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The Unpredicted Host Use of *Chamaecytisus prolifer* by the Introduced Weed Biocontrol Agent, *Bruchidius villosus*: a Retrospective Analysis and Explanation

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A thesis submitted in partial fulfilment of the requirements

for the Degree

of

Doctor of Philosophy

at

Lincoln University

New Zealand

by

M. L. Haines

Lincoln University

2004

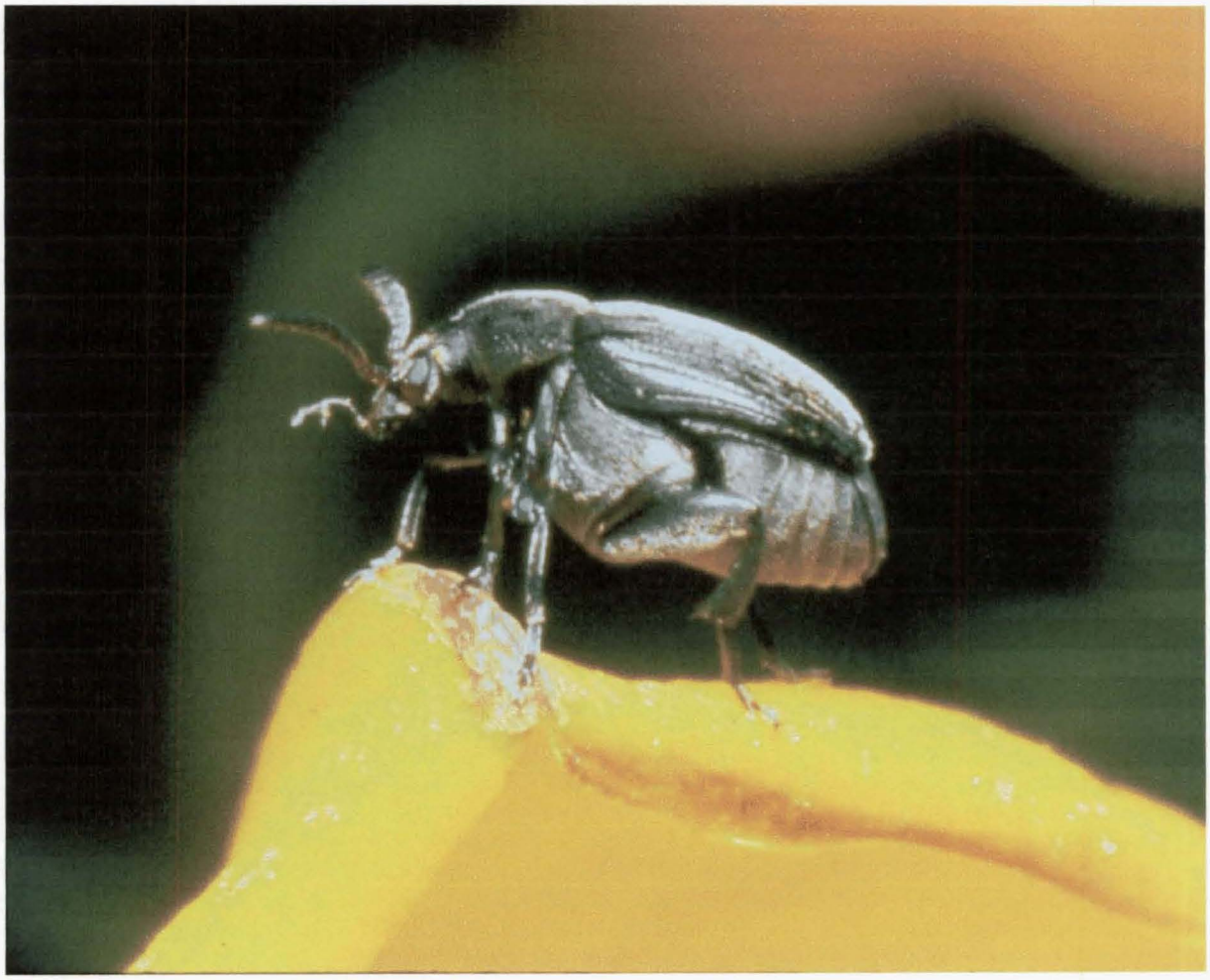
Dedicated with love

to

Nana Haines* and *Roydon

who began this journey with me in person

but ended it in spirit



***Bruchidius villosus* on a *Cytisus scoparius* flower**

Abstract of a thesis submitted in partial fulfilment of the requirements for the Degree of Ph.D

The Unpredicted Host Use of *Chamaecytisus prolifer* by the Introduced Weed Biocontrol Agent, *Bruchidius villosus*: a Retrospective Analysis and Explanation

By Melanie Haines

This study investigates unpredicted host use by a weed biological control agent in New Zealand. *Bruchidius villosus*, a seed-feeding beetle, was released into New Zealand in 1986 as a biocontrol agent to aid in the control of *Cytisus scoparius* (Genisteae), a European shrub that has become widespread in New Zealand. Although host specificity tests had predicted that *Chamaecytisus prolifer* would not be attacked, in 1999 *B. villosus* was found utilising this plant as well as *C. scoparius* in the field in New Zealand. This finding prompted a thorough re-examination of historical rearing records, which showed that *B. villosus* does in fact exhibit a wider host range than was initially thought and forecast by New Zealand and UK testing. Uncertainty over the identity of *B. villosus* (which is referred to in some literature as *B. ater* and other synonyms) appears to have contributed to the misunderstanding of its known host range. To ascertain why host specificity tests (choice tests on cut shoots) failed to predict that *Ch. prolifer* would be a suitable oviposition host for *B. villosus*, a series of field and laboratory experiments were undertaken.

The field host range of *B. villosus* in New Zealand was established: *C. scoparius* and *Ch. prolifer* were the only hosts oviposited on and no other native or exotic pod-bearing relatives of *C. scoparius* were found to be hosts in the field. However, subsequently (2003) beetles have also been collected from *Lupinus arboreus* in the field. In no-choice sleeved tests on whole plants in the field *C. scoparius*, *Ch. prolifer* and two additional species, *Cytisus multiflorus* and *Genista monspessulana*, were oviposited on,

and adults emerged from all these species except *C. multiflorus*. These results supported European literature records that *B. villosus* has a broader host range.

Mitochondrial DNA for *B. villosus* reared from a number of host plants from the UK, France, Spain, Germany, Hungary, Canada, and the USA was analysed to determine whether the current concept of *B. villosus* as a single species is valid. The alternative hypothesis was that *B. villosus* included a number of sibling species separated either geographically or by host plant. Results indicated that all beetles belong to the same species.

Performance of *B. villosus* on *C. scoparius* and *Ch. prolifer* in the field was compared. Larval survival was significantly higher and development from egg to adult significantly faster on *C. scoparius* indicating it was the more suitable host for *B. villosus*. Despite apparently being a less suitable host, *B. villosus* destroys approximately 40% of available *Ch. prolifer* seed, compared to about 90% of *C. scoparius* seed.

Aspects of the methodology of the original testing were examined to identify any feature of the experimental design that may have caused the failure of the original choice oviposition tests to detect the acceptability of *Ch. prolifer*. Firstly the effect of larval rearing host, adult food type and no overwintering period were studied. In all cases beetles oviposited on *Ch. prolifer*, though showing a strong preference for *C. scoparius*. Secondly quantity of oviposition resource, cage size and orientation of plant material were also investigated. Beetles laid eggs on *Ch. prolifer* when they were presented with equal amounts of test plant and control material in replicates identical in design to the original tests, but when the quantity of control pod resource presented in choice tests was half that of the test plant, oviposition increased on the test plants *Ch. prolifer* (significantly) and *C. multiflorus*. Cages of two different sizes and cut shoots presented horizontally or vertically had no significant effect on oviposition.

In order to determine whether a host range expansion had occurred in the New Zealand population of *B. villosus* since its introduction from the UK, a comparison of oviposition preference was undertaken between New Zealand beetles and a newly imported population from the UK. Sample size was increased from two replicates (ten female beetles) in the original 1985 tests to ten replicates (50 female beetles). Beetles newly imported from the UK laid eggs on *Ch. prolifer* as well as on *C. scoparius* which ruled out the development of a host race with a broader host range in New Zealand. In

only 40% of replicates were eggs laid on *Ch. prolifer* indicating that 20 (or less) females found *Ch. prolifer* acceptable for oviposition. Results from this experiment and from previous investigations suggested that insufficient replication could be responsible for the failure to detect oviposition on *Ch. prolifer* due to a high level of individual variation in oviposition preference among female beetles.

Oviposition preference by individual female *B. villosus* beetles, from New Zealand and UK populations, between *C. scoparius* and *Ch. prolifer* was measured in a laboratory choice test. The variation in numbers of eggs laid was high, especially on *Ch. prolifer* (CV = 148% New Zealand population, CV = 166% UK population) compared with numbers on *C. scoparius* (CV = 97% New Zealand population, CV = 50% UK population). Although both populations showed an overall preference for *C. scoparius*, New Zealand populations showed higher acceptance of *Ch. prolifer* (New Zealand population: mean number of eggs 8.6 ± 1.6 SEM on *C. scoparius*, 6.5 ± 1.8 SEM on *Ch. prolifer*; UK population: mean number of eggs 12.7 ± 2.0 SEM on *C. scoparius*, 3.9 ± 2.0 SEM on *Ch. prolifer*). Nine of 29 New Zealand beetles laid more eggs on *Ch. prolifer* compared to none of the nine UK beetles that laid eggs. A sibling experiment using three mothers showed that there were no strong maternal influences on overwintering survival, longevity, fecundity or oviposition preference of offspring.

Although as some beetles from the newly imported UK population accepted *Ch. prolifer* it was possible to rule out the likelihood of a host shift having taken place, there are indications from the differences in individual preferences shown between New Zealand and UK populations that differentiation in populations may be occurring. There seemed to be little maternal influence on preference, but further study is required to determine whether observed differences are inherited and whether host race formation is likely. Beetles reared from *Ch. prolifer* were bigger, more fecund and had higher overwintering survival. Longevity was similar for beetles reared from either host, but survival from egg to adult was higher and development faster on *C. scoparius*, the host preferred overall for oviposition. Clearly original choice host specificity tests failed to detect that *B. villosus* accepted *Ch. prolifer* because insufficient replication was used: testing 20 female beetles instead of 10 would have reduced the chance of selecting a sample that would lay no eggs on *Ch. prolifer* from 11% to 0.02%.

Lessons for host specificity testing in weed biological control are that high levels of individual variation in host preference require suitably high levels of replication to

detect non-target effects and avoid type II errors (false negative results). This will inevitably generate larger numbers of type I errors (false positives) requiring detailed follow-up, preferably with individual insects. There are also indications that preference by an imported weed biological control agent for a non-target host plant may have changed over the 15 generations since its release, suggesting that test results for plant species that are new associations should be interpreted with particular care. The release of any agent that in no-choice tests accepts and develops fully on a valued host plant, cannot be recommended for release, even if the level of attack is low.

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Chapter 1: General Introduction

1.1 This study

This study aims to determine why host specificity tests failed to predict the non-target attack of *Chamaecytisus prolifer* (L. f.) Link subsp. *prolifer* var. *palmensis* (H. Christ) A. Hansen & Sunding (Genisteae: Fabaceae) by a biological control agent introduced for control of *Cytisus scoparius* (L.) Link (Genisteae: Fabaceae). In 1986 *Bruchidius villosus* (F.) (Bruchinae: Chrysomelidae) was released in New Zealand after results of host specificity tests showed that it was specific to the target weed *C. scoparius*. However, in 1999 *B. villosus* was found emerging from seeds of the non-target plant, *Ch. prolifer*. This finding indicated that the host specificity testing procedure used for this agent had failed, as the tests predicted no non-target impact on any species outside the genus *Cytisus*.

1.2 Non-target impacts of weed biological control agents

Prior to deliberate introductions of exotic insects and other organisms for weed biological control, practitioners determine the potential host range of a new agent using established methodologies. This host specificity testing is undertaken to assist in estimating potential ecological risks to non-target plant species in the country of introduction. In weed biological control, host specificity tests are designed to ensure introduced insects will damage only the target weed (or known acceptable non-targets) when introduced to new regions (Zwölfer and Harris 1971, Cullen 1990, Harley and Forno 1992, Heard 1997, van Klinken 2000). The term host specificity is often used synonymously with the term host range breadth (van Klinken 2000). However, while host range defines the sum of plant species that are hosts, host specificity ranks hosts on their acceptability or suitability relative to one another according to the proposed terminology of van Klinken (2000). If host specificity tests fail to predict accurately host range in a new country, non-target impacts that can result have the potential to be significant (Fowler and Withers, in press). Impacts of biocontrol agents on non-target plants can have both direct and indirect ecological effects (Louda et al. 2003a) which may take time to become apparent, be difficult to quantify and be irreversible (Fowler and Withers, in press).

The problem of non-target impact has been effectively summarised by Pemberton (2000) who reported that in Hawaii, the continental USA and the Caribbean, non-target feeding, oviposition and development has been recorded on 41 native plants by 15 of 112 biocontrol insects.

In this study non-target impact is defined as any predicted or unpredicted damage to a non-target plant or other species. This includes cases where damage to a non-target plant or organism had been predicted by host specificity tests, but the level of the impact was not accurately forecast, as well as cases where no non-target attack was expected, but damage to other species did occur.

McFadyen (1998) reported on agents that have been released when some damage to non-targets was anticipated. *Tyria jacobaeae* L. (Arctiidae) was introduced to the USA and Canada to control *Senecio jacobaea* L. (Asteraceae) (Bucher and Harris 1961, Diehl and McEvoy 1990), *Croesia zimmermanii* Clarke (Tortricidae) was released to control *Rubus argutus* Link (Rosaceae) in Hawaii (Gagné 1972) and *Chrysolina quadrigemina* Suffrian (Chrysomelidae) was released to control *Hypericum perforatum* L. (Clusiaceae) in California (Andres 1986). The level of realised damage to non-target plants in the above cases has either not yet been assessed or is recorded as minimal, marginal or not significant (McFadyen 1998).

Cactoblastis cactorum (Berg) (Pyralidae) was introduced to Australia where it achieved rapid control of the target *Opuntia* species. (Dodd 1940). It was later introduced to the Caribbean to control weedy *Opuntia* species despite the results of field studies which showed it could complete development on other cacti (Dodd 1940). As predicted it damages other cacti species in its now greatly expanded geographical range, including central American native plants (Simmonds and Bennett 1966). The full ecological impacts of this agent are yet to be assessed as *Opuntia* species are a food source for some Caribbean iguanid lizards (Stiling and Simberloff 1999) and have native insects associated with them which may be adversely affected (Burger and Louda 1994). In addition, *C. cactorum* has now been accidentally introduced to Florida where it is reported to feed on native *Opuntia* species increasing the risk of extinction of a rare species *O. corallicola* (= *O. spinosissima* Mill.) (Johnson and Stiling 1996).

A well known example of a significant non-target impact by a biological control agent is *Rhinocyllus conicus* Fröl. (Curculionidae) released in North America for control

of weedy thistles (Asteraceae, Carduinae). Despite the minor non-target impact predicted by host specificity tests (Zwölfer and Harris 1984), major unpredicted damage has occurred. Populations of sparse non-economic, but ecologically significant, native thistles have been severely depressed and the abundance of a native floral feeding tephritid, *Paracantha culta* Wiedeman, is also in decline. The latter result is evidence that indirect ecological effects have also taken place (Louda et al. 1997, Louda et al. 1998, Louda and Arnett 2000, Louda et al. 2003b). Similarly, *Altica carduorum* Guer. (Chrysomelidae) was redistributed from the north-eastern USA to control *Cirsium arvense* (L.) Scop. in the western USA and British Columbia even though host specificity tests predicted some damage to non-target native thistles (McClay 1990). This agent is now having a substantial unpredicted non-target impact on native thistles including rare species (Louda and O'Brien 2002).

In many of the above studies where some effect on non-target plants was predicted the full ecological impact on these plants by the agents was not thoroughly investigated, and in others, the value of non-economic native plants was simply not recognised as important at the time. Conservation values change over time, and in previous decades the negative impacts of exotic weeds to agricultural systems were often considered more important than the conservation status of apparently non-useful native plants (Fowler and Withers in press).

The review by McFadyen (1998) also reports three cases of damage by agents where no impact on non-target plant species was predicted. *Uroplata girardi* Pic. (Chrysomelidae) and *Teleonemia scrupulosa* Stål. (Tingidae) were released against *Lantana camara* L. (Verbenaceae) in Hawaii and are causing minor damage to basil (*Ocimum basilicum* L.) and other herbs (Labiatae) (Conant and Tsuda 1992), and to *Sesamum* species (in Africa) (Greathead 1968, Harley and Kassulke 1971) respectively. More recently *Aconophora compressa* Walk. (Membracidae) was released against *Lantana camara* (Verbenaceae) in Australia and is causing significant damage to a non-target exotic tree, *Citharexylum spinosum* L. (Verbenaceae) (Palmer 2004). *Zygogramma bicolorata* Pall. (Chrysomelidae) was released to target *Parthenium hysterophorus* L. (Asteraceae) in India and is also utilising *Helianthus annuus* L. to a minor degree (McFadyen and McClay 1981, Bhan et al. 1993, Jayanth et al. 1993).

If damage to a non-target plant occurs when an insect is released in a new area, and this outcome was not predicted by host specificity testing, regardless of the status or

value of the plant, the tests are considered to have failed for their intended purpose. Similarly, if host specificity tests inaccurately predict the extent of damage that would result to a non-target plant, this is also considered a failure, although a less serious one.

Presently non-target effects are a very topical issue in biological control (McFadyen 1998, McEvoy and Coombs 2000, Louda et al. 2003a, Fowler et al. 2004, Louda and Stiling 2004, Paynter et al. 2004, Fowler and Withers in press). Non-target impacts are difficult to measure. If they are unpredicted, they are unexpected, and often little or no pre-release study data is available (Fowler et al. 2004). The information available on non-target impacts must be treated with a degree of caution as it is often subjective and variable between sources.

In the future it will be important to be able to categorically define and standardise measures of non-target impact, so the relative degrees of severity can not only be compared between regions but also between species. This means resources will need to be secured for long-term post-release monitoring of impacts on both target and non-target species (Howarth 1991). However, there is also room for the implementation of a 'prevention is better than cure' approach. Not only is post-release monitoring a requirement when a biological control agent is released, or when a non-target impact has been identified, pre-release monitoring and assessment also plays an important part in predicting impacts on target and non-target species (Louda et al. 2003a). In New Zealand, ERMA (Environmental Risk Management Authority) New Zealand, the body responsible for giving permission to release new species of biological control agents, requires a commitment for post release follow up from the party seeking its release, before permission will be given (www.ermanz.govt.nz).

Concern over the power of pre-release studies to predict non-target effects has been voiced by many authors (Howarth 1991, Simberloff and Stiling 1996, Marohasy 1998, Thomas and Willis 1998, Stiling and Simberloff 1999, Heard 2000, McEvoy and Coombs 2000, Arnett and Louda 2002, Louda et al. 2003a). Improving host specificity testing (often a large part of pre-release studies) to reduce the likelihood of unpredicted non-target impacts is currently a considerable area of interest for weed biological control practitioners. Recent work involves specific studies paying particular attention to areas such as insect behaviour (Withers 1998, 1999, Heard 2000), plant physiology (Tallamy 2000), genetics and evolution (Karowe 1990, Futuyma 2000, Roitberg 2000), and methodology, assessment, interpretation and prediction (Heard 1997, McFadyen and

Heard 1997, Withers 1997, Marohasy 1998, Briese 1999, Day 1999, Edwards 1999, Heard 1999, Hill 1999, Sheppard 1999, Withers et al. 1999, Louda and Arnett 2000, Sands and Van Driesche 2000, van Klinken 2000, Gassman and Louda 2001, Lonsdale et al. 2001, Mahr 2001, Schaffner 2001, Briese and Walker 2002, Louda et al. 2003a, Fowler et al. 2004, Paynter et al. 2004). Such studies, in conjunction with the investigation of ecological parameters (Arnett and Louda (2002) and references therein, Fowler et al. (2001)) will improve the predictability of the fundamental host range (which includes all the plant species that an insect can accept or utilise and is therefore considered as the maximal host range (Nechols et al. 1992, van Klinken 2000) and the realised host range (how the fundamental host range is expressed under certain conditions (Nechols et al. 1992, van Klinken 2000) of potential weed biological control agents, and ultimately reduce the likelihood of non-target effects.

An integral part of improving pre-release studies is to learn from past mistakes. To date, few studies have retrospectively examined the reasons for the failure of host specificity tests to predict non-target attack. The study by Arnett and Louda (2002) is one exception. In this case-study some non-target impacts were forecast by host specificity tests, but the level of the impact was not, because relevant ecological information was excluded from the initial investigations.

Host specificity tests failed to predict the non-target attack of *Chamaecytius prolifer* by *Bruchidius villosus*, even though *Ch. prolifer* was included in the testing programme (Syrett and O'Donnell 1987). The present study aims to determine why the tests failed to adequately identify the risk to the non-target plant species. Secondly, it aims to evaluate the host specificity criteria used at the time, so that useful recommendations that improve current host specificity testing methods can be made.

1.2.1 *Cytisus scoparius*

Cytisus scoparius (also known as Scotch broom or broom) is a woody leguminous shrub of European origin, which is an invasive weed in many countries including New Zealand, Australia, North and South America, in both productive and conservation lands. It was first recorded in New Zealand in 1872 (Owen 1998), it is abundant on the eastern side of both the North and South Islands, and continues to invade new areas (Syrett et al. 1997). It flowers and seeds freely under a wide range of altitudinal and climatic conditions, and grows from sea level to 1200 m (Syrett et al. 1997).

