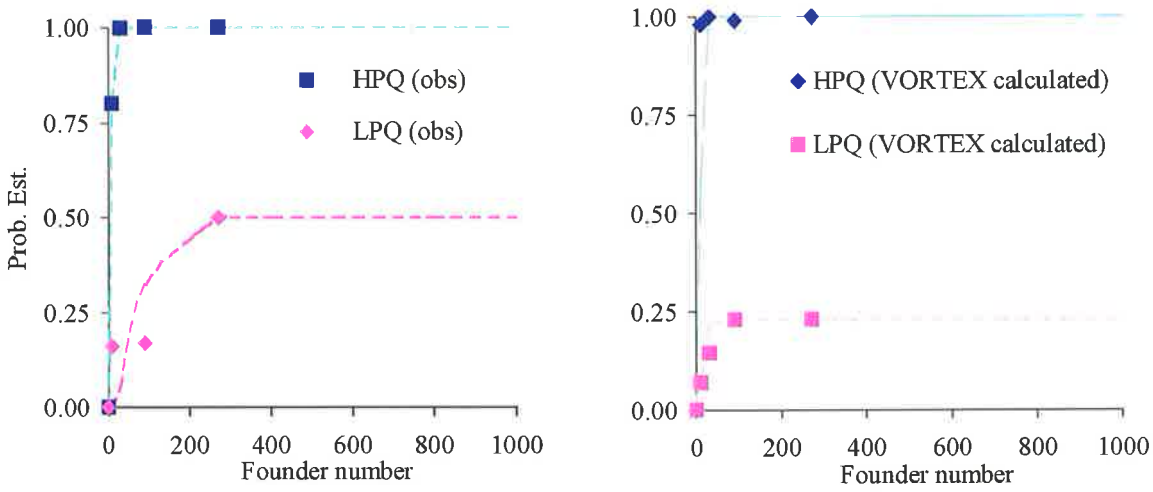

Figure 8.11. The estimated probability of horehound plume moth establishment in high (HPQ) and low plant quality (LPQ) areas for observed (obs) and VORTEX simulated populations after 4 generations. The dotted lines represent the relationship for the probability of establishment as defined by Shea and Possingham (2000).

8.4 Discussion

PVA can be used to investigate a range of conditions and scenarios in order to provide an objective basis for management decisions when combined with decision-making analysis, such as optimal

release strategies. As such, it should provide realistic reflections of how various conditions and threats will affect the risk of extinction. Despite the use of PVA as a management tool for predictive analysis in wild or managed species, it has remained largely untested in biological control programs. Yet biological control programs provide an excellent opportunity to test the strengths of PVA for predicting the likelihood of extinction. The primary objective in this chapter was to compare the empirical and simulated outcomes of releases of horehound plume moth to determine if predictive tools reflected reality. A secondary objective was to investigate the impact of inbreeding on establishment when using the generic PVA package, VORTEX.

M°Carthy *et al.* (2001) cautioned that, despite the growing use of PVA, the predictions of these models were rarely tested with field data that were not used in initially developing the model. It was also recognised that the predictions would only be useful if it was assumed that the distributions of the population growth rate and vital rates would not change in the future (Coulson *et al.* 2001). On this basis and assuming no marked change in future life cycle parameters, the PVA for the horehound plume moth described here utilized data observations made earlier by Weiss and Lippai (1996) and Clarke (2001), together with laboratory experiments, to establish the parameters that were used to predict population extinction over 4 generations for two distinctly different plant quality driven population replacement rates. The variation in the projected population levels over the time period examined in the PVA was high, as shown by the population quantiles over 4 generations for both locations, but the general outcomes for growth rates reflected the field observations, i.e., the low plant quality driven population replacement rates had slow population growth rates and high levels of extinctions, while the high plant quality driven population replacement rates had high population growth rates and low levels of extinctions.

Anecdotal accounts also indicated the PVA predictions would be valid for high and low plant quality areas other than those recorded here. Local farmers and council workers who have made releases in high plant quality areas have reported rapid population spread for the horehound plume moth. For example, a single release of 100 individuals sent to a farm on Kangaroo Island, South Australia, was estimated by the farmer to have spread at least 2 kilometres after 4 generations. This

single release had reached a density high enough for the farmer to be comfortable about re-distributing the horehound plume moth to fellow farmers and council officers. However, council officers working in the Riverland region of South Australia reported that, despite several releases of 200 individuals made where horehound plant quality was poor, no individuals or plant damage could be observed. So, while the results of PVA should always be treated with some caution, in this case the experimental results and anecdotal evidence indicated that it was a useful tool for predicting extinction events in the early stages of establishing horehound plume moth colonies.

The growth or decline of a population is strongly influenced by stochastic events (Gilpin and Soulé 1986; Boyce 1992; Lacy 1992; Lindenmeyer *et al.* 1993, Mangel and Tier 1994; Brook *et al.* 1997a 1997b; Forys and Humphry 1999). Many of these stochastic events may be correlated with each other, either positively or negatively, in a temporal sequence, for example, those events that may be induced by seasonal changes. Temperature, humidity and plant quality affect the fecundity and survival of the horehound plume moth (Clarke 2001). Consequently, it is likely that seasonal change in these factors would result in correlated stochastic events, which affect fecundity and survival over the entire population. Comparing the sinusoidal variation in the environment versus a relationship incorporating random variation for the percentage of females mating and number of adults produced per female did not produce different results for any of the scenarios investigated. But on the basis that there was no significant difference in the outcomes using either relationship, together with the assumption that stochastic events would be auto-correlated with seasonal changes, the use of sinusoidal variation was deemed a more realistic interpretation of events and all subsequent scenarios were run using this pattern of seasonal variation.

The PVA did not suggest that including a genetic component would be influence the successful establishment of the horehound plume moth. Despite markedly decreasing fecundity and the number of adults produced per female as the inbreeding co-efficient increased there was little difference in establishment rates between identical release sizes with or without inbreeding by generation 4. The smallest release size showed the largest difference in probability of establishment between simulations run with and without inbreeding. This was even more noticeable for

simulations where host plant quality was low. It is possible that, because the horehound plume moth is highly fecund the inbreeding co-efficient would not reach a high enough level to cause a significant impact unless population numbers remained very low for several generations. The importance of the genetic component on predicted outcomes cannot be ignored, particularly if restrictive mating strategies influence the expression of inbreeding depression. If PVA is to be utilized routinely as a management tool for planning biological control releases the relevance of this component should be understood.