1.2.2 Costs and benefits of *Cytisus scoparius*

In New Zealand, costs of establishing pine forests are increased by the need to clear *C. scoparius* from plantation sites, and re-invasion by the weed reduces the rate of tree growth. *Cytisus scoparius* is a serious invader of pastoral land, where it is associated with losses to agricultural production (Jarvis et al. 2003). *Cytisus scoparius* also invades conservation lands (Jarvis et al. 2003) and provides cover for predatory species in open river beds, which is detrimental to the habitat of several native bird species (Syrett et al. 1997).

The beneficial roles that *C. scoparius* plays are as a garden plant, as a pollen source for beekeepers, as a useful plant in erosion control, and as a forage source (Jarvis et al. 2003). It is also known to form a part in some successional processes (Syrett et al. 1997). However, the beneficial aspects of *C. scoparius* are considered to be strongly outweighed by its pest status (Syrett et al. 1997, Jarvis et al. 2003). *Cytisus scoparius* is one of 110 National Surveillance Pest Plants whose propagation, sale, distribution, and commercial display is prohibited by Regional Council Pest Plant Management Strategies under the Biosecurity Act (1993) of New Zealand (Syrett et al. 1997).

1.2.3 Control of *Cytisus scoparius*

Cytisus scoparius can sometimes be controlled with grazing management, but where further control is necessary, herbicides, although costly, are effective. Cutting and burning techniques are also used in some situations. In addition to *B. villosus*, two other biological control agents are present in New Zealand; the self introduced twig-mining moth *Leucoptera spartifoliella* Hübner (Lyonetidae) and the sap-sucking psyllid *Arytainilla spartiophila* (Förster) (Psyllidae) (Syrett et al. 1997).

Bruchidius villosus (commonly known as the broom seed beetle) was identified as a potential candidate for the biological control of *C. scoparius* in New Zealand in 1982 after results of studies undertaken in its native range showed it had potential to damage the seed crop of *C. scoparius* and that it was likely to be host specific to this plant (Parnell 1966, Waloff 1968). Initial host specificity tests for the New Zealand project were completed in the UK and following these *B. villosus* was imported from the UK into quarantine in New Zealand where further host specificity testing was completed (Syrett and O'Donnell 1987).

1.3 *Chamaecytisus prolifer*

Chamaecytisus prolifer (usually referred to in the New Zealand literature as *C. palmensis* (Christ) Bisby et K. Nicholls, also known as *Chamaecystisus proliferus* (L.) Link and commonly referred to as tagasaste or tree lucerne), originated in the Canary Islands, and was introduced to New Zealand and Australia last century as a forage plant and for shelter (Townsend and Radcliffe 1990). It is a leafy leguminous shrub or small tree, which grows to a height of approximately 5 m. *Chamaecytisus prolifer* is well established in New Zealand and is scattered throughout the North and South Islands, and Stewart Island. It is commonly found in coastal areas, particularly dry coastal hilly sites, but is less common in areas with heavy frosts. It is also prominent in river beds and dry waste areas (Syrett et al. 1997).

1.3.1 Costs and benefits of *Chamaecytisus prolifer*

Chamaecytisus prolifer is regarded as weedy in some places in New Zealand (Williams and Timmins 1990), but also has beneficial attributes. Benefits include, its use as a fodder crop on high country farms when there is drought (Douglas et al. 1996), the provision of an almost year-round pollen source for beekeepers (Dann and Trimmer 1986) and a supplementary food-source for the threatened native pigeon in New Zealand (McEwan 1978). It has limited use as a forage plant in areas with substantial frosts and high rainfall (Townsend and Radcliffe 1990). In a recent report which assessed the potential impact of another biological agent, *Gonioctena olivacea* Förster (Chrysomelidae) on *Ch. prolifer*, it was concluded that the agent should be introduced because the benefits from the chance of controlling *C. scoparius* was thought to be greater than the risk of damage to *Ch. prolifer* which was considered low in value (Jarvis et al. 2003).

1.4 Systematic placement and relationships of *Bruchidius villosus*

Bruchidius villosus belongs to the beetle family Bruchidae or subfamily Bruchinae of the family Chrysomelidae, and is placed in the superfamily Chrysomeloidea. Members of this family breed in every continent except Antarctica and tend to be strongly associated with plants in the family Fabaceae. The larvae of Bruchidae feed and develop only in seeds, often destroying them in immense numbers (Southgate 1979).

1.5 Identity of *Bruchidius villosus*

In the 1960s research on *B. villosus* was conducted in England and the USA, but under the name *B. ater* (Marsham). The two species are now believed to be synonymous (Aldridge and Pope 1986). There is much confusion surrounding the early historical nomenclature of *B. villosus*. A major revision of the bruchids was carried out by Hoffman in 1945, so all earlier taxonomic literature (prior to 1945) has been excluded from consideration in this study. All of the biological control literature relating to *B. villosus* is also post 1945.

B. villosus is referred to in the recent literature by the following synonyms (and within this literature further synonyms are recorded):

- *Bruchidius ater* (Marsham 1802) (Southgate 1963, Parnell 1964, Parnell 1966, Bottimer 1968, Waloff 1968, Southgate 1979, O'Donnell and Manfield 1986);
- *Bruchidius cisti* (Paykull 1800 nec Fabricius 1972) (Zacher 1952a, 1952b);
- *Bruchidius fasciatus* (Olivier 1975) (Hoffmann 1945, Steffan 1946, Zacher 1952b, de Luca 1962);
- *Bruchidius villosus* (Fabricius 1972) (Zacher 1952a, 1952b, Frick 1962, Aldridge and Pope 1986, Syrett and O'Donnell 1987, Szentesi and Wink 1991, Hosking 1992, Sheppard and Hodge 1994, Anton 1998, Isaacson and Markin 1998, Delobel and Delobel 2003, Jermy and Szentesi 2003);
- *Sparteus villosus* Bridwell (Fabricius 1792) (Bridwell 1946).

1.6 Biology and life cycle of *Bruchidius villosus*

1.6.1 Eggs

Female *B. villosus* deposit eggs on newly formed green *C. scoparius* pods (Parnell 1966, Hayes 1998). After taking up a firm position on the pod the female extrudes her ovipositor and moves it in a circular motion over the surface of the pod. A small droplet of fluid is exuded onto the pod surface by this movement, and following this an egg is deposited into the centre of the fluid, securing the egg firmly to the substrate. The female then either walks away or begins the process again by laying a second egg beside the first.

The eggs are oval in shape, and 0.54 mm (mean) in length and 0.28 mm (mean) in width (Parnell 1966), making them just visible to the naked eye as shiny spots, smaller than the size of a pinhead (Hayes 1998). They are straw yellow in colour when freshly laid, and the chorion is smooth and rigid, and maintains its shape even after the larva has emerged (Parnell 1966). Once the larvae have hatched, the egg cases are often easier to see, as they take on a whiter appearance (Hayes 1998). Eggs are normally laid around the edges of pods (Parnell 1966, Hayes 1998) and do not appear to be laid in any pattern relative to the developing seeds within the pod (Parnell 1966).

1.6.2 Larvae

The newly emerged first instar larva (which is primarily a locomotory stage) chews through the underside of the egg shell where it is firmly cemented to the pod surface, and immediately penetrates the pod wall to find its food, a soft green seed (Parnell 1966, Southgate 1979). Southgate (1979) reported that freshly hatched larvae enter the first available seed after penetrating the pod wall, but observations by Parnell (1966) indicated that a larva does not necessarily enter the nearest green seed, as single larvae have been found inhabiting seeds up to 4 cm away from their entry points.

A larva generally enters the seed through the micropore and bores into the seed to begin feeding on the cotyledons. However, if it bores through the pod wall directly onto a seed, it will penetrate the testa and commence feeding (Parnell 1966). Since the adult females attach the egg directly to the pod, the larvae only need to bore through the shell and pod wall to reach the food (Southgate 1979). Once the larva reaches a seed, little feeding occurs before the larva moults to the second instar. The second, third and fourth instars are the feeding stages which consume the entire seed embryo by the time the fourth instar is complete. The fourth instar larva chews half way through the testa before pupation, marking out the exit hole through which the adult will emerge. This is usually positioned at the end of the seed opposite to the micropore. At this point the seed testa surrounds a hollow cavity which houses a single fully grown larva along with frass and three larval exuviae (Parnell 1966).

After a short pupal period the adult hatches by pushing out the disk made by the fourth instar larva and emerges into the pod cavity where it usually stays until the pod dehisces (Parnell 1966). Adult *B. villosus* feed on the pollen of many plant species, but this is not considered a significant non-target effect, although the impact of *B. villosus*

robbing pollen from flowers of native species and the effect this has on these species has not been investigated.

1.7 Host specificity testing

Host specificity testing for the New Zealand *C. scoparius* biological control programme was initiated in the UK (the country of origin of *B. villosus*), following the criteria for selecting test plants established by Wapshere (1974). The plants used in the testing programme were a selection of important pod-bearing crop plants, plant species closely related to *C. scoparius* and some native New Zealand plants within the same family as *C. scoparius*, the Fabaceae (Syrett and O'Donnell 1987). Other testing for different biological control programmes was completed in Australia (Sheppard & Hodge, unpublished report) and the USA (Frick 1962, Isaacson and Markin 1998). All the host specificity tests measured oviposition only. *Bruchidius villosus* larvae cannot move between plants, so adult oviposition choice was tested instead. In none of the tests was complete development from egg to adult assessed.

Initial surveys are generally completed in the country or region of origin of the insect and literature is searched for information on the insect's host range prior to host specificity testing commencing in quarantine facilities of the new country (Forno and Heard 1997). Currently host specificity testing procedures follow the principles established by Wapshere (1989) where plants closely related to the target weed are included in tests which are generally conducted under glasshouse or laboratory conditions (Harley and Forno 1992).

Generally, the mechanisms of host selection by potential biological control agents are not well understood. 'No choice' tests in which insects are confined with a test plant only, are useful in discounting large numbers of plants, but generally insects feed on a broader range of plants in these tests than in natural conditions (Cullen 1990, Syrett and Emberson 1997). There are however alternative definitions for no-choice tests.
Hill et al. (1995) define a no-choice test as a test where the target host is not present, regardless of the number of test plants included. The rationale is that the insects do not have the opportunity to 'choose' a known acceptable host. No-choice tests were not favoured in the 1980s because of the strongly held view that they tended to induce false positive results (insects would oviposit on the plant species in the test when they had no choice, but when given a choice or put in a natural environment they would not oviposit

on this plant) (Shepherd 1989, Cullen 1990). 'Choice' tests in which insects are presented with material of both a test plant and a known host plant generally produce results indicative of a narrower range of acceptable plant species. These tests were thought to be more representative of natural field conditions, where insects make choices, but results can still be misleading (Cullen 1990, Syrett and Emberson 1997).

It is currently accepted that for insects such as *B. villosus* (where the adult female selects the host plant and the larva is relatively immobile), oviposition assays are necessary to establish which host plants are acceptable. Following this, acceptable hosts can be further tested to determine whether the insect can complete its life cycle on that host (Sheppard 1999). In spite of the problem with false positive results, no-choice tests are now recommended at the first stage of screening, followed by choice tests with those species scoring positives in no-choice tests to compare relative oviposition preferences between plant species (Edwards 1999).

In order to satisfactorily determine whether *B. villosus* posed a risk to non-target plants in New Zealand, the testing needed to be appropriate to the biology of the insect. It was necessary for *B. villosus* to be presented with plant species that bore pods (as previously mentioned the pod is the plant stage within which *B. villosus* completes development), at the correct phenological stage of development. Observations suggest that eggs laid anywhere but on the surface of the pod results in larval death (Parnell 1966). However, there is one documented case where a larva which emerged from an egg suspended on a hair above the pod surface, penetrated the pod wall and proceeded to normally develop (Wittenberg and Thomann 2001). There is no evidence that eggs can be manually transferred from one pod to another without adversely affecting viability.

Host specificity testing for *B. villosus* has been undertaken in Europe (source area), New Zealand, USA and Australia.

1.7.1 UK host specificity testing

1.7.1.1 No-choice oviposition tests

Various oviposition tests carried out by O'Donnell and Manfield (1986) and Syrett and O'Donnell (1987) are described below.

Five to 10 *B. villosus* (sex ratio not reported) were placed in sleeve cages on whole potted plants or branches for four weeks. Thirteen non-target plants were tested

from within the Fabaceae family, from the tribes, Genisteae, Loteae (Coronillae), Phaseoleae (Phaseolineae, Glycininae), Trifolieae, and Fabeae (Vicieae). Eggs were only laid on *C. scoparius*. The number of pods tested per plant species ranged from one (*Vicia faba* L.) to 479 (*Trifolium dubium* Sibth.). Half of the tests were carried out on only a single live host plant. Beetles used in the tests were of UK field origin (Syrett and O'Donnell 1987).

No-choice tests were also run in the laboratory using cut shoots. Twelve non-target plants were tested from within the Fabaceae, from the tribes Genisteae, Loteae (Coronillae), Phaseoleae (Phaseolineae, Glycininae), Trifolieae, and Fabeae (Vicieae). Five beetles (sex ratio not reported) were placed in containers with pod-bearing shoots of the test plants for four days. Eggs were laid on *C. scoparius*, *Genista hispanica* L., *Ulex europaeus* L. (all Genisteae) and *Vicia sativa* L. (Fabeae) (O'Donnell and Manfield 1986).

The number of replicates varied between four (*Lotus corniculatus* L.) and 18 (*C. scoparius*). Beetles used in these tests were of UK field origin.

1.7.1.2 Choice plus control oviposition tests

Two-hundred *B. villosus* (sex ratio not reported) were placed in a cage for four weeks with seven non-target plant species from within the Fabaceae, from the tribes Genisteae, Loteae (Coronillae), Phaseoleae (Phaseolineae, Glycininae), Trifolieae, and Fabeae (Vicieae), and two *C. scoparius* plants acting as the control. Eggs were laid on *C.* × *praecox* Beauverd cv 'Allgold' and *C. scoparius* (both Genisteae).

The numbers of pods tested varied from five (*Pisum sativum* L.) to 137 (*G. hispanica*). Two plants for each plant species were tested, the only exception to this was *C.* × *praecox* cv 'Allgold' of which only one plant was used. Beetles used in these tests were of UK field origin.

Two-choice tests were also run in the laboratory using cut shoots. Fourteen non-target plants were tested from the within the Fabaceae, from the tribes Genisteae, Loteae (Coronillae), Phaseoleae (Phaseolineae, Glycininae), Trifolieae, and Fabeae (Vicieae). Five beetles (sex ratio not reported) were placed in containers with pod-bearing shoots of the test plant and control (*C. scoparius*) for four days. Eggs were laid on *C.* × *praecox* cv 'Allgold', *C. scoparius* (both Genisteae) and one was laid on *V. sativa* (Fabeae) (O'Donnell and Manfield 1986). The number of replicates varied

between three (*Spartium junceum* L., *Vicia faba* L. and *Coronilla varia* L.) and 69 (*C. scoparius*). Beetles used in these tests were of UK field origin.

No New Zealand native plants were tested in the UK no-choice or choice tests, but all plants tested had either economic or ornamental value in the UK or were close relatives of *C. scoparius*. *Chamaecytisus prolifer* and New Zealand native plants within the tribes Carmichaeliae, Galegeae, or Sophorae (all within the Fabaceae family) do not produce pods under UK climatic conditions (O'Donnell and Manfield 1986), so could not be included in these tests.

1.7.2 New Zealand host specificity testing

1.7.2.1 Two-choice oviposition tests

Two-choice tests (test plant plus control) were run in the laboratory using cut shoots. Groups of ten *B. villosus* (five male and five female) were placed in containers with two water-filled vials one supporting a shoot of *C. scoparius* and the other the test plant. Both shoots bore equal numbers of pods at an equivalent stage of development. Nine plant species from the Fabaceae from the tribes Carmichaeliae, Galegeae, Genisteae or Sophorae were tested (Syrett and O'Donnell 1987). Tests were run sequentially for six or seven days and eggs were only oviposited on *C. scoparius* (Genisteae). Beetles used in these tests were of UK field origin.

1.7.3 Australian host specificity testing

In spring 1994, after *B. villosus* was released and established in New Zealand, adult beetles were imported to quarantine in Australia from New Zealand for host specificity testing (Sheppard and Hodge 1994).

1.7.3.1 Two-choice oviposition tests

Groups of 10 *B. villosus* (five male and five female) were placed in cages with cut shoots or whole plants bearing pods for five days. Twenty-eight plant species from within the Fabaceae were tested (18 Australian native and 10 exotic), from the tribes Acacieae, Bossiaeeae, Crotalarieae, Genisteae, Indigofereae, Loteae, Mirbelieae, Phaseoleae and Trifolieae. No eggs were laid on any plant species other than *C. scoparius* (Sheppard and Hodge 1994).

The number of pods tested per plant species varied from 32 (*Acacia mearnsii* De Wild) to a large number (over 607) on *C. scoparius*. Beetles used in these tests were of New Zealand origin (Sheppard and Hodge 1994).

1.7.4 USA host specificity testing

Host specificity testing of *B. villosus* commenced in the USA in the 1960s (Frick 1962), and further testing was done in the late 1980s (Isaacson and Markin 1998).

1.7.4.1 No-choice oviposition tests

Groups of ten adult *B. villosus* (sex ratio not reported) were placed in glass vials for 48 hours with cut shoots, of *C. scoparius*, *P. sativum* L. (= *P. sativa*) (Vicieae) *Phaseolus vulgaris* L. (Phaseoleae, Phaseolinae), *Robinia pseudoacacia* L. (Genisteae) or *Vicia villosa* Roth (Fabeae, Vicieae), (Frick 1962). Some species were replicated twice. Eggs were laid on *C. scoparius*, *R. pseudoacacia* and *V. villosa*. However, in total more eggs were laid on the vials (120) than on pod material (107).

In a separate test, 20 adults (sex ratio not reported) were placed in gallon jars with cut shoots of *C. scoparius*, *R. pseudoacacia* or *V. villosa* for 48 hours. Eggs were laid on pods of *C. scoparius*, one egg was laid on *V. villosa* and again a few eggs were laid on the jars (Frick 1962).

In another test ten adult *B. villosus* (sex ratio not reported) were placed in tubes with *Caragana* sp. (Galegeae) or *Genista monspessulana* (L.) L. A. S. Johnson for 17 days, but eggs were laid on neither species (Isaacson and Markin 1998). The origin of the beetles used in these tests was not reported.

1.7.4.2 Four and five-way choice oviposition tests

Thirty-four adult beetles (sex ratio not reported) were placed in a gallon jar with a single bouquet of cut shoots from *C. scoparius*, *P. sativum*, *P. vulgaris*, *R. pseudoacacia* and *V. villosa* for 48 hours. Eggs were laid on *C. scoparius* and *R. pseudoacacia*, and just two on *V. villosa*.

Several hundred adults (sex ratio not reported) were placed in a large cage for 48 hours with a single bouquet of cut shoots from *C. scoparius*, *Laburnum anagyroides* Medik. (Genisteae), *R. pseudoacacia* and *V. villosa*. Eggs were laid on pods of all plant species (Frick 1962). The origin of the beetles used in these tests is unknown.

1.7.4.3 Two-choice oviposition tests

Eight adult beetles (sex ratio not reported) were placed in a container with a cut shoot of *C. scoparius* and of a test plant for seven days. Eight plant species from the Fabaceae were tested, from the tribes Genisteae and Fabeae (Vicieae). Eggs were laid only on *C. scoparius* (Isaacson and Markin 1998). Beetles used in these tests are assumed to have been field caught in the USA.

Table 1.1: Summary of plant species oviposited on by *Bruchidius villosus* in host specificity testing conducted prior to its release in NZ in 1986. (Tests were conducted by authors with the corresponding superscript in the same row).

Plant species	Type of test	Author
GENISTEAE		
Cytisus multiflorus (L'Her.) Sweet	Choice (C/S)	(P. Syrett, unpublished report)
Cytisus × *praecox* cv 'Allgold'•	Choice (C/S[a] & W/P[b])*	(O'Donnell and Manfield 1986[a], Syrett and O'Donnell 1987[b])
Cytisus scoparius	Choice (C/S[a] & W/P[b]) & No-choice (C/S[c] & W/P[d])	(Frick 1962[ac], O'Donnell and Manfield 1986[ac], Syrett and O'Donnell, 1987[abd])
Genista hispanica	No-choice (C/S)*	(O'Donnell and Manfield 1986)
Laburnum anagyroides	Choice (C/S)	(Frick 1962)
Ulex europaeus	No-choice (C/S)*	(O'Donnell and Manfield 1986)
ROBINIEAE		
Robinia pseudoacacia	Choice*[1] & No-choice (C/S)*	(Frick 1962)
FABEAE		
Vicia sativa	Choice (C/S)* & No-choice (C/S)*	(O'Donnell and Manfield 1986)
V. villosa	Choice & No-choice (C/S)*	(Frick 1962)

Key (All choice tests included *C. scoparius*)
C/S Cut shoot
W/P Whole plant either sleeved branch or potted
* ≤ 1 egg laid per pod or replicate on test plant
*[1] ≤ 1 egg per pod was laid in one experiment on test plant, >1 egg laid in the other
• Author of name not found
NB. Plant species named from the plant names list on the USDA-ARS Germplasm Resources Information Network (www.ars-GRIN.gov)

1.8 Summary of host specificity testing results

Eggs were laid on pods other than *C. scoparius* in six separate choice tests (Frick 1962, O'Donnell and Manfield 1986, Syrett and O'Donnell 1987) and five no-choice tests (Frick 1962, O'Donnell and Manfield 1986) (Table 1.1). In the four-way choice test run by Frick (1962), *B. villosus* laid a mean of 4.3 and 30.7 eggs per pod on *L. anagyroides* and *R. pseudoacacia* respectively while a mean of 54.6 eggs were laid per *C. scoparius* pod. In the five-choice test, *B. villosus* laid a mean of 0.2 and 1.6 eggs per pod on *R. pseudoacacia* and *V. villosa* respectively whilst also laying a mean of 48.7 eggs per pod on *C. scoparius*. In the no-choice test conducted in vials a mean of 5.8 eggs per pod were laid on *C. scoparius*, and 0.1 and 3.0 eggs were laid per pod on *R. pseudoacacia* and *V. villosa*. In the no-choice tests conducted in gallon jars, *B. villosus* laid a mean of 47.0 eggs on *C. scoparius* whereas only a mean of 0.1 eggs per pod were laid on *V. villosa* (Frick 1962).