One of the questions to be examined was how environmental variation for each of the categories, high or low plant quality, would affect each population. Would the worst 'years' produce similar outcomes to the 'good' years or would there be significant differences in the response of the releases? If differences in establishment were pronounced when conditions were good, i.e. plant quality was exceptionally high, then a planned release could be timed for the optimal seasonal conditions and so increase the likelihood of establishment. Figure 8.10 showed that when plant quality was low, the populations steadily declined, despite increases in population numbers in spring and autumn. This suggested that even if releases of horehound plume moth were made in good seasons/years, the long-term likelihood of achieving population persistence was unlikely where plant quality was low unless there were favourable sites nearby that could provide immigrants to rescue populations.

The mean time to extinction in high or low plant quality areas also provided useful information. Where plant quality was high, all extinctions occurred before the second generation, irrespective of founder size. This suggested that the critical time for the released populations where conditions were good was immediately after the initial release. If the populations persisted into their second generation then successful establishment was highly probable, barring a major catastrophe. The simulated releases where plant quality was low had a mean time to extinction occurring during the second and third generation, when seasonal effects were likely to combine to be most adverse with respect to plant quality, temperature and humidity. Despite indications that long-term persistence in these areas was unlikely, it may be possible that by manipulating the local plant quality (i.e., by

irrigation), particularly in extreme conditions when the population decrease would be expected to be the most severe, horehound plume moth releases might persist.

The proportion of horehound plume moth populations establishing was used to determine the relationship defining the probability of successful establishment following the equation formulated by Shea and Possingham (2000). The optimal strategy sets defined in Shea and Possingham (2000) could then be used to formulate release strategies. For example, if there were no established field sites in high plant quality areas the initial decision would have been to make few large releases but as soon as there was at least one established site then small releases could be made. Again, using the simulated results, these releases could have consisted of as few as 10 individuals and still achieved a probability of establishment of 0.99. The observed data supported this strategy, i.e., once a secure release site was established, then small releases could be made, but that release sizes of 10 only established 80% of the time whereas release sizes of 30 established at a rate of 100%. Consequently, the management decisions would depend on the number of individuals available for release, the desired level of saturation and spread for a given geographical distribution. Factors such as travel and time may influence the decision as to whether release sizes of as small as 10 are practical or if it would be more efficient to opt for slightly larger releases but still maintaining the 'many small' approach.

Where plant quality is poor the management decisions become more complex. Based on the predicted outcomes and the optimal release strategy suggested by Shea and Possingham (2000), the only chance for successful establishment was to make a few large releases. Of greater concern for management decisions in low plant quality areas was the suggestion, supported by simulated and observed population sizes, that whatever the release size, the trend was for a gradual decline in the population numbers. This would suggest that successful establishment in these areas would be unlikely over the long term, unless the environment or populations could be manipulated to ensure population persistence.

Apart from considering such factors as the number of available insects, travel, releases established in other areas, a management decision based on the predicted outcomes may need to include the question 'is a 25% likelihood of establishment worth the effort and risk?'. The observations from the empirical data could not answer whether the populations still existing after 4 generations would persist and continue to increase to become securely established sites. However, indications based on the observations made during this project and on anecdotal evidence from other field releases, are that management decisions in the low plant quality areas would require large releases and acceptance of high extinction rates. This suggests a critical assessment of long term goals, particularly early in the release program. The ultimate decision may well be that releasing in areas where host plant quality is poor is futile, because the target species could never be controlled, even if establishment was achieved, because the horehound plume moth numbers would always remain low. In this case, the effort made rearing and releasing the moth could be directed to ensuring distribution in areas were establishment and dispersal was likely to succeed. This would provide an earlier indication of what levels of control could be identified and, because there are many more populations in the field, produce a situation where established populations could buffer neighbouring sites from extinction caused by local catastrophes.

The retrospective testing of PVA and SDP to optimise management strategies for the horehound plume moth indicated that they would have been useful tools in predicting the outcome of the biological control program for horehound plume moth released in high and low plant quality locations. While this study considered two extremes of plant quality, there is every indication that release strategies for a variety of conditions would be possible if data were available to estimate population growth rates in these conditions. There are some indications that PVA failed to reflect reality accurately when conditions were extremely variable and population numbers very low, but the outcomes did provide a general guideline for management actions, taking into consideration "all" the possible uncertainties. While there is no doubt that further testing of predictive tools is required, either retrospectively or incorporated into current/future biological control programs, there is evidence that they will become useful for presenting guidelines and general rules that could improve the efficiency and design of biological control programs.

9. Final conclusions - lessons learned from investigating optimal release strategies for the horehound plume moth in South Australia

9.1 The influence of demography, environment and genetics on the establishment of horehound plume moth in South Australia

A capability to predict the establishment or impact of an introduced biological agent for the control of a targeted pest is still in the early stages of development (Memmott *et al.* 1998; Grevstad 1999; Shea and Possingham 2000). This is despite a long history in the use of natural enemies for biological control (Simmonds *et al.* 1976). Waage (1990) argued that failure in initial establishment was as important a reason for the failure of biological control programs as the agents' inability to effect control of the target. The causes of failure are similar to those faced by small, isolated populations, and conservationists, along with biological control practitioners, have been debating the interaction and importance of many of the factors hypothesised as causes of failure for some time (Soulé 1986; Roush 1990; Hopper and Roush 1993; Caughley 1994; Soulé 1994; Barton and Whitlock 1997; Hedrick and Gilpin 1997; Hedrick *et al.* 1997).

The study undertaken here was unique in the examination of the scope of factors that may influence population establishment. These included temporal, spatial and genetic factors, as well as examining the potential for predictive decision making tools in developing optimal release strategies using empirical data. The temporal and spatial scale of the environmental factors affecting the establishment of horehound plume moth in this project were season of release and habitat location. The conclusion that environmental and demographic stochasticity would have a greater effect on the establishment rates of smaller releases is accepted in the literature (Simberloff 1983; Soulé 1986; Caughley 1994; Korn 1994) and borne out by this investigation. The differences recorded between habitats and the establishment rates for horehound plume moth populations

indicated that environmental factors and season were important, particularly when release sizes were small. Other authors have also reported seasonal influences on population growth rates of various organisms (Campbell 1976; Center *et al.* 2000; Ruohomaki *et al.* 2000; Kamata 2000). Outcrossing the two laboratory colonies seemed to produce more F1 offspring for the same number of parents than did maintaining pure lines and the field population replacement rates appeared higher for hybridised populations than for pure populations (Chapter 6). While this may have been a reflection of outcrossing vigour, it also demonstrated that demographic factors, expressed through higher fecundity and number of viable offspring, influenced the establishment and expansion of horehound plume moth populations.

Existing literature (Wilson 1960; Van den Bosch and Telford 1964; Campbell 1976; Harris 1981; Room and Thomas 1985; Clarke 2001) highlighted the importance of host quality and abundance. While not comprehensively tested this study also suggested that host quality and abundance influence establishment. At first glance it appeared that the higher the percentage of host plants present, the higher the chance of successful establishment for the horehound plume moth, but the results from the low plant quality area did not support this. Clarke (2001) investigated the use of tents as a release method for the horehound plume in order to prevent possible Allee effects and exclude parasitoids and predators. He concluded that the use of tents, when release sizes of 200 larvae were used, was detrimental to the initial population growth rate and dispersal of the plume moth. High larval mortality was observed within the release tent due to a lack of food and plant buds that gave protection to the early instar larvae. Factors influencing carrying capacity have not been investigated for horehound plume moth, although Clarke (2001) estimated that not even a very dense stand of horehound was capable of supporting 406 larvae/m^2. Together with the findings outlined in chapter 2, this suggested that inadequate resources and lack of refuges when conditions are poor decrease the probability that horehound plume moth populations could be established, particularly when environmental variation is high and plant quality poor. Hopper and Roush (1993) hypothesised the Allee effect could explain the lack of success of colonies founded by very few individuals. However, the horehound plume moth does not travel long distances over its lifetime (Menéndez and Thomas 2000; Traeger 2000) and has high population growth rates

(Clarke 2001). It also has a mate detection distance of up to 8 m (Leyson, 1999). The lack of a demonstrated Allee effect (Leyson 1999) and slow dispersal rates (Menéndez and Thomas 2000; Clarke 2001) suggested the higher rates of extinction observed for smaller releases of the horehound plume moth were due to inbreeding and/or the inability of very small releases to compensate for environmental fluctuation and localised catastrophe events.

There are many papers that indicate inbreeding does reduce population fitness and that stressful conditions exacerbate the expression of inbreeding depression (Wright 1977; Jiménez *et al.* 1994; Thornhill 1993; Charlesworth and Charlesworth 1987; Jiménez *et al.* 1994, Miller 1994; Helenurm and Schaal 1996; Frankham and Ralls 1998; Saccheri *et al.* 1998; Soulé and Mills 1998; Westemeier *et al.* 1998; Frankham 2000). Indeed, the assumption remains, that for adaptation in any population to occur, there must be a level of robustness, or immediate fitness, in the individuals plus the presence of enough genetic variation to allow selection processes (Lewontin 1974; Soulé 1987; Hedrick and Gilpin 1997). However, many populations exist that have been founded by few individuals and survived over many generations (Caro and Laurenson 1994; Caughly 1994; Quemmen 1996). It was obvious from the level of extinctions recorded for the horehound plume moth that smaller release sizes in high and low plant quality areas suffered more extinctions, and where conditions were severe these extinctions affected a wider range of population sizes. Population simulations run using VORTEX indicated that extinctions, where plant quality was high, occurred only in the released generation and the first generation in the field. This suggested that chance as well as environmental fluctuation and catastrophe were the driving influences when populations have not begun to expand, but conditions were optimal for potential establishment. The extinctions at low plant quality sites occurred over more generations, especially as the release size increased. This would suggest that low population growth rates when coupled with environmental fluctuation and catastrophe events were long term threats to establishment when conditions were poor.

Given this background, it would seem that inbreeding had no measurable effect on population establishment of the horehound plume moth when larger populations (greater than 30) were

considered. Yet the results of chapter 7 indicated that the horehound plume moth carried a high deleterious genetic load and that single pair matings, undertaken in the laboratory, caused severe inbreeding depressions over two generations of half and full sib matings, so the threat exists. Inbreeding levels for populations that went extinct during the founder and outcrossing experiments could not be measured, but smaller releases did go extinct more often than larger releases. This itself may provide indirect evidence that inbreeding depression contributed to extinction. The attempt to purge lethal alleles was certainly not an option for the horehound plume moth because the population viability for the horehound plume moth decreased at such a rate that the probability of extinction became unacceptably high. This also supported the hypothesis that inbreeding depression would have an effect when population numbers were very low. However, in larger populations (greater than 30, intensive inbreeding may be tolerated even if it results in a lower population growth rate due to the reduced viability of individuals. The conclusion being that inbreeding is a minor concern for practitioners of biological control unless the organisms being released have restrictive mating strategies that would be extremely sensitive to alterations in the sex ratios and/or adult mortalities or population growth rates become negative.

The molecular results from the AFLP study reported in chapter 4, 5 and 6 supported existing theory and observations (Nei *et al.* 1974; Ewens *et al.* 1987; Soulé 1987; Ryan and Seigfried 1994; Ouberg and Vantreuren 1994; Hedrick and Gilpin 1997) that smaller populations do lose genetic diversity when compared to their source population. The 3 successful populations investigated from the low plant quality area did not show significant decreases in the number of recorded bands present or genetic diversity compared to high plant quality populations, but data from neither location provided any evidence of a selective sweep for any band. It was impossible to separate environmental and inbreeding factors in the field, let alone investigate whether loss of genetic diversity exacerbated the extinction vortex. So the premise that inbreeding reduced population fitness or that stressful conditions exacerbate the expression of inbreeding depression and hasten the extinction vortex (Wright 1977; Jiménez *et al.* 1994; Thornhill 1993; Charlesworth and Charlesworth 1987; Frankham and Ralls 1998; Saccheri *et al.* 1998; Soulé and Mills 1998;

Westemeier *et al.* 1998; Jiménez *et al.* 1994, Miller 1994; Soulé and Mills 1998)could not be clarified.

Molecular tools such as AFLPs provide a rapid and relatively simple method that can be applied that will give much information about the variation present within a population. From a practitioner's point of view, this would provide useful data as to whether re-seeding existing sites with horehound plume moth collected from a different location or containing different rare alleles would benefit population replacement rates and prevent possible genetic drift and loss of genetic diversity from becoming a factor that contributes to failure to establish. Hence, if the potential for inbreeding becoming a risk is to be avoided, then managers must either ensure that large releases are made in order to preserve the genetic diversity or give some thought to other strategies that preserve genetic diversity and rare alleles. Some of these strategies may include outcrossing separately maintained cultures or family lines at the time of release (Roush and Hopper 1995) or adopting the concept of metapopulation dynamics with releases made close enough to each other to allow some gene flow. One other advantage of following this strategy is that time spent rearing or maintaining separate colonies or family lines could be decreased as field releases with the potential for gene flow are established.