In no-choice tests conducted by O'Donnell and Manfield (1986) a mean of less than one egg per replicate was laid on *G. hispanica*, *U. europaeus* and *V. sativa* while over ten times as many eggs were laid per replicate on *C. scoparius*. In choice tests less than one egg per replicate was laid on *C.* × *praecox* cv 'Allgold'and *V. sativa*, while 27.9 were laid on *C. scoparius* (O'Donnell and Manfield 1986). In choice tests conducted by Syrett and O'Donnell in 1985 a mean of one egg was laid per pod of *C. praecox* (Table 1.1).

In all choice tests more eggs were laid on *C. scoparius* than on the test plant except in an unpublished observation where 24 eggs were laid on *C. multiflorus* and none on *C. scoparius*. In this case it was found that *B. villosus* had been presented with *C. scoparius* pods that were too mature to be acceptable to ovipositing female beetles. The experiment was repeated with younger pods of both species and the result was reversed, all eggs were laid on *C. scoparius* and none were laid on *C. multiflorus* (P. Syrett, unpublished data).

The results of these experiments indicated that *B. villosus* was only going to oviposit on *C. scoparius* or perhaps occasionally on other species within the tribe Genisteae (Table 1.2 shows plant species tested on which no eggs were oviposited). Doubt about the validity of Frick's (1962) experiments arise because in many cases eggs were laid on the inside of the containers in which the experiments were run, and not on pods suggesting under artificial conditions these beetles behaved unnaturally.

Table 1.2: Summary of plant species not oviposited on in host specificity tests conducted prior to *Bruchidius villosus* (and synonyms section 1.5) being released in New Zealand in 1986. (Tests were conducted by authors with the corresponding superscript in the same row).

Plant species	Type of test	Author
CARMICHAELIAE		
Carmichaelia australis R. Br.	Choice (C/S)	Referred to as *Carmichaelia ovata* (Syrett and O'Donnell 1987)
Carmichaelia petriei Kirk	Choice (C/S)	(Syrett and O'Donnell 1987)
Carmichaelia williamsii Kirk	Choice (C/S)	(Syrett and O'Donnell 1987)
Chordospartium stevensonii Cheeseman	Choice (C/S)	(Syrett and O'Donnell 1987)
Notospartium carmichaeliae Hook. f.	Choice (C/S)	(Syrett and O'Donnell 1987)
GENISTEAE		
Chamaecytisus prolifer	Choice (C/S)	Referred to as *C. palmensis* (Syrett and O'Donnell 1987)
Cytisus multiflorus	Choice (C/S)	(Syrett and O'Donnell 1987)
Cytisus scoparius	Choice (C/S)	(P. Syrett, unpublished data)
Genista hispanica	Choice (C/S[a] & W/P[b]) & No-choice (W/P)[d]	(O'Donnell and Manfield 1986[a], Syrett and O'Donnell 1987[bd])
Genista monspessulana	Choice (C/S)	(Isaacson and Markin 1998)
Genista pilosa L.	No-choice (W/P)	(Isaacson and Markin 1998)
Laburnum anagyroides	Choice (C/S)[a] & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[d])
Lupinus arboreus Sims	No-choice (W/P)	(Isaacson and Markin 1998)
Lupinus nootkatensis Donn ex Sims	Choice (C/S)	(O'Donnell and Manfield 1986)
Robinia pseudoacacia	No-choice (C/S)	(Frick 1962)
Spartium junceum	Choice (C/S)	(O'Donnell and Manfield 1986)
Ulex europaeus	Choice (C/S)[a] & No-choice (W/P)[d]	(O'Donnell and Manfield 1986[a], Syrett and O'Donnell 1987[d])
GALEGEAE		
Clianthus puniceus (G. Don.) Sol. Lindl.	Choice (C/S)	(Syrett and O'Donnell 1987)
LOTEAE		
Coronilla varia	Choice (C/S)[a] & No-choice (W/P)[d]	(O'Donnell and Manfield 1986[a], Syrett and O'Donnell 1987[d])
Lotus corniculatus	Choice (C/S) & No-choice (C/S)	(O'Donnell and Manfield 1986)
PHASEOLEAE		
Glycine max (L.) Merr.	Choice (W/P[b]) & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[c], Syrett and O'Donnell 1987[bd])
Phaseolus vulgaris	Choice (C/S[a] & W/P[b]) & No-choice (C/S[c] & W/P[d])	(Frick 1962[ac], O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[bd])

SOPHOREAE		
Sophora microphylla Aiton	Choice (C/S)	(Syrett and O'Donnell 1987)
Sophora prostrata Buchanan	Choice(C/S)	(Syrett and O'Donnell 1987)
TRIFOLIEAE		
Medicago sativa L.	Choice (C/S[a] & W/P[b]) & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[bd])
Trifolium dubium	Choice (C/S[a]) & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[d])
FABEAE		
Pisum sativum	Choice (C/S[a] & W/P[b]) & No-choice (C/S[c])	(Frick 1962[ac], O'Donnell and Manfield 1986[c], Syrett and O'Donnell, 1987[b])
Vicia faba	Choice (C/S[a] & W/P[b]) & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[bd])
Vicia hirsuta (L.) Gray	Choice (C/S[a]) & No-choice (C/S[c] & W/P[d])	(O'Donnell and Manfield 1986[ac], Syrett and O'Donnell 1987[d])
Vicia sativa	No-choice (W/P)	(Syrett and O'Donnell 1987)

Key
C/S Cut shoot
W/P Whole plant either sleeved branch or potted
NB. Plant species named from the plant names list on the USDA-ARS Germplasm Resources Information Network (www.ars-GRIN.gov)

1.8.1 Critical assessment of the testing programmes

1.8.1.1 UK no-choice tests

Because eggs were laid only on *C. scoparius*, no-choice tests conducted on whole plants in the UK suggested that *B. villosus* was host specific to *C. scoparius* (Syrett and O'Donnell 1987). However, results of preliminary no-choice tests run in the UK (O'Donnell and Manfield 1986) were not published. In these tests some eggs were laid on *G. hispanica*, *U. europaeus* and *V. sativa* (Table 1.1). Despite these results, an assumption may have been made that the later whole plant tests were more reliable than those preliminary tests using cut shoots. As a result of this testing (on exotic and economically important non-target UK plants) the risk to non-targets was perceived as low.

Only a limited number of non-target plant species were screened in no-choice tests, either as cut shoots or whole plants in the UK. Controls were not set up on *C. scoparius* at the same time each plant species was tested. A control, consisting of a subset of the insects to be used in the test placed on the known host plant, provides information on the reproductive maturity of the cohort, and is useful to ensure that the test insects are behaving normally. No details of the stage of pod development presented to *B. villosus* were given. It is assumed however, that pods of the appropriate stage of development were always available to *B. villosus* in all the tests.

1.8.1.2 UK choice tests

Choice tests were conducted on both whole plants and cut shoots. In the whole-plant test, large numbers of non-target plants were tested under semi-natural conditions so that pods remained attached to whole plants and insects were not confined in sleeve cages. However, because the test was done in the UK, plants that do not form pods in UK environmental conditions were excluded. Survival of larvae was poor on the control plants, which in some instances renders results void. Only five larvae from the 351 eggs laid on *C. scoparius* were found when the experiment was completed (Syrett and O'Donnell 1987).

The phenology of the plants was not reported throughout the investigation. However it is likely that pods of the appropriate developmental stage were available for most, if not all species, as the four week duration should have allowed for differences in timing of pod set between species.

The results of the preliminary choice cut shoot tests done by O'Donnell and Manfield (1986) were not published. Some eggs were laid on *C. praecox* and *V. sativa* in these tests (Table 1.1). It may have been assumed here, as in the no-choice tests, that the later choice tests on whole plants were more accurate than the preliminary tests on cut shoots, for reasons not recorded in the unpublished notes.

1.8.1.3 New Zealand tests

The New Zealand host specificity tests were conducted primarily to establish the risk posed by *B. villosus* to non-target New Zealand native plants within the Fabaceae. The results reinforced the published UK findings. No eggs were laid on any of the test plants and so *B. villosus* was deemed host specific. There are several factors that may have contributed to the fact that no eggs were laid on the test plants.

Because a limited number of beetles were imported into quarantine, replication was low and the choice tests were run sequentially. The sequential nature of the experiments may have altered the preference of *B. villosus* by increasing the level of experience toward *C. scoparius* pods over time, and in turn reducing the acceptability of the other pod species. *Chamaecytisus prolifer* was tested early in the sequence though, so this is unlikely to explain why this species was unacceptable. Over time, beetles were dying and may have been becoming less fecund (although this is not clear), resulting in less reliable results for plants tested later in the sequence.

Bruchidius villosus adults were imported from the UK in September and were used in these tests in November without undergoing the usual overwintering period, although it was not known if the lack of a winter diapause affects oviposition behaviour. Nor was the source of the plant material reported. The possibility is that all pod material may have been collected from the same plant, removing individual plant quality and genetic variation as influential factors in the test outcome. However, it is known that pod quantity presented within tests was similar, regardless of pod size (P. Syrett, personal communication)

1.8.1.4 Other data

Along with New Zealand, Australia and the USA were interested in using *B. villosus* as a biological control agent for *C. scoparius*. *Bruchidius villosus* was already present in the USA but was restricted to North Carolina in the eastern USA, so host specificity testing was done prior to introducing it to western areas. The results from choice tests conducted

in all the countries supported the findings of published UK and New Zealand studies that *B. villosus* was host specific to *C. scoparius* (Syrett and O'Donnell 1987, Sheppard and Hodge 1994, Isaacson and Markin 1998).

The discovery that *B. villosus* readily accepted *Ch. prolifer* after its establishment in the field in New Zealand (Fowler et al. 2000) led to a reassessment of the literature records as part of the current study. The non-target impact, and the failure of tests to accurately predict host range is of real concern and is the first documented case of this in New Zealand (Fowler et al. 2004). Recent field records support earlier work of Hoffmann (1945), Zacher (1952b), Frick (1962) and Bottimer (1968) suggesting *B. villosus* is not monophagous on *C. scoparius* (Table 1.4). *Bruchidius villosus* has been recorded in the field from 15 species in 8 genera since 1991. Discrepancies between these field records and results of host specificity testing are highlighted in Table 1.6.

1.8.1.5 Discrepancies in data

In some tests, eggs were laid on cut shoots but not on whole plants of the same plant species. In other instances the same type of test gave different results and field rearing records differed from data gathered from host specificity testing.

Specifically, Szentesi and Wink (1991) reported rearing *B. villosus* from seeds of *L. anagyroides* in central Europe, a host which was oviposited on in choice tests carried out by Frick (1962) but not in those carried out by Syrett and O'Donnell (1987). Syrett and Emberson (1997) made field observations in Europe, and found *B. villosus* feeding on flowers of *S. junceum* and native *Cytisus* species. This observation is supported by rearing records in France where *B. villosus* was reared from *S. junceum* (Delobel and Delobel 2003, Jermy and Szentesi 2003, Haines et al. 2004). Frick's (1962) rearing records included *S. junceum* as a host, yet choice tests done by Isaacson and Markin (1998) and field observations by O'Donnell and Manfield (1986) did not indicate that this species was a suitable host. *Bruchidius villosus* has also been reared from *G. monspessulana* (*Teline monspessulana*) in France (Delobel and Delobel 2003, Haines et al. 2004). Again this finding contrasts with results of choice tests conducted by Isaacson and Markin (1998) where eggs were never laid on this host. However *S. junceum* and *G. monspessulana* were only presented to *B. villosus* in choice tests; no-choice tests were not carried out with these species.

1.9 Rearing records

The field observations/collections of host plants of *B. villosus* recorded prior to the host specificity testing programme illustrate that *B. villosus* (and its synonyms) had been reared from seeds of a total of 31 plants species, 26 from the tribe Genisteae (Table 1.3).

The majority of field rearing records available before 1985 were from Frick who collected data from seed confiscated by USA customs, and from Bottimer (1968) who studied *B. villosus* in the northeastern USA. Also Parnell (1966) and Waloff (1968) studied *B. villosus* in the UK and found it to be specific to *C. scoparius* (Table 1.3). Because there has been confusion concerning the identity of both the seeds, and emerging insect species, it is difficult to verify the authenticity of these records. If these rearing records had been considered reliable initially, *B. villosus* may not have been regarded as monophagous, but instead exhibiting oligophagy: the beetle was recorded developing on plant species from 12 genera in the tribe Genisteae. Such a classification as an oligophage, may have resulted in the beetle's lower priority for consideration as a biological control agent, and perhaps a more comprehensive testing programme. However, the results of host specificity testing that was carried out indicated that the beetle was, in fact, highly specific to *Cytisus* species.

Table 1.3: Summary of field records for *Bruchidius villosus* and its putative synonyms reared from pods or seeds prior to the release of *B. villosus* in NZ in 1986.

Plant species	Author
GENISTEAE	
Calicotome spinosa (L.) Link	(Zacher 1952b)
Cytisophyllum sessilifolius (L.) Láng	Referred to as *Cytisus sessifolius* L. (Steffan 1946, Frick 1962)
Chamaecytisus glaber (L. f.) Rothm.	Referred to as *Cytisus elongatus* Waldst. & Kit (Zacher 1952b)
Chamaecytisus hirsutus (L.) Link	(Frick 1962). Referred to as *Cytisus hirsutus* L. (Zacher 1952b) also *Cytisus capitatus* Scop. (Zacher 1952b)
Chamaecytisus purpureus (Scop.) Link	Referred to as *Cytisus purpureus* (Zacher 1952b)
Cytisus andreana•	(Steffan 1946, Zacher 1952b)
Cytisus austriacus (L.) Link	(Zacher 1952b)
Cytisus bolougnensis•	(Frick 1962)
Cytisus grandifolius Brot. DC.▪	(Zacher 1952b). Referred to as *Cytisus lustanicus*▪(Miller) Maire (Zacher 1952b)
Cytisus ratisbonensis (Schaeff.) Rothm.	(Zacher 1952b). Referred to as *Cytisus biflorus* L'Her.
Cytisus scoparius	(Bottimer 1968). Referred to as *Sarothamnus scoparius* (L.) Koch (Hoffmann 1945, Steffan 1946, Parnell 1964, Parnell 1966, Waloff 1968)
Cytisus striatus (Hill) Rothm.	(Zacher 1952b) also as *Cytisus pendulinus* L.f. (Zacher 1952b)
Cytisus supinus (L.) Link	(Frick 1962)
Cytisus villosus Pourr.	(Steffan 1946)
Genista anglica L. ▪	(Zacher 1952b)
Genista canadensis•	(Zacher 1952b)
Genista cinerea (Vill.) DC.	(Hoffmann 1945, Zacher 1952b)
Genista corsica DC. •	(Hoffmann 1945)
Genista hispanica	(Frick 1962)
Genista linifolia L.	Referred to as *Cytisus liniifolius* (L.) Lam (Zacher 1952b)
Genista monspessulana	*Cytisus monspessulanus* L. (Frick 1962)
Genista tinctoria L.	(de Luca 1962, Frick 1962). Synonym *Genista anxantica* Griseb., nom. illeg., referred to as *G. anxantina* (Frick 1962)
Laburnum alpinum (Mill.) J. Presl	(Zacher 1952b, Bottimer 1968)
Laburnum anagyroides	(Frick 1962). Referred to as *Laburnum vulgare* J.Presl (Zacher 1952b)
Lupinus arboreus	(A. Sheppard, unpublished data)
Petteria ramentacea (Sieber) C. Presl	(Zacher 1952b, Frick 1962, Bottimer 1968). Synonym *Cytisus weldenii*, referred to as *Cytisus weldonii* (Zacher 1952b)
Podocytisus caramanicus Boiss. & Heldr.	Referred to as *Podocytisus* sp. (Zacher 1952b)

Spartium junceum	(Hoffmann 1945, Steffan 1946, Frick 1962)
HEDYSAREAE	
Onobrychis viciifolia Scop.	Referred to as *O. viciaefolia* (Zacher 1952b), *Onobrychis sativa* Lam. (Caillol 1954)
LOTEAE	
Coronilla valentina L. subsp. *glauca* (L.) Batt.	Referred to as *Coronilla glauca* (Caillol 1954)
Coronilla juncea L.	(Caillol 1954)
ROBINIEAE	
Robinia pseudoacacia	Referred to as *R. pseudaccacia* (Hoffmann 1945), *R. pseudacacia* (Zacher 1952b)

Key
• Author of name not found
▪ Plant species name not recorded on GRIN, or other databases consulted
Plants were names from the plant names list on the USDA-ARS Germplasm Resources Information Network (www.ars-GRIN.gov).

Table 1.4: Additional field rearing records for *Bruchidius villosus* since its release in New Zealand in 1986. (All records unless noted are from Europe).

Plant species	Author
GENISTEAE	
Cytisophyllum sessilifolium	(Delobel and Delobel 2003, Kergoat et al. 2004). Referred to as *C. sessifolius* (Jermy and Szentesi 2003).
Chamaecytisus hirsutus	(Delobel and Delobel 2003, Jermy and Szentesi 2003). Referred to also as *Cytisus supinus* (Jermy & Szentesi 2003).
Chamaecytisus prolifer	Referred to as *C. palmensis* (Syrett 1999[1], Wittenberg and Thomann 2001)
Cytisus cantabricus (Willk.) Rchb. f.	(Hosking 1992, Syrett and Emberson 1997)
Cytisus × praecox	(Haines et al. 2004)
Cytisus scoparius	(Hosking 1992, Syrett and Emberson 1997, Delobel and Delobel 2003, Jermy and Szentesi 2003)
Cytisus striatus	(Syrett and Emberson 1997, Haines et al. 2004)
Cytisus villosus	(Delobel and Delobel 2003)
Genista monspessulana	(Haines et al. 2004). *Teline monspessulanus* (Delobel and Delobel 2003)
Genista stenopetala Webb & Berthel.	Referred to as *Cytisus racemosus* Marnock (Wittenberg and Thomann 2001, Haines et al. 2004)
Genista tinctoria	(Jermy and Szentesi 2003, Haines et al. 2004)
Laburnum alpinum	(A. Szentesi, unpublished data)
Laburnum anagyroides	(Szentesi and Wink 1991, Delobel and Delobel 2003, Jermy and Szentesi 2003)
Petteria ramentacea	(Jermy and Szentesi 2003)
Robinia pseudoacacia	(Szentesi and Wink 1991)
Spartium junceum	(Delobel and Delobel 2003, Jermy and Szentesi 2003, Haines et al. 2004)

Key
[1] Common field host in New Zealand

Table 1.5: Additional host specificity test records for *Bruchidius villosus* post 1986 where no eggs were laid on test plant species.

Plant species	Type of test	Author
ACACIEAE		
Acacia dealbata Link	Choice (C/S)	(Sheppard and Hodge 1994)
Acacia mearnsii	Choice (C/S)	(Sheppard and Hodge 1994)
Acacia melanoxylon R.Br.	Choice (C/S)	(Sheppard and Hodge 1994)
Acacia rubida A. Cunn.	Choice (C/S)	(Sheppard and Hodge 1994)
BOSSIAEEAE		
Bossiaea buxifolia A. Cunn.	Choice (W/P)	(Sheppard and Hodge 1994)
Goodia lotifolia Salisb.	Choice (C/S)	(Sheppard and Hodge 1994)
Hovea acutifolia A. Cunn. Ex G. Don	Choice (C/S)	(Sheppard and Hodge 1994)
CROTALARIEAE		
Crotalaria cunninghamii R. Br.	Choice (W/P)	(Sheppard and Hodge 1994)
GENISTEAE		
Ch. prolifer	Choice (C/S)	Referred to as *C. palmensis* (Sheppard and Hodge 1994)
C. multiflorus	Choice (C/S)	Referred to as *C. alba* cv 'Bridal Veil' (Sheppard and Hodge 1994)
C. scoparius	Choice (C/S & W/P)	(Sheppard and Hodge 1994)
G. monspessulana	Choice (C/S)	Referred to as *G. monspessulanus* (Isaacson and Markin 1998)
Genista stenopetala	Choice (W/P)	Referred to as *Cytisus racemosus* (Sheppard and Hodge 1994)
Laburnum watereri (Wettst) Dippel	Choice (C/S)	(Sheppard and Hodge 1994)
Lupinus angustifolius L. 'Illyarrie'	Choice (W/P)	(Sheppard and Hodge 1994)
Lupinus arboreus	Choice (C/S)	(Isaacson and Markin 1998)
Lupinus arboreus x rivularis	Choice (C/S)	(Isaacson and Markin 1998)
Lupinus littoralis Douglas	Choice (C/S)	(Isaacson and Markin 1998)
GALEGEAE		
Caragana sp.	Choice (C/S)	(Isaacson and Markin 1998)
INDIGOFEREAE		
Indigofera australis Willd.	Choice (C/S)	(Sheppard and Hodge 1994)
LOTEAE		
Lotus pendunculatus Cav.	Choice (W/P)	Referred to as *Lotus pendiculatus* (Sheppard and Hodge 1994)
MIRBELIEAE		
Chorizema cordatum Lindl.	Choice (C/S)	(Sheppard and Hodge 1994)
Daviesia mimosoides R. Br.	Choice (C/S)	(Sheppard and Hodge 1994)
Eutaxia cuneata Meis.	Choice (C/S)	(Sheppard and Hodge 1994)
Oxylobium ellipticum (Vent.) R. Br.	Choice (W/P)	(Sheppard and Hodge 1994)

Pultenaea daphnoides Wendl.	Choice (C/S)	(Sheppard and Hodge 1994)
Pultenaea subternata Williamson	Choice (W/P)	(Sheppard and Hodge 1994)
PHASEOLEAE		
Glycine clandestina J. C. Wendl.	Choice (W/P)	(Sheppard and Hodge 1994)
Hardenbergia violacea (Schneev.) Stearn	Choice (C/S)	(Sheppard and Hodge 1994)
TRIFOLIEAE		
Medicago polymorpha L.	Choice (C/S)	(Sheppard and Hodge 1994)
Medicago truncatula Gaertner	Choice (W/P)	(Sheppard and Hodge 1994)
Trifolium subterraneum L.	Choice (C/S)	(Sheppard and Hodge 1994)
FABEAE		
P. sativa	Choice (C/S)	(Isaacson and Markin 1998)

Key
C/S Cut shoot
W/P Whole plant, either sleeved branch or potted

Table 1.6: Discrepancies between results of host specificity testing and field records for *Bruchidius villosus*.