Simulation models have been used extensively in biological conservation (Brook *et al.* 1997,b; Lacy and Lindenmeyer 1995; Lindenmeyer *et al.* 1995; Novellie *et al.* 1996). They have also found their way into the disciplines of biological control (Legaspi *et al.* 1995, Memmott *et al.* 1998, Grevstad 1999; Shea and Possingham 2000). Will these models provide insights into suitable release rates and the effects of different points of release on the degree of success? Many of the models that have been developed and used are site-specific tools to address specific conditions. Some of these models also include spatio-temporal dimensions but, as far back as 1981, Shaffer suggested that both demographic and genetic factors determined the minimum size of a viable population. One of the key questions addressed in this study on horehound plume moth focused upon how useful a generic, not a species or site specific, population viability analysis model would be as a predictive tool for planning release strategies for classical biological control agents. It was

also a requirement that a genetic component was incorporated in the model when selecting PVA software.

The simulations run using VORTEX suggested that inbreeding was not a key parameter when estimating the probability of establishment for horehound plume moth populations at the release sizes considered. If demographic parameters and carrying capacities can be used to simulate inbreeding depressions for newly released populations, including a genetic component in PVA models would appear to be unnecessary. The results of this study indicated that populations with a positive growth rate over 4 generations did establish, and it could be assumed they would continue to expand unless random catastrophe events caused extinction. Unfortunately, even for large releases, the empirical and simulated data for horehound populations with very low or negative growth rates suggest that stochastic variation and catastrophe events would result in a failure to persist over time.

Promisingly, the predictions of VORTEX for rates of horehound plume moth establishment were similar to the empirical field results. This was true for both high and low plant quality locations. If such generic PVA software can be shown to be useful across a range of biological control agents and conditions, then it would become a useful tool in scientific decision-making and in setting priorities for management activities. VORTEX has been used to investigate populations ranging from a bird (Brook *et al.* 1997b) to a large mammal (Novellie *et al.* 1996), but until now had not been used to investigate the release strategy of an insect biological control agent such as the horehound plume moth. While the interaction of the factors that contributed to a population decline for the horehound plume moth are interesting, it may be far more important for practitioners planning optimal release strategies for a biological control agent to identify the factors that most affect the rate of establishment. The horehound plume moth study indicated that key parameters were host quality and environment variation. To my knowledge combining PVA and SDP models for determining optimum release strategies for a biological control agent had not been tested with empirical data until this study. The results indicated that utilising PVA or Shea and Possingham's

(2000) SDP model for optimal release strategies would have resulted in decision-making strategies supported by the empirical field results.

9.2 Including cost in the decision making process

When planning the field distribution of a biological control agent, effective management decisions should balance the release size that returns the highest number of successful establishments and the most effective use of available funds for distributing the agent. Chapter 2 outlined the development of optimal release strategies based on a simple risk assessment. This approach, proposed by Memmot *et al.* (1998), was useful in that it highlighted the necessity of including risk assessment for different environments and host plant quality. But such an assessment was limited in that it remained generalised. For example, the recommendations were that releases of 30 or more horehound plume moth larvae would establish with minimum risk where plant quality was high, but where plant quality was poor large release sizes were necessary to achieve any likelihood of establishing a population of horehound plume moth. The decisions on release strategies were further developed in chapter 8, using the method outlined by Shea and Possingham (2000). Here it was found that the shape of the sigmoid curve defining the probability of population establishment could be used to decide strategies based on the presence or absence of already existing colonies, as well as on the environmental conditions likely to be encountered. In this case the decision for high plant quality areas would have been to make small release sizes once a colony was securely established. The low plant quality locations, on the other hand, would require large releases under most circumstances and even then success would be limited.

The inclusion of cost-benefit analysis may provide further information for managers when deciding decision-making strategies. The release strategies already identified as the optimum for horehound plume moth were considered in combination with a set of fixed costs for rearing and associated variable costs for distribution. Fixed costs included rearing and maintenance of horehound plants, rearing and maintaining the horehound plume moth colony for one generation and travel to an initial starting point from which releases would commence. These costs where regarded as being

the same for either plant quality areas under investigation, i.e., it was assumed that the distance to an initial starting point was identical for either low or high plant quality locations. No consideration for the initial cost of identifying, selecting, testing or importing a suitable agent was included in the cost-benefit analysis. It was also assumed all releases would radiate out from the first release site, with 1 km between each release. Using these assumptions the cost of a making a single release was estimated at $9.75 and costs were incremental for a number of initial releases varying from 1 to 220. But the fixed costs for rearing and travel to the initial starting point were $4 498.50 (Appendix: Table A.5). The analyses also made the assumption that suitable patches of horehound existed that were large enough to permit the maximum possible number of releases. The numbers of insects available for release were those outlined in chapter 2. The observed probabilities of establishment for different release sizes were calculated using the following relationships (determined in 2.3.1):

High plant quality $$P_{est} = P_{\max}\left(1 - e^{-0,0082*x^2}\right)^2$$ (Chapter 2, equation 5)

Low plant quality $$P_{est} = P_{\max}\left(1 - e^{-0,000005*x^2}\right)^2$$ (Chapter 2, equation 6)

where P_{est} = proportion of populations establishing, P_{max} = maximum likelihood of establishment and x = release size. The total possible releases for each release size were multiplied by the probabilities of establishment to give the proportion of horehound plume moth populations expected to be extant at 12 months. Ratios of established populations for each release size over total costs (fixed plus release costs) or the release costs alone were calculated to provide standardised cost-benefit figures for the releases in high or low plant quality locations (Figures 9.1 and 9.2).