Plant species	Results which support wider host range	Results which do not support wider host range
GENISTEAE		
Chamaecytisus hirsutus	Field host in Hungary (Jermy and Szentesi 2003)	Not reared from seed in Italy (Hosking 1992)
Chamaecytisus prolifer	Referred to as *C. palmensis.* Host in field in New Zealand (Syrett 1999), Europe field records (Wittenberg and Thomann 2001)	Referred to as *C. palmensis*. No eggs laid in choice cut shoot tests in quarantine in New Zealand and Australia (Syrett and O'Donnell 1987, Sheppard and Hodge 1994)
Cytisus multiflorus	Eggs laid in choice cut shoot test when *C. scoparius* pods presented were at an advanced stage of development (P. Syrett, unpublished data)	Referred to as *C. alba* cv 'Bridal veil'•, no eggs laid in choice cut shoot test (Sheppard and Hodge 1994)
Genista hispanica	Eggs laid in no-choice cut shoot test (O'Donnell and Manfield 1986)	No eggs laid in choice cut shoot test (O'Donnell and Manfield 1986) and choice and no-choice test whole plant test (Syrett and O'Donnell 1987)
Laburnum anagyroides	Eggs laid in choice cut shoot tests (Frick 1962). Field host in central Europe (Szentesi and Wink 1991)	No eggs laid in no-choice test on whole plants (Syrett and O'Donnell 1987)
Lupinus arboreus	Field records from France (A. Sheppard, unpublished data)	No eggs laid in no-choice test on whole plants or UK field trial (O'Donnell and Manfield 1986)
Robinia pseudoacacia	Eggs in choice and no-choice cut shoot tests (Frick 1962)	No eggs laid in cut shoot no-choice test (Frick 1962)
Spartium junceum	Field host in central Europe (Jermy and Szentesi 2003)	No eggs laid in choice cut shoot tests or in the field on whole potted plants (O'Donnell and Manfield 1986)
Genista monspessulana	(Haines et al. 2004). Referred to as *T. monspessulanus* (Delobel and Delobel 2003)	Referred to as *G. monspessulanus*, no eggs laid in choice cut shoot tests (Isaacson and Markin 1998)
Genista tinctoria	Field host in Europe (Jermy and Szentesi 2003) and in field trial in France (Haines et al. 2004)	No eggs laid in field trial in UK (O'Donnell and Manfield 1986) or in field in France (Wittenberg and Thomann 2001)
Ulex europaeus	Eggs laid in no-choice cut shoot test (O'Donnell and Manfield 1986)	No eggs laid in no-choice cut shoot test (O'Donnell and Manfield 1986) or no-choice whole plant test (Syrett and O'Donnell 1987)
VICIEAE		
Vicia sativa	Eggs laid in no-choice and choice cut shoot test (O'Donnell and Manfield 1986)	No eggs laid in no-choice whole plant test (O'Donnell and Manfield 1986)

Key
• Author of name not found

1.10 Summary of experimental approach

The aim of this study was to test the validity of a number of possible explanations and hypotheses for the discrepancies (Table 1.6) between the results of host range tests, field observations and rearing data. Specifically the aim was to determine why the host specificity tests conducted prior to the release of *B. villosus* in New Zealand failed to predict the colonisation of the alternative host *Ch. prolifer*. The majority of the study was undertaken in the field and laboratory at Landcare Research, Lincoln, Canterbury, New Zealand. *Bruchidius villosus* beetles were also collected from the UK, Hungary, Spain, France, USA and Canada for testing during this work.

There were eight parts to the study, described in the following nine Chapters of this thesis. Chapter 2 investigates the possibility that the field host range of *B. villosus* in Europe (origin) is different from that in New Zealand.

The objective for Chapter 3 was to determine whether the species known as *B. villosus* around the world is genetically one species with a broad host range, or a group of distinct genetic sub groups with narrower host ranges, by analysing DNA of beetles from selected sites in the Northern and Southern Hemispheres.

As a consequence of the results from Chapter 3, individual aspects of the host specificity testing procedure used in New Zealand were re-examined (Chapter 4, 5 and 6). The specific objectives were to:

- Investigate the effect that seed size has on *B. villosus* oviposition
- Determine the effect that pod developmental stage has on *B. villosus* oviposition and survival
- Determine the effect that larval rearing host and previous feeding experience has on *B. villosus* oviposition
- Examine the effect that no overwintering period has on *B. villosus* oviposition
- Investigate the effect that quantity of oviposition resource, cage size and orientation of test plant material has on *B. villosus* oviposition

The findings from these experiments led to the replication, as closely as possible, of the original New Zealand host specificity tests (Chapter 7). The objective was to determine the oviposition preference of groups of *B. villosus* imported from the UK under

increased replication. The hypothesis was if British beetles still refused to accept *Ch. prolifer* for oviposition, while their New Zealand progeny now accepted it, then a host range expansion would be the most likely explanation.

The findings in Chapter 7 indicated that oviposition preferences of individual beetles should be investigated, as all testing had previously been done on groups of beetles. The objective for experiments described in Chapter 8 was to determine oviposition preferences of individual *B. villosus* beetles.

In the final part of the study (Chapter 9) the likelihood that host race formation is in the process of occurring in New Zealand, following the release of the beetles was investigated by comparing the fitness of *B. villosus* reared from *C. scoparius* with beetles from *Ch. prolifer*.

The implications of the findings of the investigations in this study for host range testing and biological control practices are discussed in Chapter 10.

Linking page: Chapter 2

The field host records of *Bruchidius villosus* are inconsistent (Chapter 1). Confusion has occurred as a result of taxonomic issues surrounding plant and insect names. In an attempt to clarify some of the historical records and determine the full host range of *B. villosus* in New Zealand, trials were set up in the field with plants closely related to the target weed *Cytisus scoparius* as well as native New Zealand plants. The hypothesis for Chapter 2 was that if *B. villosus* did not oviposit (or complete its lifecycle) on plants, known to act as hosts in Europe, other than *C. scoparius* and *Chamaecytisus prolifer* the assumption would be that 1) the population introduced to New Zealand was different (phenotypically) from that on which the historical host records were based, or 2) that it had changed since its introduction.

Chapter 2: Does the New Zealand population of *Bruchidius villosus* have a similar field host range to populations in Continental Europe?

2.1 Abstract

Host specificity investigations conducted with *Bruchidius villosus* in the field in Canterbury, New Zealand are summarised. In sleeved no-choice tests, adult *B. villosus* laid eggs on and emerged from pods of *Cytisus scoparius*, *Chamaecytisus prolifer* and *Genista monspessulana* and laid eggs on *Cytisus multiflorus*. No eggs were laid on *Laburnum anagyroides*, *Lupinus arboreus*, *Spartium junceum*, *Sophora microphylla* and *Sophora prostrata*. In an open field trial *B. villosus* emerged from *C. scoparius* and *Ch. prolifer*. In the field trial no eggs were found on *Clianthus puniceus, C. multiflorus*, *G. monspessulana*, *S. junceum*, and *So. microphylla* trees surrounding the trial. It was found that differences in plant phenology might exclude some species from attack by *B. villosus* in some seasons. No eggs were laid on any New Zealand native plant species in these experiments.

2.2 Introduction

Host specificity testing determines fundamental host range but not necessarily realised host range. Field surveys in the native range plus host specificity tests provide crucial information on realised host range (Louda et al. 2003a). van Klinken (2000) describes the host range of an insect as the sum of plant species that are hosts. Host range which is demonstrated by experiments is frequently broader than that which occurs in the field (Sheppard and Hodge 1994, Olckers 1999). There are two distinct parts to the host range of an insect, defined as the fundamental and realised host ranges. In simplified terms the fundamental (Nechols et al. 1992, van Klinken 2000) or physiological (Cullen 1990, McEvoy 1996, Olckers 1998, Louda et al. 2003a) host range includes all the plant species that an insect can accept or utilise and is therefore considered as the maximal host range. The realised (Nechols et al. 1992, van Klinken 2000), behavioural (Cullen 1990) or ecological (Louda et al. 2003a) host range is how the fundamental host range is expressed

under certain conditions. In field conditions the realised host range is often a subset of the fundamental host range of the insect because oligophagous insects utilise fewer species than they are physiologically capable of using (Wapshere 1989, Cullen 1990, Harris and McEvoy 1992).

The benefits and limitations of open field testing have been raised previously. Some benefits of field testing are: 1) it is a realistic method for assessing host range allowing full expression of host selection behaviour as agents are not restricted by cages which can elicit unnatural behaviour, and 2) it can work as a support tool to confirm or negate findings in other types of tests on the same agent. Some limitations are: 1) achieving an adequate density of agents can be difficult (field tests in the native range can be marred by natural enemies (White 1997)), 2) testing is often limited to areas where the insect is either native or introduced, 3) ethical difficulties occur if exotic plants or insects need to be imported to an area outside their current native or introduced range for testing, and 4) the process can be expensive (Rizza et al. 1988, Cullen 1990, Dunn and Campobasso 1993, Blossey 1995, Clement and Cristofaro 1995, Briese 1999).

Some more sophisticated methods of field testing have been proposed. Briese (1999) recommended a two-phase experimental design using equal numbers of all test plant species to avoid possible false negative results due to suppression of ovipositing response. In some trials, oviposition response could be suppressed as a result of sensory domination which can occur when a high proportion of one particular plant species is present (Marohasy 1998). This is often the case with weed infestations, as they are often the dominant vegetation in their habitat (Briese et al. 2002).

In the first 'choice' phase the agent is introduced to the trial and the population is given time to build in size. During this time the insect has a choice between target and non-target plants. The second, no-choice phase, involves killing or removing the target weed to expose the insect's response to high densities of non-target plants when the target weed is not present. This type of test allows examination of host choice when insects are deprived of the target weed, and mimics the situation when a successful agent locally destroys its host and migrates to host free areas. This design takes account of two aspects, firstly that of lowered responsiveness to other plants in the presence of the target weed, and when the target plant is absent, time-dependent changes in the behaviour of the insect can be examined.

Marohasy (1998) also proposes a set design to reveal hosts when the target weed is reduced in abundance. In this design, the target weed plays a minor part in the plant species composition used in the experiment. This ensures the host choice behaviour is not influenced by sensory domination and realised host range can be identified.

In contrast, no-choice development tests are likely to identify more accurately the range of hosts the insect is physiologically and behaviourally capable of using, but the breadth of this range may be reduced when the insect is given a choice in experiments or in the field. For plant hosts to be utilised in the insect's realised host range they must be phenologically synchronised with the insect. The weevil *Pachycerus cordiger* Germar a biological control agent for *Heliotropium europaeum* L. for example, needs a rootstock in summer, so all winter annual species are excluded from its realised host range, even though oviposition and larval development can occur in its fundamental host range when plants are maintained unnaturally in an artificial environment (Cullen 1989).

Bruchidius villosus was introduced to New Zealand as a biological control agent for *Cytisus scoparius* after results of host specificity tests predicted that it was host specific to this plant (Syrett and O'Donnell 1987). *Bruchidius villosus* is proving to be a successful biological control agent in New Zealand where it destroys up to 90% of available *C. scoparius* seed at some sites (M. Haines, unpublished data). However, it also destroys up to 40% of available seed on the alternative host *Chamaecytisus prolifer* at a field site in Canterbury (M. Haines, unpublished data), a finding that was not predicted by host specificity tests.

Preliminary field data from pods collected from various pod-bearing plants suggested that *Ch. prolifer* was the only non-target host in New Zealand. No adults were found emerging from pods of *Laburnum anagyroides*, *Spartium junceum* or native New Zealand Fabaceae, *Clianthus puniceus, Sophora microphylla* and *So. prostrata,* collected from the field (M. Haines, unpublished data). Upon examination of field records in Europe, the native range of *B. villosus*, many plants are recorded as hosts of *B. villosus* (Table 1.3 and 1.4), including *L. anagyroides* (Szentesi and Wink 1991) and *S. junceum* (A. Sheppard, unpublished data). Numbers of seeds destroyed in the beetle's native range in Europe are significantly fewer than in New Zealand (M. Haines, unpublished data) as the population is likely to be regulated by the presence of natural enemies in its native range (White 1997).

No-choice tests on sleeved branches were conducted in the UK in 1985 on a selection of pod forming crop plants and plant species closely related to *C. scoparius*. In these tests, eggs were laid only on *C. scoparius*. The aim of this investigation was firstly to compare the host range in New Zealand determined by the no-choice tests on sleeved branches with that of an open field trial. Six common plant species were present in both test types. A further aim was to determine whether the field host range of *B. villosus* in New Zealand is different from that in Europe.

If the host ranges are different, one theory to explain this phenomenon could be that when the UK separated from Europe in the late Pleistocene, *B. villosus* populations became separated and consequently there was little or no gene flow between them because the English Channel acted as a barrier. Different selection pressures may have occurred in the different areas because of host plant availability, which could have driven the host preferences of the two populations apart. There are many fewer native pod-bearing plant species in the Genisteae in the UK as compared to southwest Europe. In the UK there are three species of each *Ulex* and *Genista* and one species of *Cytisus* (Clapham et al. 1987), while in Europe there are seven *Ulex*, 56 *Genista* and 23 *Cytisus* species plus 49 other native pod-bearing species within the Genisteae. Of these 135 species, 70 occur on the Iberian Peninsula (Tutin et al. 1968). Therefore, *B. villosus* may have adapted to *C. scoparius* and exhibit a narrower host range in the UK than in Europe where it is exposed to a wider range of species and has adapted to those.

New Zealand beetles were sourced from the UK population, so if this mechanism was acting, we might expect that the beetles in New Zealand would have a narrower host range than European *B. villosus*. Beetles are not exposed to *Ch. prolifer* in the UK, as it does not grow there. All recorded field hosts to date (apart from *C. scoparius*) are from continental Europe, except for *Ch. prolifer*, which occurs naturally only on the Canary Islands (section 1.3.1). In field trials (open and caged) in the south of France (Haines et al. 2004) European adult *B. villosus* emerged from *C. scoparius*, *C. striatus*, *C.* × *praecox*, *C. racemosus*, *Genista tinctoria*, *G. monspessulana*, *S. junceum* and *Ch. prolifer*.

This Chapter describes field studies of *B. villosus* in New Zealand where the host range of *B. villosus* was assessed, fundamental host range by no-choice sleeve tests and realised host range by a field trial. The overall aim of this experiment was to determine

the range of hosts on which *B. villosus* can complete its life cycle and to investigate how replication and pod density affects oviposition by *B. villosus*.

2.3 Methods

2.3.1 No-choice tests on sleeved branches

Nine plant species within the tribes Genisteae, Galaegeae, and Sophorae were tested in order to ascertain the host range of *B. villosus*. To prevent contamination by the natural field population of *B. villosus* during the investigation, 90 branches each of *Ch. prolifer* and *C. scoparius* bearing fertilised flowers and immature pods were sleeved in the field prior to *B. villosus* becoming active in early spring. Two weeks later the sleeves were checked to ensure no eggs had been laid on the pods. Before the beetles were added to the sleeve cages excess pods were removed at random from the sleeves and the remaining pods were measured and seeds within them counted.

Beetles were field collected from *C. scoparius* plants the day before the tests were set up and were held in Petri dishes with bee pollen and cotton rolls soaked in honey water for 24 hours. Ten beetles (five male and five female) were confined to each of three replicates of each treatment, within 300 mm diameter by 500 mm long, 1 mm-mesh sleeve cages; each replicate was set up on a different plant.

The initial experiment design was a 8 (plant species) × 3 (resource levels) factorial but because the beetles only laid eggs on three plant species the design was reduced to a 3 (plant species) × 3 (resource levels) factorial for the analysis. Treatments for resource level 1 consisted of three sleeved branches of each test plant species, each paired with three sleeved branches of *C. scoparius* (control), each with two pods per sleeve. Treatments for resource level 2 consisted of three sleeved branches of each test plant species, paired with three sleeved branches of *C. scoparius* (control), each with five pods per sleeve. Treatments for resource level 3 consisted of three sleeved plants of each test plant species, paired with three sleeved branches of *C. scoparius* (control), each with ten pods per sleeve.

Bee pollen was added to each sleeve cage every seven days. After four weeks the beetles were removed from the sleeves and the numbers of eggs were counted. The plants were then re-sleeved and the pods allowed to mature. On maturation of the pods, all the plants were re-examined, pods were harvested and taken to the laboratory where

emerging adults were counted. Tests were conducted in the field between October and January, when pods were available in the field and judged to be almost of the correct phenological condition. Plant species were not all tested at the same time due to differences in phenology between species.

2.3.2 Field trial

The aim of this investigation was to determine the host range of *B. villosus* in the field in New Zealand.

In March 2000 an existing stand of *C. scoparius* (six-eight years old), comprising 30 rows of seven plants in a 17 × 100 m plot, was modified by removing some plants and replanting the cleared area with new plants. *Cytisus scoparius* plants around the perimeter of the plot were left in place and, within this perimeter, four trial blocks were planted. Each block consisted of three rows of test plants (six plants per row, with 2 m between plants) and two rows of existing *C. scoparius* plants (five plants per row, with 2.3 m between plants). Two rows of existing *C. scoparius* plants (five plants per row, with 2.3 m between plants) separated each block and one row of existing *C. scoparius* plants (five plants per row, with 2.3 m between plants) separated blocks one and four from the perimeter row (Figure 2.1). The six test plant species used in the trial; *C. scoparius*, *C. multiflorus*, *S. junceum*, *Ch. prolifer*, *Cl. puniceus* and *G. monspessulana* were all one year old.

The existing stand of *C. scoparius* acted as a source of a field population of *B. villosus*. In the summer of 2002/2003 300 pods per plant of all test species in the trial were harvested and the numbers of emerging adult *B. villosus* were recorded. A method discussed by Sedcole (1977) was used to calculate the sample size required to detect at least one individual at a theoretical infestation rate of 1% with 95% confidence. The formula $n \geq \log(1-p)/\log(1-q)$, where p is the probability (0.95) of detecting a single pod that is infested and q is the theoretical infestation rate (0.01) per plant. Using the Sedcole (1977) method it was determined that a sample size of approximately 300 pods per plant were required for dissection. Theoretical infestation rates less than 1% would be virtually undetectable, requiring much larger numbers of pods to be dissected which was beyond the scope of this study. In cases where plants bore fewer than 300 pods, all available pods were collected. Three hundred pods from 12 randomly selected plants of old *C. scoparius* in the perimeter of the field trial and *So. microphylla* in the area

surrounding the field trial were also dissected. *Sophora microphylla* was excluded from being planted in the trial due to its extended time to maturity in relation to the other plants. During dissection, pods were examined for the presence of eggs.

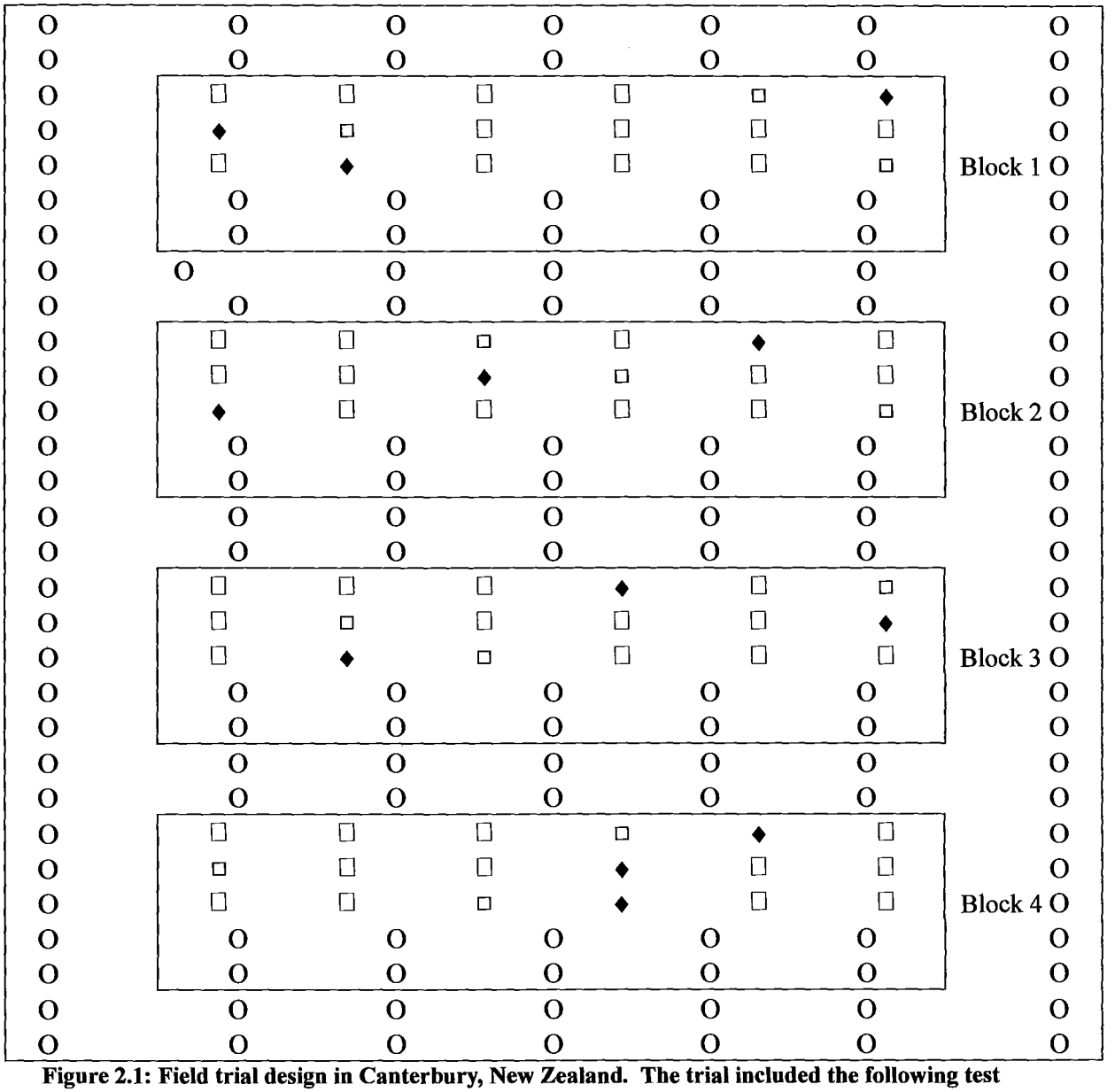


Figure 2.1: Field trial design in Canterbury, New Zealand. The trial included the following test plants: O - Existing *Cytisus scoparius* (Old), □ - *Cytisus scoparius* (Young), □ - *Cytisus-multiflorus*, □ - *Spartium junceum*, □ - *Chamaecytisus prolifer*, ♦ - *Clianthus puniceus*, □ - *Genista monspessulana*.

2.4 Analysis

All data was tested for normality using the Kolmogorov-Smirnov test and homogeneity of variances using Levene's test. A 3 × 3 factorial (3 plant species × 3 resource levels) ANOVA was used to determine the effect of plant species and pod density on number of eggs laid in no-choice tests by *B. villosus*. The control plant *C. scoparius* was included as a comparative control and was removed from the analysis of variance of the test plants. A separate ANOVA was carried out on the control treatments. ANOVA was also used to determine differences in the number of seeds per pod and pod length between each plant

species tested. Fisher's LSD (Zar 1984) was used for comparisons between individual means.

A generalised linear regression model for an unbalanced design was used to analyse the field trial data. All statistical analysis was done using GenStat (2002).

2.5 Results

2.5.1 No-choice tests on sleeved branches

Only three of the eight test plant species (*Ch. prolifer*, *C. multiflorus* and *G. monspessulana*) were oviposited on by *B. villosus* in the no-choice tests on sleeved branches. No eggs were laid on five species, *L. anagyroides*, *Lu. arboreus*, *S. junceum*, *So. microphylla* and *So. prostrata*. Overall, a grand mean of 14.68 eggs per adult per pod were laid.

In the reduced analysis there was a significant interaction between the mean number of eggs laid on the three test plants (that received eggs) and number of pods ($F = 105.1$, $df = 2$, $P < 0.001$). A significantly higher average number of eggs were laid on *Ch. prolifer* at all pod densities (two, five and ten) than on *C. multiflorus* or *G. monspessulana*. The mean number of eggs laid on *Ch. prolifer* increased significantly when number of pods per sleeve increased, but there was no significant difference between eggs laid at the different densities of pods of the other species (5% LSD, 21.6) (Table 2.1).

The ANOVA on the control treatments showed a significant interaction between the mean number of eggs laid per control sleeve of *C. scoparius* and number of pods ($F = 36.3$, $df = 7$, $P < 0.001$). In all species except *Lu. arboreus* and *S. junceum* mean number of eggs laid on the control increased significantly when a higher number of pods were available (5% LSD, 21.8) (Table 2.1). There was a significant difference in the mean number of eggs laid per *C. scoparius* pod between replicates over time ($F = 75.8$, $df = 2$, $P < 0.001$).

Table 2.1: Mean number (no.) of eggs laid per sleeve (2, 5 or 10 pods) by *Bruchidius villosus* on test plants in no-choice tests on sleeved branches. Controls were set up on *C. scoparius* at the same time.

Test plant species	Mean no. of eggs laid per sleeve on each test plant and control (*C. scoparius*) containing					
	2 pods		5 pods		10 pods	
	Test plant	Control	Test plant	Control	Test plant	Control
Ch. prolifer	29.3 b	32.7 b	62.3 b	90.7 b	168.3 b	206.7 cd
C. multiflorus	4.7 a	29.7 b	14.3 a	91.0 b	23.0 a	214.3 d
G. monspessulana	2.3 a	23.7 b	12.3 a	105.7 b	15.7 a	294.7 e
L. anagyroides	0	38.3 b	0	107.7 b	0	209.0 c
Lu. arboreus	0	3.7 a	0	3.7 a	0	3.7 a
S. junceum	0	1.7 a	0	2.3 a	0	7.7 a
So. microphylla	0	31.7 b	0	97. 0 b	0	224.0 d
So. prostrata	0	36.7 b	0	94.0 b	0	189.7 bc

Values with the same letter within columns are not significantly different (test plant 5% LSD, 21.6, control 5% LSD, 21.8).

There was significant variation in mean number of seeds ($F = 114.1$, $df = 2$, $P < 0.001$) and pod length ($F = 281.4$, $df = 2$, $P < 0.001$) among test plant species. Mean number of seeds ranged from 0 to 7.7 seeds per pod and pod length from 9.6 mm to 38.4 mm (Table 2.2). *Cytisus scoparius* (control) pods on average contained a significantly higher number of seeds and were significantly longer than all test plant species (Table 2.2). Despite these differences correlation analysis showed no clear relationships between these variables and the number of eggs laid in previous work (M. Haines, unpublished data).

Table 2.2: Mean number (no.) of seeds per pod and mean pod length of *Cytisus scoparius* (control) and test plants in no-choice tests on sleeved branches. Measurements were made before experimentation commenced.

Test plant species	Mean no. seeds	Mean pod length (mm)
C. scoparius	9.8 f	44.6 g
Ch. prolifer	7.7 e	37.0 e
C. multiflorus	0.0 a	9.6 a
G. monspessulana	4.3 c	17.8 b
L. anagyroides	4.7 c	38.4 ef
Lu. arboreus	4.5 c	32.9 d
S. junceum	6.7 d	37.4 e
So. microphylla	6.3 d	40.1 f
So. prostrata	3.7 b	27.4 c

Sample size 51 pods per test plant, 408 pods for control
Values with the same letter within columns are not significantly different (seeds 5% LSD, 0.6, pod length 5% LSD, 2.4).

2.5.1.1 Emergence and survival data

A total of 126 eggs were laid on *C. multiflorus* by *B. villosus* but no seeds were infested with larvae as the pods contained no seeds. *Bruchidius villosus* laid 780 eggs on 394 available seeds of *Ch. prolifer*, and adults emerged from 60.9% of the seeds. Beetles laid 91 eggs on 219 available seeds of *G. monspessulana*, and 30.0% of eggs developed through to adults. In the control, *B. villosus* laid 6419 eggs on pods of *C. scoparius*, which contained 4009 seeds, and 77.2% of the eggs successfully developed through to adults. There was no significant difference in adult emergence between the different pod densities (two, five and ten) of *Ch. prolifer* ($F = 0.9$, $df = 9$, $P > 0.05$) and *G. monspessulana* ($F = 1.0$, $df = 9$, $P > 0.05$) and the control *C. scoparius* ($F = 0.9$, $df = 9$, $P > 0.05$).

Overall 74.3% of the beetles on the controls and 57.4% of the beetles on the test plants were alive at the end of the four week period indicating a reasonable level of survival over the duration of the experiment.

2.5.2 Field trial

In the field trial *B. villosus* emerged only from seeds of *C. scoparius* and *Ch. prolifer*. No *B. villosus* emerged from pods of *C. multiflorus*, *G. monspessulana*, *S. junceum* *Cl. Puniceus*, or *So. microphylla* surrounding the trial.

In the field trial there were significant differences in levels of *B. villosus* infestation between plant species (F = 17.1, df = 2, P < 0.001). Old *C. scoparius* had a significantly higher proportion of infested seeds than young *C. scoparius* and *Ch. prolifer* (approximate 5% LSD, 0.1). Estimated infestation on old *C. scoparius* was over two times higher than new *C. scoparius* and over five times higher than *Ch. prolifer* (Table 2.3).

Table 2.3: Estimated mean number of *Bruchidius villosus* per pod (± SEM) and estimated percentage infestation of young and old *Cytisus scoparius* and *Chamaecytisus prolifer* plants in the field in New Zealand. Estimated means are from the GLM analysis.

Plant species	Mean number of seeds per pod	Estimated mean number of *B. villosus* per pod	Estimated percentage of seeds infested with *B. villosus*
C. scoparius (old)	8.9 ± 1.4	5.6 ± 0.04 c	62.5 c
C. scoparius (young)	9.6 ± 1.5	2.7 ± 0.05 b	27.7 b
Ch. prolifer	4.1 ± 1.4	0.9 ± 0.03 a	22.6 a

Values with the same letter within columns are not significantly different (without covariate – approximate 5% LSD, 0.1, seed covariate – approximate 5% LSD, 0.1).

2.6 Discussion

Control sleeves were set up at the same time as test plant sleeves to ensure the reproductive activity of *B. villosus* was equivalent for all plant species, but as a result of differences in plant phenology, test plant sleeves were set up over a ten week period and beetles were collected from the field at the appropriate time for each test plant species. The significant differences between numbers of eggs laid in the control sleeves set up at different times on *C. scoparius* (Table 2.1) are likely to reflect these differences in set-up times in relation to beetle reproductive activity.

Lupinus arboreus and *S. junceum* were the last two test plants in the testing sequence, tested in mid-late January, and it is evident from the significantly lower number of eggs laid on the controls that reproductive activity of *B. villosus* was declining severely by this time. It is also a possibility that some newly emerged unreproductive beetles were inadvertently used in this investigation, which may account for some of the low reproductive activity. From late December to early January in New Zealand, the bulk of the new *B. villosus* population emerges from *C. scoparius* and mixes with the generation from the previous year. These beetles cannot be distinguished, except upon dissection, where a difference in fat body content can be observed (Parnell 1966). Newly emerged beetles require four to six weeks feeding on pollen before they become reproductively active (section 5.3.1.2), so if some were present within the sleeve cages it is unlikely that they would have been mature enough to contribute any eggs at that time.

It appears that, in some seasons at least, *Lu. arboreus* and *S. junceum* could avoid attack from *B. villosus* due to their phenology. In New Zealand both of these plant species bear pods late in the season when reproduction in *B. villosus* is declining. *Lupinus arboreus* (A. Sheppard, unpublished data) and *S. junceum* are hosts to *B. villosus* in Europe (Delobel and Delobel 2003, Jermy and Szentesi 2003). Beetles were collected from pods of *Lu. arboreus* in Canterbury in the summer of 2003/2004 when pods were abundant earlier than usual and *B. villosus* was still reproductively active. It is likely that this scenario could also be possible for *S. junceum* in some years. Phenological synchrony (or temporal distribution) is a factor that can affect field host use (Cullen 1990).

It is not known why *L. anagyroides* is unacceptable to *B. villosus* (this Chapter, Chapter 6 and 7) in New Zealand as it has been reported as a field host in Europe (Zacher 1952b, Frick 1962, Szentesi and Wink 1991, Delobel and Delobel 2003, Jermy and Szentesi 2003). The beetles laid good numbers of eggs in control sleeves set up on *C. scoparius* at the same time as those set up on *L. anagyroides* (Table 2.1), so declining reproductive activity is unlikely to be an adequate explanation for this finding. There is a possibility that the plant species in New Zealand could be genetically different from those tested elsewhere.

Eggs were laid on *C. multiflorus* but no *B. villosus* emerged. No seeds developed within *C. multiflorus* pods, though, so potentially this plant is a host. Lack of seed production could be due to inadequate pollination, as seed does usually set in this species in New Zealand.

The average numbers of eggs laid per sleeve were significantly affected by pod density. There was a trend toward a higher number of eggs when pod density was highest. Redmon et al. (2000) found a significant effect of female density on the proportion of seeds infested with *B. villosus* in a field cage experiment. In that study female densities were 0, 1, 4 and 8 on 8-18 pods per cage. When higher numbers of beetles were caged per pod, the proportion of seed infested with larvae increased by 30%. This investigation used methodology differing to that used by Redmon et al 2000 where pod density was variable and number of females fixed. No difference in infestation levels was found between pod densities for all species from which adults emerged. This may suggest the number of pods per female used was too low for any differences to be detected.

The average number of eggs laid per pod was relatively consistent over all host densities suggesting that the number of eggs laid per pod may be influenced by the amount of resource available (Table 2.1). Females may be able to detect the presence of eggs oviposited by other females, and oviposition decisions may be affected by egg-load per pod. Lower numbers of eggs per pod reduce offspring competition. Recognition of potential competitors is profitable for bruchids that develop on discrete resource packages. *Callosobruchus* species (Coleoptera: Bruchidae) have been reported to distribute their eggs uniformly among seeds (Mitchell 1975, Messina and Renwick 1985). They are known to discriminate between seeds that are egg laden or egg free (Messina et al. 1992b) and to avoid oviposition on plants that already bear conspecific eggs or larvae (Messina and Renwick 1985).

Most *Callosobruchus* species lay their eggs directly onto the surfaces of seeds, not pods like *B. villosus*, and the host seeds can usually accommodate more than one larvae, so it is not known whether the selection criteria for oviposition is similar. Levels of survival were recorded when the experiment was completed at four weeks. Exactly how many females were alive and actively laying eggs throughout the investigation period is unknown. This may have affected the number of eggs laid in some instances.

In no-choice tests on sleeved branches conducted in the UK *B. villosus* laid a mean of 0.63 eggs per adult per pod (Syrett and O'Donnell 1987), whereas in the New Zealand tests a grand mean of 14.68 eggs per adult per pod were laid (section 2.5.1). Both tests were run for a duration of four weeks, so this suggests that the New Zealand beetles may have either been more fecund than UK beetles, survival was higher or the pods presented to them may have been more suitable for oviposition than those presented in the UK.

In the field trial, the average number of *B. villosus* beetles emerging from *C. scoparius* pods was almost two (young bushes) and five (old bushes) times higher than those emerging from *Ch. prolifer*. *Bruchidius villosus* infestation levels were significantly higher on the old *C. scoparius*, more than double that observed on young *C. scoparius* or *Ch. prolifer* (Table 2.3). Another way to compare infestation levels in the test plants (of the same age) would have been to remove the old *C. scoparius* plants during summer leaving the established beetle population behind. Set-design experiments, which incorporate equal numbers of target and non-target plants are less likely to produce

false negative results than experiments in which a relatively small number of test plants are interspersed in a natural population of the target weed (Briese 1999).

However it was decided here that it was preferable not to remove the old *C. scoparius* plants from the trial for two reasons. Firstly, by removing the old *C. scoparius* plants the food source for the *B. villosus* population would be removed along with potential oviposition sites. If this abundant source of pollen was removed it was unknown whether the pressure to feed and oviposit on the test plants in the trial would in fact increase or not. There was a possibility that upon removal of the most abundant food source the beetles could migrate to other abundant pollen sources in nearby areas outside the trial resulting in the non-targets within the field trial being exposed to less pressure.

In the summer of 1999/2000, 90% of seed at the site was destroyed by *B. villosus* and pods on average carried in excess of two eggs per seed (M. Haines, unpublished data). On planting the trial 60 established *C. scoparius* plants were removed, so the pressure on the test plants was expected to increase as a result of this, coupled with the fact that the pods appeared to be over carrying capacity (had more eggs laid on them than available seeds) in previous seasons. It was concluded that enough pressure would be placed on the test plants within the trial even with the old *C. scoparius* bushes present. Introduction of a no-choice phase to an open-field test, through removal of the target plants, mimics the situation when an agent does destroy its food resource (Marohasy 1998). Here it was decided to conduct no-choice field tests by confining beetles to test plants growing in the field using sleeve cages.

In the no-choice tests on sleeved branches *B. villosus* laid eggs on four plant species (*Ch. prolifer*, *C. multiflorus*, *C. scoparius* and *G. monspessulana*) and adults emerged from three of these (section 2.5.1). In the open field trial *B. villosus* laid eggs on, and adults emerged from, two plant species (*C. scoparius* and *Ch. prolifer*) (section 2.5.2). The no-choice tests showed that *G. monspessulana* is within the fundamental host range of *B. villosus* even though it was not a host in the simulated realised range of the field trial. This is likely to be because *B. villosus* is more deprived in a no-choice situation and therefore accepts lower ranked hosts.

A variety of factors could influence field host use of potential host plants by *B. villosus*, for example host abundance and phenological synchrony. In this study if the preferred hosts *C. scoparius* and *Ch. prolifer* are absent in a stand, then *G. monspessulana*

and *C. multiflorus* may be accepted as hosts in New Zealand. In Europe, in certain geographic areas there is only one suitable plant species available (M. Haines, personal observation). In southern France and northern Spain the natural vegetation of Genisteae is predominantly *S. junceum* and *G. monspessulana* respectively so these plant species are the hosts in these areas (M. Haines, personal observation). It also seems likely that in some seasons when pod availability and oviposition period are synchronised better, *Lu. arboreus* and *S. junceum* could be utilised as hosts in New Zealand.

It has been suggested by Fowler et al (2000) that the use of *Ch. prolifer* by *B. villosus* in the field in New Zealand may be due to seasonal asynchrony. This remains a possibility because *Ch. prolifer* sets flowers and pods before *C. scoparius* so feeding and oviposition by *B. villosus* commences earlier on this host. Differences in commencement of egg laying by *B. villosus* between seasons has been observed. In two consecutive seasons *Ch. prolifer* flowered up to a month earlier than *C. scoparius*. In one season egg laying began on *Ch. prolifer* earlier than on *C. scoparius* and in the other season both hosts received eggs at the same time (M. Haines, unpublished data). Phenological asynchrony was found to be playing a significant role in the non-target impact of *Rhinocyllus conicus* and *Larinus planus* (F.) on native American thistles (Louda et al. 2003a).

As a result of these experiments it does not appear likely that the UK and New Zealand populations of *B. villosus* have different preferences from their European counterparts as a result of isolation. This is backed up with data from choice tests conducted in the laboratory (section 7.5). From these findings it appears that *B. villosus* could potentially utilise hosts other than *Ch. prolifer* and *C. scoparius* in the field, ie. *C. multiflorus* (when it sets seed), *G. monspessulana* (when preferred hosts are absent or not abundant), *S. junceum* and *Lu. arboreus* (in seasons when oviposition and pod availability are well synchronised). These results correspond well with results obtained from the south of France where adult *B. villosus* emerged from the following species, which were common to both trials *C. scoparius*, *G. monspessulana*, *S. junceum* and *Ch. prolifer* (Haines et al. 2004).

2.7 Conclusion

As a result of the no-choice tests on sleeved branches, it was concluded that three or four plant species are likely to be hosts to *B. villosus* in New Zealand, *C. scoparius*,

Ch. prolifer and *G. monspessulana* and *C. multiflorus* (when it bears seeds). In the field trial *B. villosus* utilised both *C. scoparius* and *Ch. prolifer* which supports previous observations. *Cytisus scoparius* was found to be utilised more than *Ch. prolifer*. New Zealand native plants are unlikely to be hosts in the field, no eggs were laid on native species in either test. It seems possible that *S. junceum* and *Lu. arboreus* may escape attack by *B. villosus* in some seasons due to asynchrony between the beetle oviposition period and plant phenology. This study indicates that *L. anagyroides* is not likely to be a suitable host, but it is unclear why eggs were not laid on pods of this species which is a reported field host in Europe (Zacher 1952b, Frick 1962, Szentesi and Wink 1991, Delobel and Delobel 2003, Jermy and Szentesi 2003).

Linking page: Chapter 3

Chapter 2 showed that at least three or four plant species are likely hosts of *Bruchidius villosus* in New Zealand thus ruling out the suggestion that *B. villosus* was significantly different (phenotypically) from the European population, or had changed since its introduction to New Zealand. Consequently a genetic analysis was conducted to determine if *B. villosus* is one species with a wide host range or a group of sibling species with narrower host ranges. The hypothesis for Chapter 3 was that if *B. villosus* is a group of sibling species with different host plant ranges, this could explain the failure of the original tests to predict *Chamaecytisus prolifer* as a host and inconsistencies in the field host records.