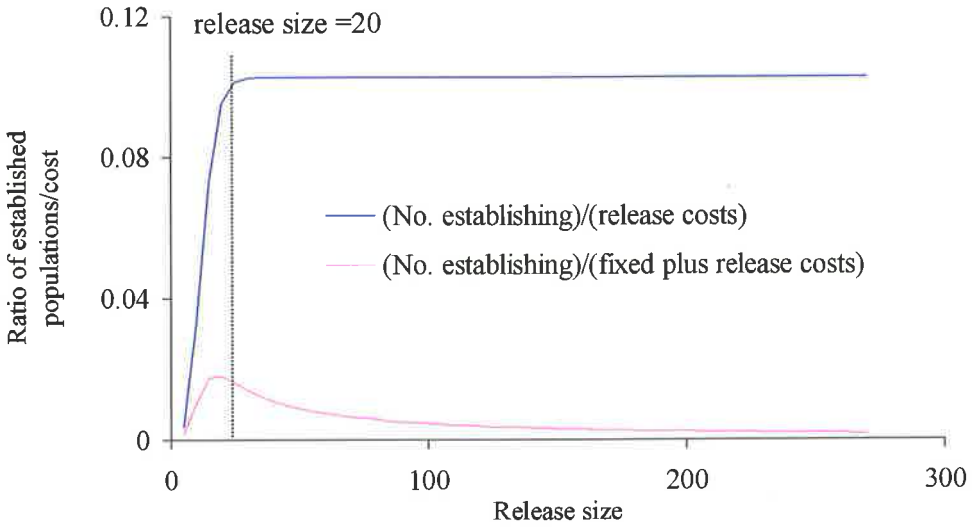

Figure 9.1. The ratio of the estimated number of horehound plume moth populations after 12 months over the projected cost of making the initial releases for high plant quality release sites.

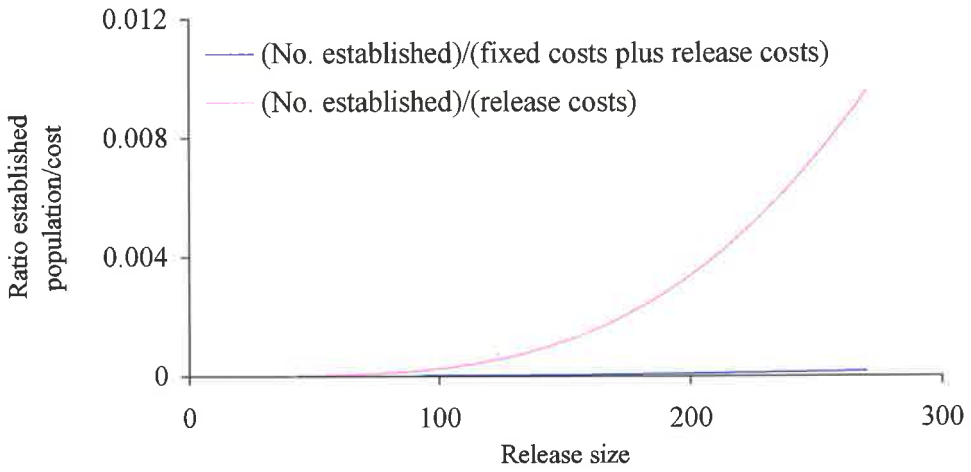

Figure 9.2. The ratio of the estimated number of horehound plume moth populations after 12 months over the projected costs of making the initial releases for low plant quality release sites.

Even though this cost analysis was simplified, the results provide useful information for managers. For high plant quality areas, the release size that provides the maximum number of returns for the cost outlaid is 20 individuals while in the low plant quality areas the costs for benefit ratio did not reach a maximum (Figure 9.3). The probability of establishment for releases of size 20 in the high plant quality area was calculated to be 0.93. Consequently, using the assumptions outlined above, releases of 20 and upward would be acceptable in these areas.

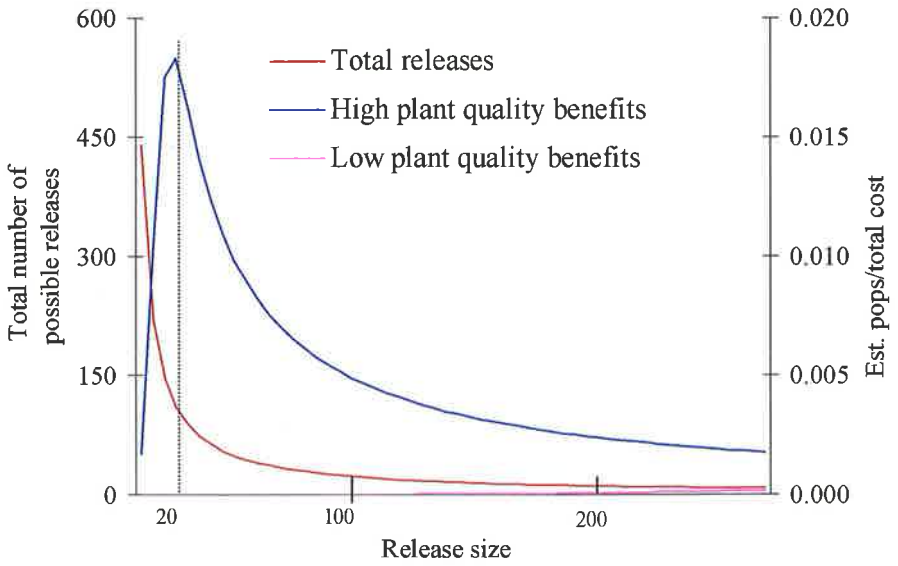

Figure 9.3. The number of total possible releases for 2 200 horehound plume moth larvae for varying release sizes and the ratio of established horehound populations at 12 months over the total costs for the number of initial releases for high and low plant quality locations.

The outcome of combining the optimum releases strategies reached using Memmott's *et al.* (1998), or Shea and Possingham's (2000), approach and the cost evaluation indicated that making releases in low plant quality areas would be risky business. Extrapolating the relationship defining the probability of establishment, and calculating the ratio for established populations at 12 months over the total costs, it is not until release sizes of 680 are reached that releases any reasonable probability of establishment could be achieved. At this release size the probability of establishment was estimated to be 0.81, with an establishment to total cost ratio of 0.00058, and 3 releases. The question here would clearly be whether the effort and size of release are of benefit until distribution and control of the target has been observed for high plant quality locations.

For those areas where the establishment of horehound plume moth was low, the resulting population growth rates indicated that continued population survival would remain tenuous and susceptible to catastrophe events. Consequently, if risk assessment is included in the decision-making then making many small releases in the low plant quality area, as suggested by Grevstad (1999), could be detrimental to a biological control program. The investigation into optimal release strategies outlined in chapter 2.5.1 did not include whether mixed release sizes would be a useful

option in high or low plant quality areas. It merely suggested that releases as small as 10 were possible when plant quality was high and that releases in excess of 270 were required when plant quality was very low. The economics of allowing a nursery site to establish and redistributing from a well established sites, thereby reducing the rearing costs, was not investigated although Shea and Possingham (2000) suggested that various release strategies existed, depending on the relationship that defined the probability of establishment and whether or not there were successful sites already existing. Using their approach and the above information on costs versus release sizes, it would seem that, once a successful field site was established, then releases as small as 20 were suitable for high plant quality locations but large releases would be optimal, if considered at all, in low plant quality areas.