Chapter 3: Is *Bruchidius villosus* a single species or a complex of sibling species?

3.1 Abstract

Historical and more recent records on the host range of *Bruchidius villosus* are inconsistent. A study of mitochrondrial DNA (COI) was conducted on *B. villosus* to determine whether it is a single species that has a broad host range within the Genisteae, or if the nominal species is composed of distinct genetic sub groups with narrower host ranges. *Bruchidius villosus* specimens were collected from eight countries and five plant species. Results showed a low level of sequence polymorphism suggesting that *B. villosus* is one single species with a broader host range than was predicted by some studies, and that there is no genetic variation associated with either host plant or geographical location. Further research is necessary to establish the reasons why the host rearing records are inconsistent.

3.2 Introduction

Bruchidius villosus, commonly known as the broom seed beetle, is native to the UK and Europe but has been introduced for biological control of *Cytisus scoparius* in North America, New Zealand and Australia. Much confusion surrounds the host range of this insect as there are many inconsistencies in the historical rearing and oviposition records. Some studies report that the host range of *B. villosus* includes many different pod-forming plants throughout the tribe Genisteae (Frick 1962, Bottimer 1968, Szentesi and Wink 1991, Hosking 1992, Syrett and Emberson 1997, Isaacson and Markin 1998, Syrett 1999), yet others suggest that it is more or less host specific to *C. scoparius* (Waloff 1968, O'Donnell and Manfield 1986, Syrett and O'Donnell 1987, Isaacson and Markin 1998).

There has also been uncertainty regarding the identity of *B. villosus* (Fabricus) (Zacher 1952a, 1952b), which has been referred to as *Bruchidius ater* (Marsham) (Southgate 1963, Parnell 1964, Parnell 1966, Bottimer 1968), *Bruchidius fasciatus* (Oliver) (Hoffmann 1945, Frick 1962), *Sparteus villosus* (Fabricus) (Frick 1962), and *Bruchidius cisti* (Paykel) (Zacher 1952a). It now appears that all of these names refer to the species currently known as *B. villosus*.

In studies carried out in the 1950s *B. villosus* was recorded on several different hosts within the Genisteae (Zacher 1952a, 1952b) and in the 1960s (then referred to as *B. ater*) was recorded attacking *C. scoparius* in the field in the northeastern USA and was also reportedly reared from 12 field hosts (all in the Genisteae) (Frick 1962, Bottimer 1968). Another two hosts (within the Genisteae) had eggs laid on them in simplified laboratory assays (Frick 1962). *Bruchidius villosus* was also studied in its native range in the UK where it was reported to be damaging to the seed crop of *C. scoparius* and to be host specific to this plant (Parnell 1966, Waloff 1968).

In 1985 host specificity tests were conducted in both the UK and in quarantine in New Zealand, as *B. villosus* was seen as a potentially useful biological control agent against *C. scoparius*, which is a widespread weed in New Zealand. These tests seemed to confirm that it was host specific to *C. scoparius* and as a result *B. villosus* was released into New Zealand (Syrett and O'Donnell 1987). Additional testing in Australia in 1994 came to the same conclusion and *B. villosus* was released there in 1995 (Hosking et al. 1996). However, recent field records from data collected in New Zealand and Europe (M. Haines, unpublished data, A. Sheppard, unpublished data) show that beetles identified as *B. villosus* are in fact utilising other plants in the Genisteae.

Field observations and initial data gathering in 1999 (M Haines, unpublished data) and in 2000 (Wittenberg and Thomann 2001) have suggested there is phenotypic plasticity in body size and colour of *B. villosus* depending on the host plant seed in which they develop. Adults emerging from seeds of *Spartium junceum* and *Chamaecytisus prolifer* may be larger and sometimes more gold or brown in colour than those emerging from *Cytisus scoparius* which are smaller and blacker in colour.

This study aimed to measure the genetic variability of *B. villosus* to determine whether the beetle is in fact a single species with a broad host range, or whether there are distinct genetic entities within the nominal species that use different hosts.

As a first step a 'species' level investigation was carried out to determine whether or not there were cryptic species within the usual concept of *B. villosus*. The mitochondrial DNA gene region cytochrome oxydase subunit 1 (COI) was used for this characterisation as it has been a useful marker in previous studies of inter- and intra-species variation (Caterino et al. 2000). The association of *B. villosus* and the

various host plants were mapped as a result of the genetic characterisation and the findings and implications are discussed.

3.3 Methods

3.3.1 Insect material

Insects were collected from different locations and host plants to represent as much geographic and host variation as possible (Table 3.1). Beetles (all identified as *B. villosus* based on morphology) were collected from *C. scoparius* in the south of France, England, Germany, Canada, USA and New Zealand and from *Genista monspessulana* in Northern Spain. Additional samples from *S. junceum* in southern France, *Laburnum anagyroides* in Hungary and *Ch. prolifer* in New Zealand were also analysed to assess genetic variability associated with host plant and geographic range. Specimens of *Bruchidius seminarius* (F.) (collected from *Coronilla glauca*) and from *C. emerus* (L.) in southern France and *Bruchidius lividimanus* (F.) (collected from *G. monspessulana* in Northern Spain), both recognised as distinct species, were also examined. Finally, sequences of other chrysomelid species were extracted from GenBank and used as outgroups to polarise substitutions along the phylogenetic tree of *Bruchidius* genetic entities (Table 3.1).

Table 3.1: Collections of *Bruchidius* used for molecular characterisation (COI) in this investigation.

No.	Taxon	Host Plant	Country	Date	GenBank Accession
1	*B. villosus*	*L. anagyroides*	Hungary	08/01	DQ351960
2	*B. villosus*	*G. monspessulana*	Spain	06/01	DQ351969
3	*B. villosus*	*G. monspessulana*	Spain	06/01	DQ351967
4	*B. villosus*	*Ch. prolifer*	New Zealand	12/01	DQ351959
5	*B. villosus*	*C. scoparius*	New Zealand	11/01	DQ351962
6	*B. villosus*	*C. scoparius*	Germany	06/01	DQ351963
7	*B. villosus*	*C. scoparius*	England	08/01	DQ351965
8	*B. villosus*	*C. scoparius*	France	07/01	DQ351968
9	*B. villosus*	*C. scoparius*	France	07/01	DQ351964
10	*B. villosus* (black)	*S. junceum*	France	07/01	DQ351958
11	*B. villosus* (gold)	*S. junceum*	France	07/01	DQ351970
12	*B. villosus*	*C. scoparius*	Canada (B.C.)	08/03	DQ351966
13	*B. villosus*	*C. scoparius*	USA (Oregon)	08/03	DQ351961
1	*B. lividimanus*	*G. monspessulana*	Spain	06/01	DQ351971
2	*B. lividimanus*	*G. monspessulana*	Spain	06/01	DQ351972
1	*B. seminarius*	*C. glauca*	France	07/01	DQ351973
2	*B. seminarius*	*C. emerus*	France	07/01	DQ351974
	Diabrotica	-	-	-	AF278547
	Acalymma	-	-	-	AF278542

3.3.2 Laboratory procedures

For the extraction of total DNA, a modified CTAB (hexadecyltrimethylammonium bromide) (Doyle & Doyle 1987) procedure was used. The individuals were homogenized

in 500µl of buffer, containing Tris 100mM, pH8.0, EDTA 20mM, NaCl 0.7M, CTAB 2%, 1% PVP 360w/v and 0.2% 2-mercaptoethanol. For digestion, 10µl Proteinase K ($10mg.\mu l^{-1}$) was added and the samples were kept in a water bath at 37°C for one hour. The samples were then purified using chloroform-isoamylic alcohol protein elimination, followed by a series of phenol-chloroform and chloroform wash steps. DNA was precipitated by adding 1/10 volume of NaAc 3M and 3 volumes of cold Ethanol (–20°C). The samples were then kept at –20°C for about one hour, followed by a centrifugation at 10,000 rpm for 15 min. The DNA was subsequently vacuum-dried and stabilised in ultra pure water.

For the PCR reaction, a mix of 2µl DNA extract, 0.5µM of each primer, 0.4µM dNTP, 1 unit of Taq polymerase and 1X Taq buffer was prepared. PCR amplifications were done with a Hybaid PCR Express device. Cycling parameters were: 5 cycles of 94°C denaturation (1 min), 48°C annealing (1 min), 62°C extension (1 min), followed by 35 cycles of 94°C denaturation (1 min), 52°C annealing (1 min), 62°C extension (1 min) with an initial denaturation of 2 min and a final extension of 5 min. Primers used for COI amplification were C1-J-2183 and TL2-N-3014 as defined in Simon et al. (1994). From each population under investigation, one or two individuals were randomly chosen and from each approximately 800 bp of COI were amplified using the above described primers. The amplified segments were sequenced with an automated sequencer (Genome Express SA).

3.3.3 Data analysis

The sequences obtained were first checked by eye then aligned using the software ClustalX (Thompson et al. 1997). The sequences were then individually verified for protein coding frame-shifts to avoid pseudogenes (Zhang and Hewitt 1996) and aligned. Mega 2.1 (Kumar et al. 1993) was used to estimate all the statistics relative to the sequences (base composition, nucleotide diversity, distance within and between groups and coefficient of differentiation) and the neighbour-joining clustering algorithm was applied (Saitou and Nei 1987) on a Jukes-Cantor distance to account for multiple substitutions. The bootstrap test (1000 replicates) was used to evaluate the reliability of the topology obtained (Felsenstein 1985).

3.4 Results

A total of 475 base pairs was available for all taxa and provided 119 variable sites in the data set, 91 of which were parsimony-informative. No bias in base composition was observed (chi-square test with $\alpha = 5\%$ $P = 0.014$), while all sequences were A-T rich, which is typical in insects (Lunt et al. 1996). The distance, based on percentage of divergence between pairs of sequences computed among putative *B. villosus* individuals ranged from null (100% identity between two individuals found on *G. monspessulana* (Spain) and *C. scoparius* (France), *L. anagyroides* (Hungary) and *C. scoparius* (USA), and *C. scoparius* (England and Canada)) to 0.008 (three mutations) with nucleotide diversity (Nei 1987) $\pi = 0.004 \pm 0.002$ SEM. These data are compatible with intra-specific variation in Coleoptera and other insects (Simon et al. 1994, Hebert et al. 2003). Further, calculation of the mean percentage of divergence between specimens of *B. villosus* and representatives of *B. lividimanus* ($D = 0.08 \pm 0.01$ SEM) and *B. seminarius* ($D = 0.13 \pm 0.02$ SEM) clearly indicate differentiation of the three recognised entities based on this mtDNA marker (coefficient of differentiation $D = 0.97 \pm 0.01$ SEM).

The tree reconstruction based on Neighbour-joining shows that *B. villosus* is a monophyletic clade strongly supported by bootstrap proportions (BP = 100), with *B. lividimanus* falling as a sister group (BP = 99) (Figure 3.1). The tree was rooted using sequences of two other chrysomelids (*Diabrotica* and *Acalymma*). Within *B. villosus* there is no significant trend to cluster sequences according to host plants or geographic locations, except perhaps for specimens found on *S. junceum* that cluster together with moderate support (BP = 68) (Figure 3.1, No. 10 and 11).

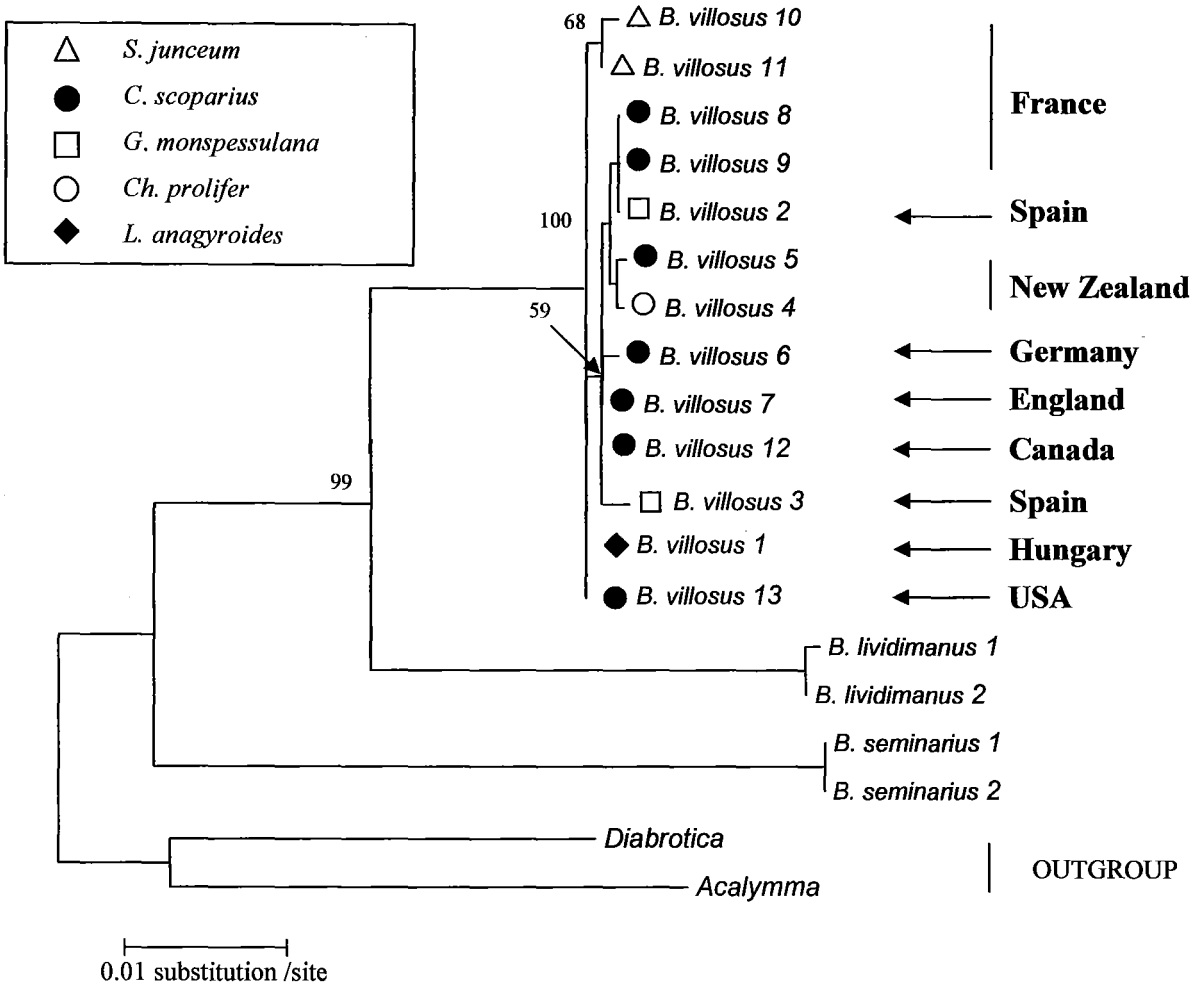


Figure 3.1: Neighbour-joining phylogram for *Bruchidius villosus* reared from various host plants from different locations. Number of changes includes all nucleotide mutations. Numbers following species names refer to collections listed in Table 3.1.

3.5 Discussion

Bruchidius villosus from *S. junceum* which cluster together with moderate support (BP = 68) (Figure 3.1, No. 10 and 11) could indicate current phenological separation of the host plant population represented by these individuals but further testing should be done at the population level, which is outside the scope of this study. There is evidence that *B. villosus* is a mobile species (L. Hayes, unpublished data) and with suitable hosts available throughout its range in Europe (Delobel and Delobel 2003, Jermy and Szentesi 2003, Haines et al. 2004), distinct geographical forms are highly unlikely to occur.

The most important feature in the context of this study is that there is no significant differentiation between any of the sequences of individuals recognised as *B. villosus* (section 3.4). Therefore, the species rank cannot be challenged (on the basis of these sequence data) even though the morph found on *S. junceum* appears as a separate

cluster among other *B. villosus* samples. However, very few characters (mutations) support this grouping as the selected marker focuses on species level information. The significance of host races of beetles amongst plants could be examined using more variable genes or DNA fingerprinting techniques.

Developmental polyphenisms occur when phenotypic variation (or plasticity) is produced by differences in environmental conditions rather than by differences in genetic constitution. For example differences in colour forms of *Nemoria arizonaria* (Grote) (Geometridae) caterpillars occur as a result of larval diet alone (Greene 1996, 1999), diet alters horn length-body size allometry in the beetle *Onthophagus acuminatus* Har. (Scarabaeidae) (Emlen 1994), and differences in body weight of the beetle *Stator limbatus* (Horn) (Bruchidae) are affected by the size of the seed from which individuals emerge (larger seeds produce beetles larger than those developing in smaller seeds) (Fox 1997). Larval diet may therefore explain the small differences in phenotype observed between individuals of *B. villosus* reared from different host seed.

3.6 Conclusion

In conclusion, *B. villosus* is a single species with a broader host range than was predicted by a number of studies. The reason for the discrepancies in these latter studies is unclear and requires further research. The information reported here now allows us to investigate other possibilities with confidence that we are in fact dealing with a single species and not a collection of distinct genetic entities.

Linking page: Chapter 4

In Chapter 3 *Bruchidius villosus* was shown to be a single species, not a group of sibling species. This means that *B. villosus* has a wider host range than was predicted by host specificity tests and that *Chamaecytisus prolifer* is within the fundamental and realised host range of *B. villosus* in New Zealand. The hypothesis for Chapter 4 was that since its introduction, *B. villosus* had colonised *Ch. prolifer* in the field because it was a suitable host for larval development and survival compared with *Cytisus scoparius*. To test this hypothesis experiments were conducted in the field and laboratory.

Chapter 4: How do pod stage and seed size affect oviposition and survival of *Bruchidius villosus*?

4.1 Abstract

Bruchidius villosus utilises the exotic species *Chamaecytisus prolifer* as a host in New Zealand as well as its host plant *Cytisus scoparius*, but the extent, and the suitability of *Ch. prolifer* pods for oviposition and beetle development was not known. In a no-choice test on excised pods no significant difference in number of eggs laid was found between green pods containing seeds of four levels of maturity (size categories; 1 mm^2, 2 mm^2, 3-4 mm^2 or 5 mm^2 and larger) of either *C. scoparius* or *Ch. prolifer*. Examination of the phenology of the plants in Canterbury showed that *Ch. prolifer* initiates flowering and pod production four weeks earlier in spring than *C. scoparius*. However, pods of *Ch. prolifer* dehisce approximately two to three weeks after *C. scoparius* in summer. *Bruchidius villosus* oviposited on both species when mean pod length was approximately 35 mm. Eggs hatched 15 days after being laid on both species. Egg to adult survival indicated that *Ch. prolifer* pods are less suitable for development than *C. scoparius* pods. Stage five pods (those which are green and $\geq$ 20 mm in length) were the most suitable phenological stage for *B. villosus* development in both *C. scoparius* and *Ch. prolifer* with adults emerging from 88% and 52% of available seeds respectively. Development from egg to adult emergence was approximately 15 days shorter on *C. scoparius* than *Ch. prolifer*. This clearly suggests that *C. scoparius*, is a more suitable host (faster development and higher survival) for *B. villosus* than *Ch. prolifer*. *Bruchidius villosus* has colonised *Ch. prolifer* because it is a suitable and acceptable field host.

4.2 Introduction

According to the preference – performance hypothesis (Jaenike 1978) oviposition preference should correlate with host suitability for offspring development in insects. Female oviposition choices are critical to survival of offspring, particularly in species in which the larval stage is immobile, because survival is ultimately determined by the oviposition decision of the mother. Not only is the plant species selected for oviposition

important in these cases, but oviposition must be synchronised with the presence of the appropriate plant part on which growth will proceed, for example, pods, flower buds and fruit. Such structures must also be at a suitable stage of development. Larvae of *Bruchidius villosus*, a seed feeding beetle, cannot move from the pod where they were deposited as an egg (Parnell 1966), so their feeding site is selected by the ovipositing female. Therefore the most suitable pod stage for larval development should be the preferred oviposition site.

Many studies have demonstrated the effect of host phenologies on host selection by specialist insects (Holdren and Erlich 1982, Wood 1982), and oviposition preferences for certain plant parts at specific stages of development (Straw 1989, Lalonde and Roitberg 1992, Kouki 1993). Results of a study conducted by Smyth et al (2003a) indicated that phenological stage of plant development had a significant effect on relative oviposition preference and larval performance (Smyth et al. 2003b) of the moth *Crocidolomia pavonana* (F.). The beetle, *Chrysomela scripta* F., exhibits a distinct preference for leaves of a specific developmental age for oviposition (Bingaman and Hart 1993), while the developmental stage of *Ulex europaeus* affected oviposition preference of the seed weevil *Apion ulicis* (Förster) (Hoddle 1991).

Bernays and Chapman (1994) point out that during development plants vary in their suitability for insect use and that temporal limitations in food availability can have important effects on population development. In their chapter on evolution in host range Bernays and Chapman refer to the importance of synchrony of tree-feeding caterpillars with their hosts. Those caterpillars that hatch too early have no food on which to feed and those that hatch too late are faced with sub-optimal feeding material.

Messina et al. (1992a) and Torgerson How et al. (1993) in studies of beetles and flies have clearly shown phenology of host plants affects oviposition success such that if insects are not well synchronised with their host plants host shifts may occur. For these reasons host plant and insect phenologies are important factors in host specificity testing procedures when assessing potential biological control agents (Heard 1995b, Louda 1998).