9.3 Future directions

Many species have been accidentally or deliberately introduced into areas outside their original range. Some of these species have successfully established and it is becoming apparent there are general rules of thumb that predict success (Duncan *et al.* 2001). Introduction effort was a key predictor for successful establishment (Hopper and Roush 1993, Williamson 1996; Memmott *et al.* 1998; Duncan *et al.* 2001). Given the time, effort and, sometimes, the heartbreak that goes into this area of biological control, it is understandable that any information that assists in increasing the rate of successful establishment will be of use. Even so, all introductions must be evaluated on a case-by-case basis and the parameters are intrinsic to the species being investigated as host or natural enemy.

Despite the history of using natural enemies for biological control the capacity to predict successful establishment for a biological control agent is still in its infancy (Memmott *et al.* 1998, Grevstad 1999; Shea and Possingham 2000). This study on the horehound plume moth indicated that PVA could provide a useful addition to the scientific tools utilized by practitioners of pest management. Applying PVA to the various release strategies used in the founder experiment allowed estimates of establishment to be made for various release sizes, environmental conditions and resource

availability. It also suggested that for a polygamous, fecund agent with a short life span, inbreeding effects would not limit the rate of establishment. Tools such as PVA require testing on a larger range of agents in order to determine whether the assumptions upon which the models are formulated can be applied to species with widely varying life cycles. The inclusion of bioeconomics as part of such decision-making tools would be of benefit to managers as it may provide a clearer picture to any limitations imposed by the costs of rearing and distributing a natural enemy. While there is no magic formula that would guarantee the final choice, introduction and integration of a successful biological control agent, the relationships between the above factors need further study if predictive models are to become decision-making 'tools of the trade'.

The relationship between resource quality and successful establishment has been given cursory treatment in biocontrol literature, with few studies addressing the influence of habitat and habitat manipulation on the success of establishment when introducing biocontrol agents (Campbell 1976; Room and Thomas 1985; Pratt 2000). Yet many authors have highlighted that high host quality and host abundance are necessary if natural enemies are to successfully establish (Wilson 1960; Van den Bosch and Telford 1964; Campbell 1976; Harris 1981; Room and Thomas 1985; Pratt 2000). Two earlier studies recognised the effect of resource quality on the horehound plume moth (Menéndez and Thomas 2000; Clarke 2001), which were subsequently confirmed by census data collected for populations released in different plant quality locations and at different seasons over the course of the experiment reported in chapter 2. It is possible that an important factor contributing to the past poor success rate for establishing natural enemies in a new country has been that insufficient attention was given to the resource requirements of the agent. This link between habitat quality and successful establishment suggests that classical biological control may more often reach its full potential when coupled with habitat manipulation techniques, which assist in providing optimal resource requirements. Data obtained while investigating the release strategy for the horehound plume moth (Chapter 2) indicated that making releases in spring, when host plants were growing vigorously, achieved higher percentages of establishment than releases made in summer. While selecting optimal seasonal conditions is a simple technique for improving the probability of establishment, further investigation into methods that provide the best conditions for

establishment may prove useful. These investigations should also address the overall cost effectiveness of implementing such strategies.

The loss of genetic diversity reduces the ability of populations to cope with environmental change and may exacerbate the expression of an inbreeding depression in natural populations (Frankham 2000). The results of this study on the horehound plume moth suggested that reduced genetic diversity was associated with lower population growth rates in field releases. Other authors have reported this association between loss of fitness and inbreeding levels (Wright 1977; Charlesworth and Charlesworth 1987; Thornhill 1993; Jiménez *et al.* 1994; Frankham and Ralls 1998; Saccheri *et al.* 1998; Soulé and Mills 1998; Westemeier *et al.* 1998; Frankham 2000). Practitioners of biological control seem to accept that inbreeding should be avoided if successful establishment is to be achieved, but seem to be divided on what the most practical and effective methods to minimise risks from inbreeding. Some of these methods involve the maintenance of separated family lines or complicated breeding programs in the laboratory (Hopper *et al.* 1993; Fu *et al.* 1998; Wang 2000). The expression of inbreeding depression for the horehound plume moth in the laboratory indicated that the species carried a high genetic load that would suggest selection for heterozygotes in a naturally breeding population, but that successful establishment could still occur if habitat conditions were favourable particularly if larger releases are made. But larger releases limit the distribution of a newly released natural enemy and increase the risk that a single colony could be destroyed by a catastrophe event. Whether the long-term effect of decreased genetic diversity on the persistence of a biological control agent is important has not been studied, nor has the relevance of adopting the concept of metapopulation dynamics to establishing a classical biological control agent. This last option may overcome the problems and costs of laboratory rearing, but also provide the potential to create genetically optimal populations structures (Wright 1931) and overcome some of the issues of patch extinction and re-colonisation. The strategy of establishing many small releases, where conditions are deemed suitable, provides the opportunity to create a situation where a number of small populations establish. These populations would presumably be based, not only on the selection of fit individuals, but also on the selection of populations that are adapted to localised environmental conditions. Such a structure would provide a population network, among

which a slight, but persistent gene flow would eventually occur (Wright 1931; Futuyma 1979) and re-colonisation from neighbouring, successful populations is possible (Saccheri *et al.* 1998; Menéndez and Thomas 2000). However, determining release strategies based on mixed population structures also requires an understanding of dispersal and mobility characteristics of the natural enemy in relation to available habitat patches. Investigating such complex situations has been undertaken in the context of conservation of rare and endangered species and habitat fragmentation (Ryan and Seigfried 1994; Ouborg and Vantreuren 1995; Lindenmeyer and Lacy 1995; Saccheri *et al.* 1998). The usefulness of hybridising techniques between the disciplines of conservation ecology and biocontrol cannot be underestimated, as insights from studies on rescuing rare species can provide guidance for practitioners of biocontrol attempting to improve the success rate of introduced natural enemies.

One of the most interesting aspects to emerge from this study was the effect of outcrossing vigour, with such horehound plume moth populations showing far higher population growth rates than corresponding pure releases. This effect has been noted for other species and has received much attention from plant breeders (Allard 1965; Ouberg and Vantreuren 1994; Ruckelshaus 1995; Smitherson *et al.* 1996; Carr and Dudash 1997). Yet outcrossing is often only given lip service by practitioners of classical biological control, possibly because of the fears that hybridisation may alter the host range expression. Outcrossing can restore genetic variation and improve individual fitness. The effect of a 50 fold increase in population replacement rates that was achieved by outcrossing the horehound plume moth cannot be ignored. Such a rapid increase in colony size would minimise the risk of extinction due to low population numbers, assist in maintaining the genetic diversity that was present in the original release and provide the potential for more rapid dispersal and control of the targeted pest. Further study is warranted in determining how such benefits can be achieved for other species. Is it achieved by merely collecting colonies from different geographical locations? If so, what are the risks that sympatric lines have become so divergent that an outcrossing depression results? Would maintaining separate family lines achieve similar results? There are many such unanswered questions, some of which could be answered by collaboration with plant breeders who have already investigated the effects of outcrossing (Allard

1965; Ouberg and Vantreuren 1994; Ruckelshaus 1995; Carr and Dudash 1997). Others will require further work in outcrossing and monitoring of biocontrol agents in the field to establish rigorous 'rules of thumb' before the possible advantages that outcrossing can increase the rate of successful introductions and become part of the 'tool-box' for practitioners of biological control.

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Appendix 1

Table A.1. Details of founding lines used to produce late instar larvae for field releases, and the size of each field release for hybridised populations surveyed in chapter 6.

Population	Description	Number of F1 instars released	Site
Spanish female x Spanish male	SXS	30	Yorke Peninsular
New French female x New French male	NFxNF	30	Yorke Peninsular
Spanish female x New French male	SFxNFM	30	Yorke Peninsular
Spanish female x New French male	SFxNFM	30	Yorke Peninsular
Spanish female x New French male	SFxNFM	21	Yorke Peninsular
New French female x Spanish male	NFFxSM	30	Yorke Peninsular
New French female x Spanish male	NFFxSM	11	Yorke Peninsular

Table A.2. Solutions used in DNA extraction.

Solution	Final concentration of ingredients in MilliQ water
Extraction buffer	100 mM Tris-Cl (pH 8.0) 100 mM NaCl 50 mM EDTA (pH 8.0) 200 mM sucrose sarkosyl to 0.5% final concentration MilliQ water
TE	10 mM tris-Cl (pH 8.0) 1 mM EDTA (pH 8.0)
TAE	40 mM Tris-acetate (pH 8.0) 1 mM EDTA (pH 8.0)
TBE	89 mM Tris-borate(pH 8.0) 2 mM EDTA (pH 8.0)

Table A.3. Composition of buffers and solutions used in the transformation of E. coli competent cells.

Glucose, 2M (500 mL)

180.16g glucose

Add distilled water to 500 mL, filter sterilize through a 0.2 μm filter unit and store in aliquots at -20°C. Stable for 1 year.

IPTG, 0.1M stock solution

1.2g IPTG (Promega cat # V3955)

Add water to 50 mL final volume. Filter and sterilize through a 0.2 μm filter unit and store in aliquots at 4°C.

LB medium with ampicillin (1L)

10g Bacto®-Trptone

5g Bacto®-Yeast Extract

5g NaCl

Adjust the pH to 7.5 with NaOH. Allow the autoclaved medium to cool to 55°C and add ampicillin to a final concentration of 100μg/mL. For LB plates, include 15g agar prior to autoclaving.

X-gal

Available from Promega (cat # V3941) at a concentration of 50mg/mL in dimethylformamide.

Mg^{++} stock solution, 2M (500mL)

101.5g $MgCl_2.6H_2O$

123.3g $MgSO_4.7H_2O$

Add distilled water to 500 mL and filter sterilize a 0.2 μm filter unit. Filter sterilizing units should be rinsed with distilled water prior to use.

SOC medium (100mL)

2.0g Bacto®-Tryptone

0.5g Bacto®-Yeast Extract

1mL 1M NaCl

0.25mL 1M KCl

1mL 2M Mg^{++} stock, filter sterilized

1mL 2M glucose, filter sterilized

Add Bacto®-Tryptone, Bacto®-Yeast Extract, NaCl and KCl to 97mL distilled water. Stir to dissolve. Autoclave and cool to room temperature. Add 2M Mg^{++} stock and 2M glucose stock, each to a final concentration of 20mM. The final pH should be 7.0.

20 x SSC (1L)

175.3g NaCL

88.2g sodium citrate

Add 800 mL of distilled water. Stir to dissolve and adjust to pH 7.0 with NaOH then make to 1 L with distilled water.

Table A.4. Matrix of Slatkins linearised F_{ST} as T/m = F_{ST}/(1-F_{ST}), M=n for haploid data, M=2n for diploid data. L=low plant quality, H=high plant quality.