The flowering phenology of *Cytisus scoparius* was determined by Harman (1999) and preliminary experiments in this present study indicated that stage 5 (soft green pod ≥ 20 mm in length) was the optimal oviposition stage for *B. villosus* for both plant species. The developmental stages for *C. scoparius* are as follows:

1) Green flower bud

2) Bud showing yellow (open)

3) Flower fully open

4) Green pod < 20 mm

5) Green pod ≥ 20 mm

6) Pod partially black

7) Pod completely black

8) Pod dehisced

This classification can also be applied to *Chamaecytisus prolifer*. Harman (1999) stated that female *B. villosus* prefer to oviposit on larger, immature green pods. In the field it was observed that *B. villosus* laid eggs when green pods of *C. scoparius* and *Ch. prolifer* (the alternative host) were ≥ 20 mm in length (stage 5) (Harman 1999). Buds, flowers, black or dehisced pods were never laid on as *B. villosus* cannot survive on these developmental stages (M. Haines, personal observation). Despite the clear preference for green pods ≥ 20 mm, this includes developmental stages that vary from those bearing tiny seeds that are only just visible to the naked eye to pods that bear large seeds that fill the seed chamber and protrude against the pod wall. Clearly, it is important to compare *B. villosus* larval survival on seeds of different sizes and among pods of different development stages at the time of oviposition, between *C. scoparius* and *Ch. prolifer* to determine their suitability.

In this study the effect of seed maturity (approximate sizes: 1 mm^2, 2 mm^2, 3-4 mm^2 or 5 mm^2 and larger at the time of oviposition) on oviposition, and the effect of phenologically defined pod growth stages (4, 5 and 6 at the time of oviposition) of *C. scoparius* and *Ch. prolifer* on larval survival and development in *B. villosus* was examined.

4.3 Methods

4.3.1 Oviposition preference

Green pods at developmental stage five (Harman 1999) containing seeds of four different sizes were collected from *C. scoparius* and *Ch. prolifer* plants in the field. The pods were initially allocated to categories based on the estimated surface area presented by the seeds. Pods were allocated to the following categories based on seed lateral surface area: one: seeds ≈ 1mm^2, two: seeds ≈ 2mm^2 , three: seeds ≈ 3-4mm^2 four: seeds ≈ 5mm^2 or larger. Ten pods containing seeds of each size category were placed on a moistened Oasis® block inside a Perspex® (500 x 500 x 500 mm) cage with a sponge door (365 × 360 × 50 mm), and treatments were replicated three times for both species. Each cage contained pods (40) of a single plant species and four oasis blocks which each contained pods (10) bearing one of the four seed sizes. Ten reproductively active female *B. villosus* were added to each cage and allowed to oviposit for 72 hours. The measures of seed thickness for pods of each category were also used to obtain a seed size for each category.

4.3.2 Larval survival and development

The purpose of the following section was to ensure an even number of eggs per pod stage and host species to directly compare survival and development.

In a field site at Lincoln in September 2001, forty sleeve cages (300 mm diameter by 600 mm long, 1 mm-mesh) were each secured around branches of *C. scoparius* and *Ch. prolifer* with fully open flowers. Sleeves were secured before pod development in order to protect the developing pod from unwanted oviposition by the natural population of *B. villosus*. However, sometimes cages were not sufficiently secure to prevent beetle ingress. When small green pods ≥ 20 mm were present within the sleeve cages the pods were examined for evidence of any unwanted eggs. Those found were removed with tweezers. Sleeves were then checked at three-day intervals until pods of developmental stages 4, 5 and 6 (section 4.2) were present. Pods were re-checked to determine that all pods were free of eggs, and 30 adult *B. villosus* (15 male and 15 female) collected from *C. scoparius* in the field and deprived of oviposition sites by caging in the laboratory with honey water and pollen for 48 hours, were added to each of 10 sleeve cages on each plant species and left to oviposit for 72 hours. The remaining 10 sleeve cages on each species acted as control treatments.

After egg laying the beetles were removed from the sleeve cages and the egg-loads per pod were adjusted down to two eggs per seed (the maximum number permitted). Excess eggs were removed with tweezers. Pods were individually labelled with a tag attached to the stalk. The length of each pod and the number of seeds it contained were recorded in both the treatment and control sleeves. Sleeves were again secured around the branches to ensure no further eggs were laid by *B. villosus*. The eggs were examined after five days and daily thereafter, to ascertain the day of maximal egg hatch (start of egg eclosion). When embryo larvae reach maturity, two distinct black lines are evident through the transparent egg shell. Soon after the appearance of the lines, larvae proceed to burrow through the underside of the egg shell into the pod wall. At this point the burrowing larvae are no longer visible from the outside of the pod, and only an empty egg shell remains on the pod surface (M. Haines, personal observation).

In this study, days to egg eclosion was defined as the number of days between egg laying and larval penetration of the pod wall. Pods were harvested when they turned black (before dehiscence), stored in separate vials for, at most, 120 days and the day of beetle emergence from each seed was recorded. Pod length and width, dry weight, number of seeds, seed weight, length, width and thickness were measured from pods within the control sleeves.

4.4 Analysis

The data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. Seed size from excised pods and other pod measurements, including the number of eggs laid per pod, were analysed using a 2-way factorial ANOVA (Systat 1999). Because the egg count data was not normally distributed egg counts were log transformed before analysis. Comparisons in larval survival and adult emergence between *C. scoparius* and *Ch. prolifer* at different stages of pod development were analysed using two-sample t-tests (Minitab 2000).

4.5 Results

4.5.1 Oviposition preference

4.5.1.1 Seed size

Seed thickness differed significantly between the pod species ($F = 726.8$, $df = 1$, $P < 0.001$) and between each of the four seed size categories of *C. scoparius* and *Ch. prolifer* ($F = 1229.0$, $df = 3$, $P < 0.001$) (Table 4.1).

Table 4.1: Mean seed thickness and number of *Cytisus scoparius* and *Chamaecytisus prolifer* seeds within excised pods presented to *Bruchidius villosus* in a no-choice oviposition test. Thirty pods of each seed size category (1-4) were measured for each plant species.

Pod factors		*C. scoparius*	*Ch. prolifer*
Number of pods		120	120
Number of seeds		1139	995
Mean seed thickness (mm): Category	1	0.3 a	0.8 a
	2	1.3 b	1.6 b
	3	1.7 c	2.3 c
	4	2.0 d	3.1 d

Values with the same letter within columns are not significantly different (seed thickness - 5% LSD, 0.09)

4.5.1.2 Oviposition

Bruchidius villosus females laid a mean of 16.0 and 12.8 eggs each over the four seed size categories on *C. scoparius* and *Ch. prolifer* respectively. On *C. scoparius* one egg was laid by *B. villosus* for every 2.4 seeds available, and on *Ch. prolifer* one egg was laid for every 2.6 seeds available.

There was no significant difference in log mean eggs laid per pod between species ($F = 1.5$, $df = 1$, $P = 0.2$) or seed size category ($F = 2.5$, $df = 3$, $P = 0.06$) (Figure 4.1 and 4.2). However the P value of 0.06 for the difference in mean number of eggs laid per seed size category between species is significantly different at the 10% level. Pods of *C. scoparius* in seed size category 2 (seed surface area 2 mm^2) had the highest mean number of eggs laid on them.

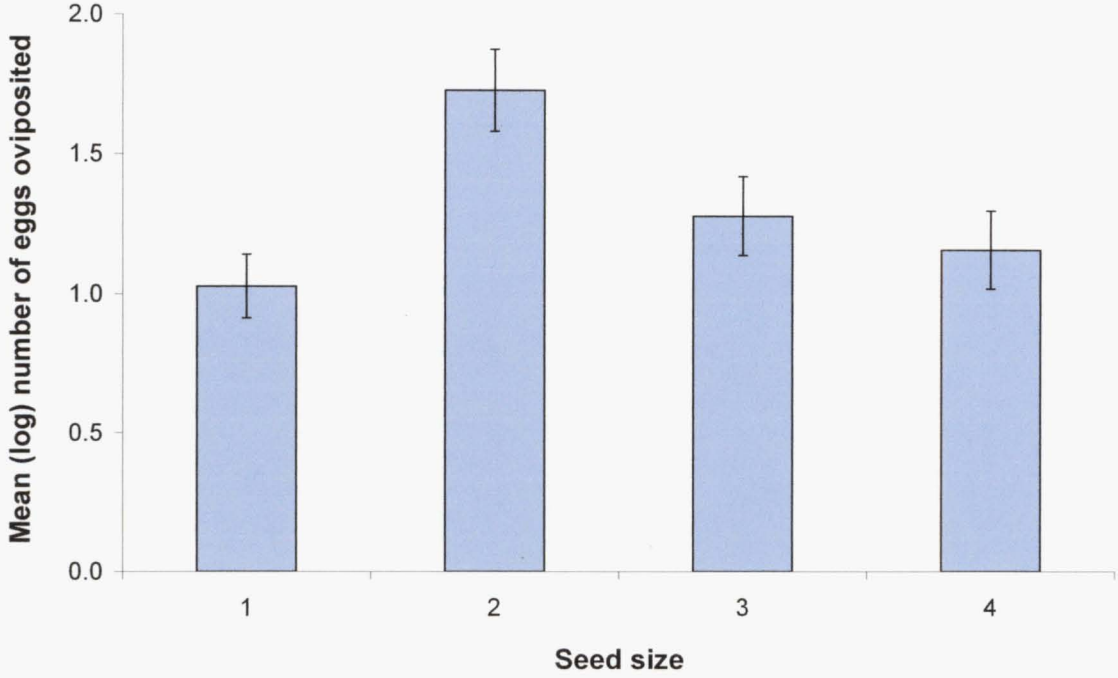


Figure 4.1: Log number of eggs laid by *Bruchidius villosus* (± SEM) in a no-choice test on excised pods of *Cytisus scoparius* grouped into four seed size categories (1-4).

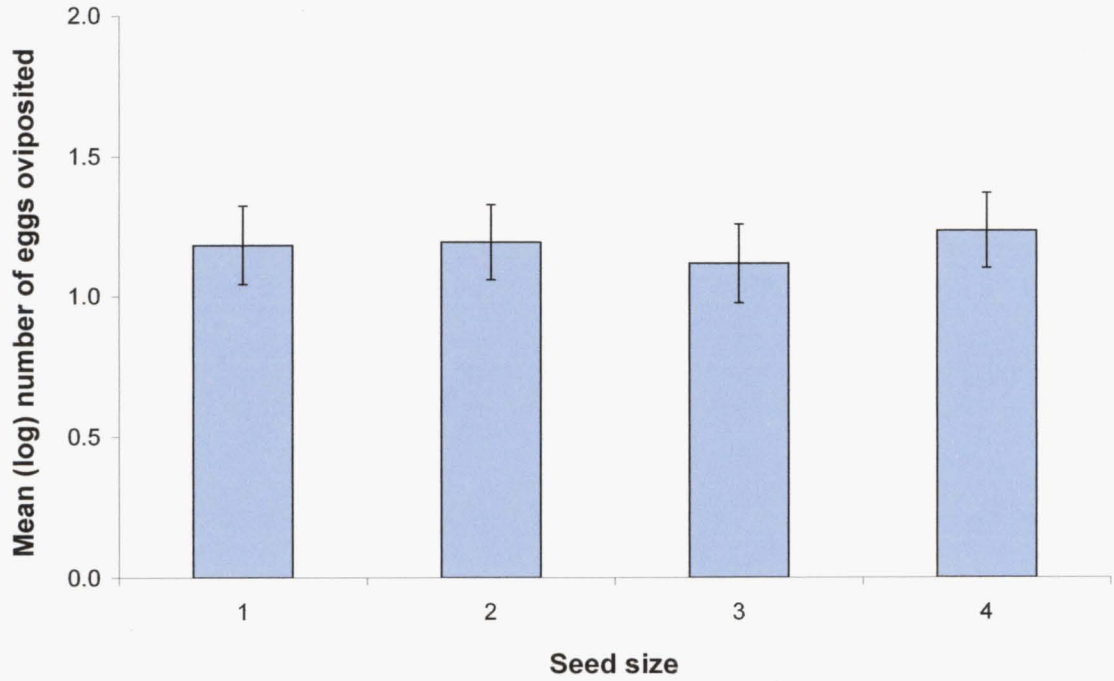


Figure 4.2: Log number of eggs laid by *Bruchidius villosus* (± SEM) in a no-choice test on excised pods of *Chamaecytisus prolifer* grouped into four seed size categories (1-4).

4.5.2 Larval survival and development

4.5.2.1 Host plant comparisons

The mean numbers of seeds per pod varied significantly between the two plant hosts ($t = 3.8$, $df = 111$, $P > 0.05$) but there was no significant difference in pod length at oviposition ($t = 1.4$, $df = 164$, $P > 0.05$) (Table 4.2).

Table 4.2: Mean number of days from oviposition to egg eclosion and to adult emergence of *Bruchidius villosus* (± SEM) from developmental stage 4 and 5 pods of *Cytisus scoparius* and *Chamaecytisus prolifer*.

Oviposition and Pod factors	*C. scoparius* ± SEM	*Ch. prolifer* ± SEM	P value
Mean pod length (mm)	32.5 ± 1.5	35.1 ± 1.2	> 0.05
Mean number of seeds per pod	8.8 ± 0.4	7.1 ± 0.2	< 0.001
Mean days from oviposition to egg eclosion	14.8 ± 0.1	15.1 ± 0.1	< 0.05
Mean days from egg eclosion to adult emergence	57.5 ± 0.7	72.7 ± 0.6	< 0.001
Mean days from oviposition to adult emergence	72.1 ± 0.7	87.9 ± 0.6	< 0.001

Harvested mature pods of *Ch. prolifer* were significantly longer and heavier than those of *C. scoparius* but were not significantly different in width. Seeds of *Ch. prolifer* were also significantly heavier, longer, wider and thicker than those from *C. scoparius*. The average number of seeds per pod was significantly higher in *C. scoparius* compared with *Ch. prolifer*. The average difference in weight between seeds within the same pod was 0.3 µg and 0.5 µg for *C. scoparius* and *Ch. prolifer* respectively. The ranges for all measurements were smallest for *C. scoparius* except for seed number which was equal to that of *Ch. prolifer* (Table 4.3).

Table 4.3: Average pod and seed measurements of harvested mature control pods of *Cytisus scoparius* (± SEM) (n = 462) and *Chamaecytisus prolifer* (± SEM) (n = 558) respectively.

Pod factors	*C. scoparius*	Range interval	*Ch. prolifer*	Range interval	P value
Pod length (mm)	37.2 ± 0.3	29.7	43.0 ± 0.33	39.5	<0.001
Pod width (mm)	8.8 ± 0.1	4.2	8.8 ± 0.01	5.1	>0.05
Pod dry weight (g)	0.14 ± 0.00	0.2	0.3 ± 0.01	0.7	<0.001
Seed number per pod	4.2 ± 0.1	11.0	3.4 ± 0.10	11.0	<0.001
Seed weight (g)	0.01 ± 0.00	0.01	0.03 ± 0.00	2.0	<0.001
Seed length (mm)	3.1 ± 0.02	2.2	4.3 ± 0.04	3.5	<0.001
Seed width (mm)	2.2 ± 0.01	1.8	3.2 ± 0.03	4.0	<0.001
Seed thickness (mm)	1.4 ± 0.01	1.5	2.0 ± 0.02	2.7	<0.001

4.5.2.2 Development of *Bruchidius villosus*

Egg eclosion commenced on *C. scoparius* and *Ch. prolifer* approximately 15 days after eggs were laid. Statistically there was a significant difference between the average number of days to egg eclosion for eggs laid on the two host plant species ($t = 3.0$, $df = 132$, $P < 0.05$) but while this is a statistically significant result it is not biologically

significant. Larvae eclosed after a mean of 14.8 on *C. scoparius* and 15.1 days on *Ch. prolifer* which when rounded equates to 15 days on both species. There was a significant difference in mean number of days from egg eclosion to adult emergence for beetles developing on the two plant species ($t = 16.6$, $df = 106$, $P < 0.001$). Adults reared from *C. scoparius* emerged on average 16 days earlier than those on *Ch. prolifer* (Table 4.2). In 90% of cases on *C. scoparius* and 85% on *Ch. prolifer* respectively, variation in adult emergence within the same pods was no more than four days.

4.5.2.3 The effect of pod stage on development

There were large differences in the number of pods of each developmental stage available for oviposition within the sleeve cages. Large differences in number of eggs laid on and adults emerging from these pods was also observed (Table 4.4).

Table 4.4: Number of eggs laid and number of *Bruchidius villosus* adults emerging from, pods of *Cytisus scoparius* and *Chamaecytisus prolifer* at three different stages of development (4, 5, 6).

Pod developmental stages	4		5		6	
	C. scoparius	*Ch. prolifer*	*C. scoparius*	*Ch. prolifer*	*C. scoparius*	*Ch. prolifer*
Number of eggs	44	46	760	776	56	15
Number of pods	11	10	51	72	8	14
Number of pods laid on	5	5	51	72	8	7
Number of seeds in pods which were laid on	*22	*34	440	513	70	56
Eggs per seed	2	1.35	1.70	1.51	0.8	0.27
Number of mature adults	12	0	388	274	0	0

*Seeds in pods which were not oviposited on were too small to count with a naked eye

Days to emergence varied significantly between pod developmental stage four and five in *C. scoparius* ($F = 4.8$, $df = 1$, $P < 0.05$). *Bruchidius villosus* from stage five pods emerged on average 4.7 days earlier than those from stage four.

At the time of egg removal, egg counts were made, revealing that *B. villosus* oviposited on 50% of *C. scoparius* and 40% of *Ch. prolifer* stage four pods, and 100% and 50% of stage six pods respectively. *Bruchidius villosus* oviposited on 100% of stage five pods of both plant species (Figure 4.3). Fifty-one percent of stage 5 and 45% of stage four *C. scoparius* pods and 20% of stage five *Ch. prolifer* had more than two eggs per seed laid on them, so the excess eggs were removed.

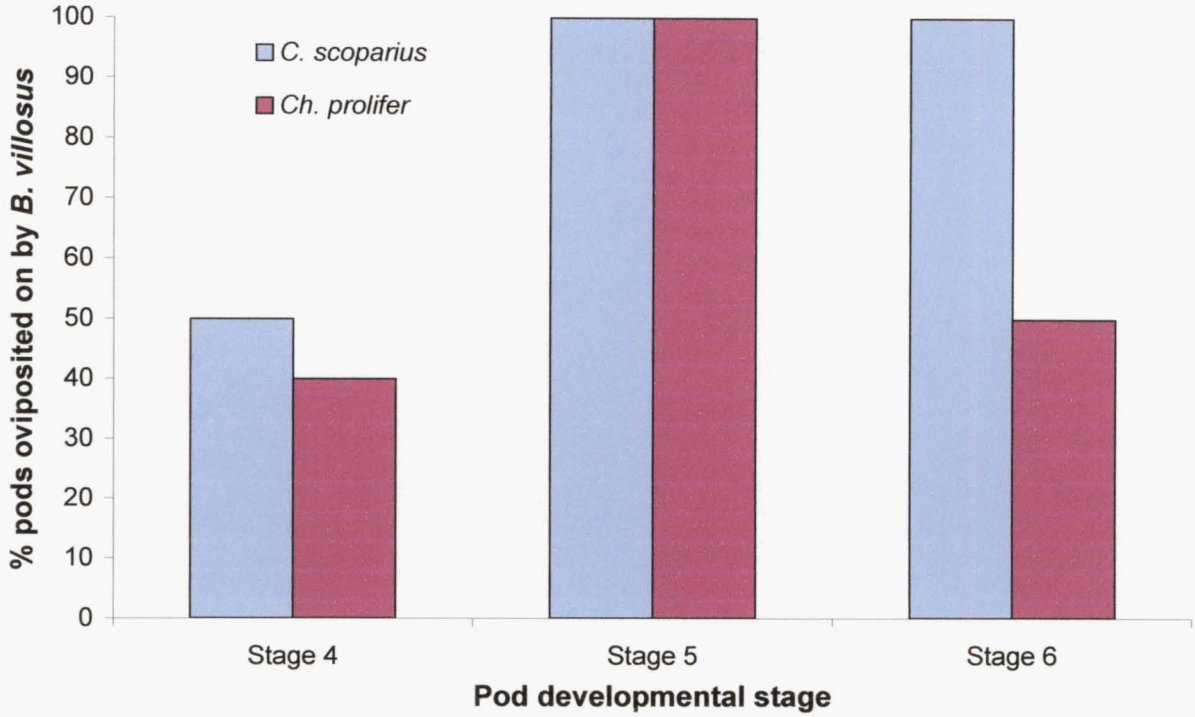


Figure 4.3: Percentage of *Cytisus scoparius* and *Chamaecytisus prolifer* pods at three different stages of development (4, 5, 6) oviposited on by *Bruchidius villosus* in a no-choice test on sleeved branches.

Bruchidius villosus adults emerged from 55% of the total number of *C. scoparius* seeds at developmental stage 4 while no adults emerged from *Ch. prolifer* seeds of this stage. Eggs laid on pod developmental stage 5 resulted in the highest percentage emergence of adults from both plant species, with adults emerging from 88% of total *C. scoparius* seeds and 53% of *Ch. prolifer* seeds. No adults emerged when eggs were laid on pod developmental stage 6 in either plant species (Figure 4.4).