	Source	L, B90	L, B270	L, J10	L, J270	L, Y270	H,C10	H,C30	H,C90	H,C 270	H,P42, 90	H,P1, 10	H,P,30	H,P13, 10	H,P15, 30	H,P22 30	H,P23, 270	H,P27 3,30	H,P3, 90	H,P4, 270	H,P73, 90	H,P84, 270	H,PP 10	H,PP 30	H,PP 90	H,PP 270	H,T1, 10	H,T1, 30	H,T1, 90	H,T1, 270	H,T2, 10	H,T2 30	H,T2, 90	H,T2, 270
Source	0.00																																
L, B90	0.08	0.00																															
L, B270	0.06	0.08	0.00																														
L, J10	0.06	0.20	0.02	0.00																													
L, J270	0.04	0.10	0.07	0.06	0.00																												
L, Y270	0.05	0.07	0.02	0.04	0.05	0.00																											
H,C10	0.09	0.12	0.05	0.14	0.19	0.06	0.00																										
H,C30	0.07	0.16	0.07	0.08	0.05	0.05	0.08	0.00																									
H,C90	0.10	0.06	0.01	0.12	0.01	0.05	0.08	0.06	0.00																								
H,C270	0.07	0.15	0.16	0.19	0.05	0.12	0.08	0.09	0.16	0.00																							
H,P42,90	0.02	0.07	0.09	0.15	0.00	0.03	0.19	0.06	0.03	0.11	0.00																						
H,P1,10	0.13	0.10	0.06	0.19	0.10	0.06	0.10	0.17	0.08	0.12	0.11	0.00																					
H,P,30	0.06	0.12	0.09	0.03	0.05	0.01	0.07	0.06	0.14	0.06	0.10	0.16	0.00																				
H,P13,10	0.09	0.09	0.00	0.13	0.08	0.05	0.04	0.11	0.01	0.20	0.06	0.07	0.17	0.00																			
H,P15,30	0.07	0.08	0.06	0.04	0.03	0.06	0.15	0.06	0.06	0.11	0.09	0.18	0.03	0.13	0.00																		
H,P22 30	0.05	0.12	0.02	0.11	0.13	0.02	0.04	0.09	0.06	0.14	0.09	0.13	0.14	0.03	0.10	0.00																	
H,P23,270	0.00	0.05	0.00	0.06	0.08	0.00	0.00	0.00	0.00	0.11	0.00	0.09	0.08	0.00	0.04	0.00	0.00																
H,P273,30	0.05	0.02	0.01	0.05	0.09	0.02	0.01	0.09	0.06	0.12	0.10	0.11	0.02	0.03	0.00	0.03	0.00	0.00															
H,P3,90	0.02	0.06	0.02	0.08	0.02	0.03	0.04	0.08	0.02	0.08	0.00	0.05	0.10	0.00	0.10	0.04	0.00	0.04	0.00														
H,P4,270	0.08	0.10	0.03	0.14	0.04	0.05	0.13	0.14	0.08	0.07	0.08	0.00	0.11	0.06	0.10	0.10	0.02	0.07	0.04	0.00													
H,P73,90	0.06	0.02	0.05	0.21	0.10	0.06	0.09	0.11	0.07	0.10	0.03	0.01	0.13	0.03	0.13	0.08	0.01	0.05	0.04	0.00	0.00												
H,P84,270	0.02	0.02	0.00	0.04	0.01	0.00	0.00	0.03	0.00	0.06	0.03	0.04	0.03	0.03	0.04	0.01	0.00	0.01	0.00	0.02	0.02	0.00											
H,PP10	0.06	0.14	0.01	0.03	0.02	0.00	0.11	0.08	0.05	0.12	0.03	0.08	0.09	0.05	0.06	0.04	0.00	0.08	0.00	0.04	0.13	0.02	0.00										
H,PP30	0.12	0.14	0.04	0.16	0.18	0.07	0.04	0.12	0.06	0.24	0.11	0.13	0.16	0.08	0.20	0.01	0.00	0.08	0.07	0.18	0.12	0.03	0.11	0.00									
H,PP90	0.11	0.05	0.02	0.11	0.15	0.07	0.00	0.14	0.03	0.20	0.16	0.10	0.13	0.07	0.12	0.05	0.01	0.03	0.07	0.16	0.12	0.00	0.13	0.01	0.00								
H,PP270	0.02	0.09	0.07	0.04	0.00	0.03	0.13	0.09	0.05	0.09	0.00	0.13	0.01	0.09	0.03	0.06	0.02	0.02	0.03	0.07	0.11	0.03	0.04	0.12	0.11	0.00							
H,T1,10	0.02	0.03	0.01	0.06	0.00	0.00	0.04	0.04	0.00	0.06	0.00	0.05	0.03	0.02	0.02	0.00	0.00	0.02	0.00	0.03	0.04	0.00	0.01	0.05	0.03	0.00	0.00						
H,T1,30	0.03	0.08	0.04	0.08	0.05	0.04	0.02	0.13	0.06	0.07	0.05	0.05	0.08	0.05	0.13	0.04	0.00	0.02	0.00	0.06	0.06	0.02	0.05	0.07	0.03	0.01	0.02	0.00					
H,T1,90	0.03	0.08	0.08	0.10	0.03	0.05	0.03	0.08	0.07	0.01	0.05	0.07	0.03	0.11	0.08	0.07	0.03	0.05	0.01	0.08	0.07	0.02	0.05	0.13	0.10	0.02	0.01	0.01	0.00				
H,T1,270	0.05	0.03	0.04	0.14	0.01	0.01	0.05	0.09	0.02	0.06	0.03	0.00	0.10	0.03	0.11	0.07	0.00	0.06	0.00	0.01	0.04	0.00	0.04	0.11	0.06	0.04	0.00	0.03	0.02	0.00			
H,T2,10	0.08	0.16	0.04	0.03	0.03	0.01	0.12	0.08	0.07	0.20	0.03	0.12	0.09	0.05	0.12	0.07	0.00	0.06	0.05	0.09	0.12	0.02	0.01	0.09	0.12	0.03	0.02	0.06	0.10	0.07	0.00		
H,T2,30	0.07	0.16	0.12	0.17	0.00	0.09	0.16	0.03	0.08	0.02	0.01	0.14	0.07	0.14	0.10	0.13	0.08	0.16	0.09	0.09	0.08	0.05	0.09	0.20	0.20	0.06	0.03	0.09	0.03	0.07	0.11	0.00	
H,T2,90	0.11	0.10	0.17	0.15	0.04	0.09	0.18	0.16	0.16	0.10	0.10	0.18	0.00	0.18	0.00	0.24	0.17	0.06	0.12	0.13	0.13	0.12	0.14	0.31	0.23	0.05	0.09	0.13	0.08	0.13	0.17	0.11	0.00
H,T2,270	0.09	0.13	0.11	0.20	0.03	0.08	0.18	0.10	0.11	0.01	0.05	0.04	0.14	0.15	0.13	0.13	0.07	0.16	0.05	0.03	0.08	0.04	0.05	0.21	0.21	0.10	0.03	0.10	0.02	0.01	0.14	0.02	0.15	0.00

Table A.5. Budget and assumptions used for determining the fixed and variable costs associated with releasing horehound plume moth in a novel environment.

Assumptions: cost of rearing 2 200 individuals is the same irrespective of where or how the releases are made; there are enough founders to rear 2 200 in one generation; labor costs are at technician level $18.50/hour (no patrol tax, superannuation, administration etc); 1 km between releases; all costs getting to the first release site, irrespective of plant quality, are identical.

Item	Time or number required	Cost per item ($)	Cost ($)
Fixed costs:			
Cost of rearing room	10 weeks	1500 per annum	288.50
Cost of soil and gravel	2 tonne	450 per tonne	900.00
Cost of fertilizer	1 kilo	3 per kilo	3.00
Cost of pots	400	0.85 ea	
Travel to and from release area	500 km	500 x 0.42 per km	
Collecting plants	32 hours	18.50 per hour	592.00
Maintenance of rearing rooms	100 hours	18.50 per hour	1850.00
Collecting allocating insects to releases	40 hours	18.50 per hour	40.00
Labour costs driving to and from release area	40 hours	18.50 per hour	740.00
Overnight stay	1 night	60 per night	60.00
Food allowance	1 day	25 per day	25.00
Total fixed costs			4498.50
Costs per release:			
Travel	1 km	0.42 per km	0.50
Labour costs	0.5 hour	18.50 per hour	9.25
Total cost per release			9.75