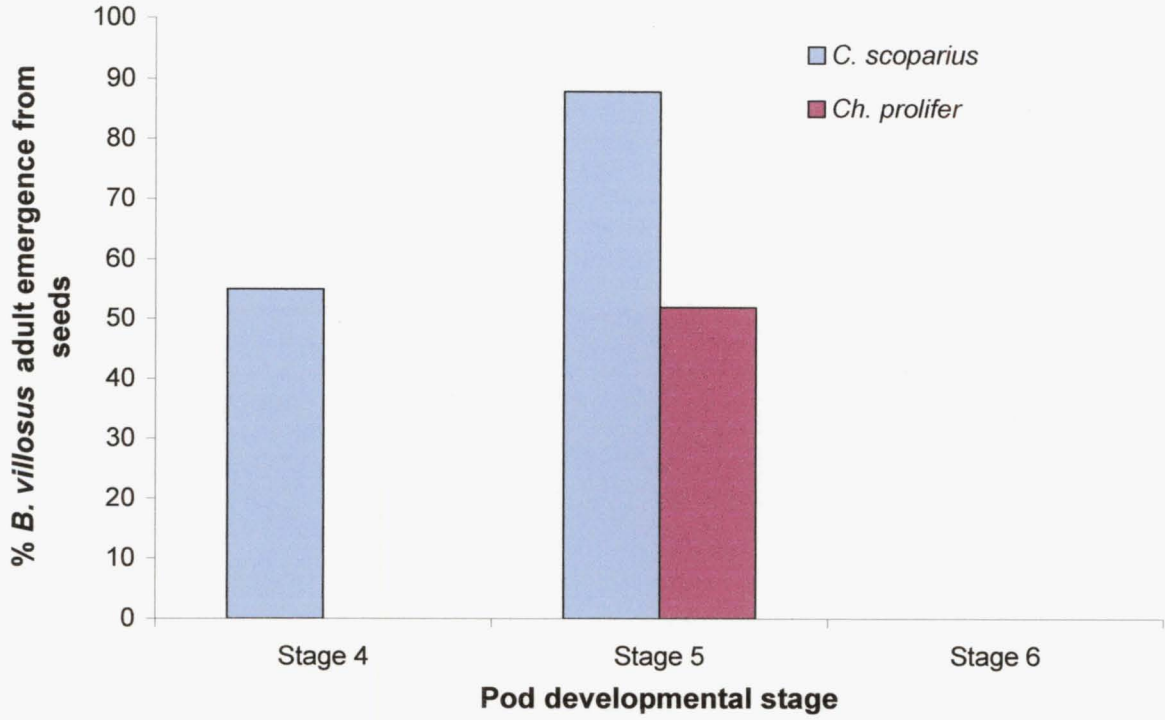


Figure 4.4: Percentage of adult *Bruchidius villosus* emerging from seeds of *Cytisus scoparius* and *Chamaecytisus prolifer* within pods at three different stages of development (4, 5, 6).

A Chi-square test of independence was done on the number of eggs laid by *B. villosus* on each pod developmental stage for each species. The calculated χ^2 values were larger than the tabulated values for both *C. scoparius* (χ^2 =35.2, df = 2, P < 0.01) and *Ch. prolifer* (χ^2 = 41.1, df = 2, P < 0.001) and both species combined (χ^2 = 62.45, df = 2, P < 0.001). The individual χ^2 value for developmental stage 5 was large (more adults emerged) and contributed most to the overall value, so the conclusion is that the beetles survived significantly better on this stage than the others.

4.6 Discussion

4.6.1 Oviposition preference

4.6.1.1 Seed size

Seed thickness varied significantly between *C. scoparius* and *Ch. prolifer* at the time of oviposition (Table 4.1). *Chamaecytisus prolifer* seeds are therefore a significantly larger food source for individual *B. villosus*. In the second part of this investigation (Table 4.3) this finding was supported; it was found that *Ch. prolifer* seeds were significantly bigger in all dimensions than *C. scoparius* seeds at maturity. Because seeds of *Ch. prolifer* were significantly larger overall at the end of development, these results suggest that the seeds

of both species were in fact at an equivalent stage of development despite the differences in seed thickness between the seed size categories.

The mechanism by which beetles assess pod or seed developmental stage is unknown. Egg number was found to be correlated with pod length and seed number in *B. villosus* (Redmon et al. 2000). Studies done on the bruchid, *Callosobruchus maculatus* (F.) suggest that females assess relative mass of seeds better than surface area (Cope and Fox 2003). However, *B. villosus* eggs are laid on seed pods containing immature seeds, whereas *C. maculatus* oviposit directly onto mature, individual seeds so the mechanisms in selection are likely to differ.

4.6.1.2 Oviposition

The results suggest that all seed sizes within stage 5 presented to *B. villosus* were equally acceptable for oviposition (Figure 4.1 and 4.2). However this is only relevant if seed size is the mechanism which initiates oviposition in *B. villosus*. It is unclear whether *B. villosus* can distinguish among seeds of different sizes, but it may be significant that *B. villosus* does seem better able to distinguish between seed size in *C. scoparius* than *Ch. prolifer*. It may however be safer to conclude that any green pod ($\geq$ 20 mm) is suitable for oviposition regardless of seed size.

In this investigation, which was a no-choice test in relation to host species, there was no significant difference between the number of eggs laid between *C. scoparius* and *Ch. prolifer* (section 4.5.1.2). All other investigations in this study were choice tests and significantly more eggs were laid on *C. scoparius* overall in every case (Chapter 5, 6, 7, 8 and 9). This suggests that *C. scoparius* is the preferred host when both plants are presented together, but in the absence of *C. scoparius*, *Ch. prolifer* is equally acceptable. It also needs to be taken into consideration however, that this was the only experiment in which excised pods were used. In all the choice tests cut shoots of the plant material bearing pods were used. It is not known whether these different treatments could affect oviposition in *B. villosus*.

4.6.2 Larval survival and development

4.6.2.1 Development of *Bruchidius villosus*

Numbers of days to egg eclosion did not differ biologically between species, which indicates that developmental conditions were similar between both plant species (Table

4.2). Days to egg eclosion could not be measured on stage 6 *C. scoparius* pods because all pods were completely black by the time the eggs matured, so that it was impossible for larvae to burrow into the pod wall. Egg development in insects is known to be influenced by temperature and photoperiod (Hill and Hodkinson 1996). Seasonal differences are therefore expected to be influential, but if the temperature rises, an increase in both the rate of pod growth, and egg and larval development would be expected, and the effect may be minimal.

Variation in time of adult emergence from seeds within the same pods was minimal, with 90% of the adults from *C. scoparius* and 85% of those from *Ch. prolifer* emerging within four days of each other. Some of this variation includes the length of the oviposition period at the commencement of the investigation. If seed size governs development time, this finding would suggest that the seeds were relatively uniform in size within pods.

Overall, *C. scoparius* seeds produced 32% more adult beetles than *Ch. prolifer* seeds (calculated using values in Table 4.4). This may be partially attributable to the fact that *C. scoparius* pods had a significantly higher mean number of eggs per seed from the outset which may have increased the chances of adult emergence. However only one larva can develop per seed. On the other hand the extended development time in *Ch. prolifer* may have resulted in increased mortality compared with beetles developing in *C. scoparius* seeds.

Development time of *B. villosus* from egg to adult was significantly shorter on *C. scoparius* than *Ch. prolifer* (Table 4.2). Development times are generally shorter on more favourable hosts (van Huis and de Rooy 1998). However, this difference may be explained by the small seed size of *C. scoparius* compared with those of *Ch. prolifer*. Seed weight, length, width and thickness were all significantly higher in *Ch. prolifer* (Table 4.3), which means larvae feeding on seeds of this host have more resource to consume prior to pupation if they do in fact continue to feed until all the seed is consumed, which may lengthen the development process. Beetles reared off *Ch. prolifer* are heavier than those reared from *C. scoparius* (section 9.4.1.1).

Dissection of *Ch. prolifer* seed after adult emergence appears to indicate that *B. villosus* sometimes consume the entire contents (M. Haines, personal observation).

Alternatively the extended development time may indicate that *Ch. prolifer* is a less suitable host (Fox et al. 1996).

In this study it was observed that some small pods 20 mm or less in length produced adults (Table 4.4 and Figure 4.4). This is possible because over 15 days (mean time for egg eclosion) the pods have time to grow to an appropriate length and begin seed fill. However, the likelihood of eggs surviving on these very small young green pods is less than on longer green pods because the small pods have more of a tendency to senesce prematurely (M. Haines, personal observation). Clip cages have been shown to promote senescence symptoms in leaves (Crafts-Bradner and Chu 1999), and if sleeve cages create a similar microclimate for the plant material enclosed within them, the pods may be similarly affected. Under natural conditions, therefore, these small pods may not senesce.

4.6.2.2 Oviposition and pod stage of development

Fewer seeds were counted in stage four pods of *C. scoparius* than in the other stages (Table 4.4). The majority of small pods on which eggs were laid tended to have larger but fewer seeds than the small pods on which no eggs were laid, indicating a difference in pod age. While pods are categorised in the same group by length, there appears to be variation in biological age. A more accurate way to assess pod development may involve taking a measure of age, such as seed thickness and width through the pod wall as well as pod length.

Ovipositing *B. villosus* provide their progeny with a limited food resource. Female *B. villosus* preferentially lay eggs on stage five pods on *C. scoparius* and *Ch. prolifer* and survival from egg to adult was highest on this stage (Table 4.4 and Figure 4.4). At this stage of development the pod wall and seeds are soft and green and easily penetrated by hatching larvae. There is also a large amount of food material available for the hatching larvae which feed inside an individual seed. Pods at this stage also allow adequate time for larvae to develop into adults before the pod dehisces.

Differences in plant phenology resulted in large differences in pod numbers at the different developmental stages. In this experiment, differences between the phenology of the two plant species made it difficult to synchronise pod development. In general *Ch. prolifer* flowers four to six weeks before *C. scoparius* in the field. This means that green pods develop on *Ch. prolifer* much earlier, making it difficult to obtain pods of both species at equivalent stages of development at the same time. In addition, within each

species it was difficult to arrange pods at each developmental stage because plants were required to be sleeved at the end of flowering to avoid contamination by the natural population. To include all three developmental stages from small green pod to partially black pod, (spanning approximately six weeks development time) within the same sleeve was problematic. The alternative would have been to select developed pods from the field and remove the eggs from them prior to commencing the experiment. This would not have only been a time consuming exercise, but also removal of eggs by scraping them off the pods with tweezers can damage the soft green pod walls resulting in bruising of tissue that may affect the suitability of pods for oviposition. It is unknown if epideictic pheromones are used by *B. villosus* to deter conspecific females from laying eggs on the same pod. Clearly, if some form of chemical marking is used, any that remains on the surface of the pod after the egg is removed may alter ovipositional responses.

Van Huis and de Rooy (1998) found that *C. maculatus* reared on pigeonpea (*Cajanus cajan* (L.) Millsp.) exhibited a longer development time and a higher mortality than those reared on cowpea (*Vigna sinensis* (L.) Savi ex Hassk.) and chickpea (*Cicer arietinum* L.). This indicated that pigeonpea was the least favourable host. Fox et al. (1996) found that in the seed beetle *Stator limbatus*, larger seeds produced larger beetles than smaller seeds but there was no detectable effect of seed size on development time. This suggests that the slower development time on *Ch. prolifer* (Table 4.2) may be due to it being a relatively unsuitable host rather than a result of larger seed size. However, Fox (1997) found that in the presence of an environmental food stressor, such as a food shortage (resulting from intense larval competition), *S. limbatus*, emerged sooner and at a smaller size. Fox et al (1994) suggest that differences in development time of *S. limbatus* on two different hosts reflected genetic differences in the ability to utilise the host.

When the pods within the sleeves turned black, they were collected from both plant species and stored in vials in the laboratory at temperatures between 26-30°C during the day and 20-24°C at night. In addition to the consistently high storage temperatures and handling impact (as a result of harvesting), the fact that these pods had recently experienced rain, may have contributed to pods opening prematurely. Mature pods of *C. scoparius* and *Ch. prolifer* tend to open rapidly when they are exposed to moisture and stored in a confined space (M. Haines, personal observation). In this experiment no beetles were found in the pod cavity in dehisced pods. This is unusual as adult beetles are

found in the field waiting within the pod cavity (outside of the seed on which they developed) to be released when the pod dehisces. However (as in this experiment), some pods do dehisce prior to beetle emergence from seeds, and seeds containing beetles fall to the ground. Pod dehiscence is a major factor influencing adult emergence from pods, if pods do not dehsice to release the beetles or seeds it ultimately leads to beetle death. Clearly, beetle development time and pod dehiscence are not always well synchronised. During winter, long after the majority of pods have dehisced and beetles are overwintering, beetles can still be found inside undehisced pods on both *Ch. prolifer* and *C. scoparius* (M. Haines, personal observation).

4.7 Conclusion

Seed size did not appear to influence oviposition decisions in *B. villosus* as all green pods within developmental stage 5 (≥ 20 mm in length) containing four different seed sizes received eggs. *Bruchidius villosus* has a shorter development time and higher rate of survival on *C. scoparius* which indicates that it is a more suitable host plant.

Linking page: Chapter 5

Experiments in Chapter 4 showed that *Cytisus scoparius* is the most suitable host for *Bruchidius villosus*. Development was faster and survival higher than on *Chamaecytisus prolifer*. One explanation why historical tests failed to detect *Ch. prolifer* as a host is that some aspect of the original host specificity testing procedure was at fault. In the original tests it is possible that beetles imported from the UK were pre-conditioned to *C. scoparius*, as they were reared from, and maintained on this species as adults. In addition, *B. villosus* imported to New Zealand did not undergo a natural overwintering period. One hypothesis tested in this chapter was that the original cut-shoot choice tests failed to predict the acceptability of *Ch. prolifer* because *B. villosus* was conditioned to prefer the host from which it was reared as a larva and which provided food (pollen) as an adult. An additional hypothesis tested was that lack of overwintering may have affected the beetles' oviposition preferences.

Chapter 5: Does larval rearing host, pollen type fed by adults or lack of overwintering influence oviposition preference by *Bruchidius villosus* between *Cytisus scoparius* and *Chamaecytisus prolifer*?

5.1 Abstract

Possible causes for a lack of oviposition on lower ranked hosts in the original host specificity tests on cut shoots conducted in 1985 were examined. Adult *Bruchidius villosus* all became ovipositionally mature in spring whether provided with *Cytisus scoparius* flowers, *Chamaecytisus prolifer* flowers, *Genista monspessulana* flowers or bee pollen. However, naive *B. villosus* adults fed bee pollen laid significantly more eggs on both plant species than *B. villosus* adults fed the other pollen types. Lack of overwintering did not significantly affect *B. villosus* oviposition behaviour, so overwintering conditions were unlikely to have caused the false negative results in 1985. *Cytisus scoparius* was the most preferred host for oviposition for both naive and experienced beetles regardless of larval rearing host. Larval rearing host had no significant effect on mean number of eggs laid and therefore is also unlikely to have contributed to the wider host range expressed by beetles now established in New Zealand.

5.2 Introduction

Adult nutrition is an important factor in insect reproduction where it clearly affects egg laying ability and egg fertility as has been demonstrated in such species as *Pieris brassicae* (L.) (Romeis and Wackers 2002). Gilbert (1972) was one of the first to show that nutrients provided by pollen are of major importance to adult maintenance and reproductive activity in *Heliconius* butterflies. Food quality has often been shown to be essential for egg maturation and increased egg production in many insects. Good (1933) showed in a study on the beetle *Tribolium confusum* Jacquelin du Val, that this species

laid an average of 521 eggs when fed on whole wheat flour, 333 on bran, and 187 on white flour. Egg production of the seed-feeding bug *Dysdercus superstitiosus* (F.) was shown to be enhanced when the natural food, cotton seeds, were consumed, compared with other seed types (Geering and Coaker 1961).

Studies that report the effect of host experience on oviposition in insects are abundant. Of significance in the literature is the Hopkin's Selection Principle that proposes the acclimatisation that can occur between larvae and host can affect subsequent adult behaviour (Thorpe 1939, Jermy et al. 1968). However, this theory has now largely been discounted, because despite many studies, there has been little positive evidence that adult behaviour is affected by larval experience (Tabashnik et al. 1981, Jaenike 1982, Papaj and Rausher 1983, Diehl and Bush 1984, Futuyma and Peterson 1985, Prokopy et al. 1988, Via 1991). The influence of early adult experience, that may in fact be of the larval rearing host, on oviposition selection is now more favoured by researchers (Courtney and Kibota 1990 and references therein).

The seed-feeding beetle *Bruchidius villosus* is attracted to *Cytisus scoparius* when it flowers in spring and beetles then become reproductively mature some time after feeding on its pollen (Parnell 1966, Waloff 1968, Harman 1999). In host specificity tests conducted in New Zealand in 1985 *B. villosus* was imported from the UK and arrived in New Zealand in September, where they were fed and put straight into tests without overwintering (Syrett and O'Donnell 1987). Normally, following emergence, *B. villosus* feeds on pollen in the field to build up fat reserves and then proceeds to hibernate during autumn, remaining inactive over winter until the temperatures rise in spring. Mating and oviposition occur soon after adults emerge from hibernation. In the original tests *B. villosus* laid eggs only on *C. scoparius*, no eggs were laid on *Chamaecytisus prolifer* (Syrett and O'Donnell 1987).

In biological control, insects are often transported to countries of reverse seasonality for host specificity testing. In many cases these insects are not exposed to a full overwintering period, and in some cases they are not given an overwintering period at all (Syrett and O'Donnell 1987) due to biological constraints such as providing test plant material for oviposition trials out of season. However, the effect of the absence of an overwintering period on subsequent oviposition behaviour has rarely been studied.

The objective of this study was 1) to establish whether *C. scoparius* pollen is required for the initiation of oviposition in *B. villosus*, or if alternative sources of pollen can also initiate oviposition, 2) to determine the effect of adult food source and larval rearing host on subsequent oviposition behaviour and, 3) to determine whether a period of overwintering is necessary for *B. villosus* to display oviposition behaviour that would be expected under natural field conditions.

5.3 Methods

5.3.1 The effect of adult food source and larval rearing host on oviposition

5.3.1.1 Overwintering

Four hundred *B. villosus* were reared from pods of *C. scoparius* and 400 from pods of *Ch. prolifer* from plants grown out of doors at Lincoln, New Zealand in January 2000. Each group was subsequently overwintered in the laboratory in separate Perspex® cages (500 × 500 × 500 mm) with sponge doors (365 × 360 × 50 mm), according to larval rearing host plant, without access to host plants. The beetles were supplied with cotton rolls soaked in honey water and held for 10-12 weeks at 22-16°C day-night temperatures with a 14:10 h L:D regime. The temperature was gradually lowered to 8-3°C day-night with a 10:14 h L:D regime for 20 weeks to replicate the seasonal changes experienced by beetles in the field. The beetles were gradually exposed to rising temperatures representative of spring temperatures and were maintained at 22-16°C day-night temperatures with a 14:10 h L:D regime. Humidity levels were kept at approximately 70% throughout.

5.3.1.2 Pretreatment

The experiments were started when *C. scoparius*, *Genista monspessulana* and *Ch. prolifer* were beginning to flower in the field in September. The overwintered *B. villosus* from each larval rearing host plant were split into four equal sized groups (four groups of 90 beetles (after accounting for mortality) and placed in eight Perspex® cages, with continual access to freshly-cut shoots of foliage and flowers of one of the following: *C. scoparius*, *G. monspessulana*, *Ch. prolifer* or mini Petri dishes containing bee collected pollen. All pods were removed from the shoots prior to their being introduced to the

cage. After about six weeks of feeding experience by these laboratory-reared beetles, pods of *C. scoparius* and *Ch. prolifer* were beginning to form in the field. A subsample of 20 beetles from the pretreatment which consisted of *B. villosus* reared from *C. scoparius* and presented with *C. scoparius* flowers, was removed from their cage and was given access to fresh pods for 24 hours to check for oviposition activity. Eggs were laid during this period so the entire cohort of beetles was judged to be reproductively active and the choice tests began.

5.3.1.3 Two-way choice tests

Naive beetles

These beetles had undergone overwintering and pretreatment in the laboratory (section 5.3.1) prior to being introduced to these tests. They were oviposition naive, ie. they had not oviposited before. Two shoots from each of *C. scoparius* and *Ch. prolifer*, each shoot bearing five pods of the preferred developmental stage (green, ≥ 20 mm length, Figure 4.3) were placed into water in vials. A vial was placed in each corner of a Perspex® cage systematically. There were three replicate cages each containing ten pairs (five male and five female) of naive beetles for each treatment (Table 5.1). The numbers of eggs laid by the naive beetles on pods of both plant species were counted after 48 hours and the numbers of seeds per pod, as well as the lengths and descriptions of the pods were recorded. At the conclusion beetles were then placed in a holding cage for 24 hours with only the same pollen source they had experienced previously, either *C. scoparius*, *G. monspessulana*, *Ch. prolifer* or bee pollen.

Experienced beetles

The beetles that had gained oviposition experience in the above tests (section 5.3.1.3) were then placed in a second two-way choice test with fresh pod-bearing shoots of *C. scoparius* and *Ch. prolifer* following the same methodology as outlined above. The numbers of eggs laid by these beetles on pods of both plant species were counted after 48 hours and the numbers of seeds per pod, as well as the lengths and descriptions of the pods were recorded.

Table 5.1: Summary of methods to determine the effect of adult food source (*Cytisus scoparius*, *Chamaecytisus prolifer*, *Genista monspessulana* and bee pollen) and larval rearing host (*C. scoparius* and *Ch. prolifer*) on oviposition in *Bruchidius villosus*.

		Treatment							
Pretreatment	**Duration**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Larval rearing host plant	approx. 2 -3 months	*C. scoparius*	*C. scoparius*	*C. scoparius*	*C. scoparius*	*Ch. prolifer*	*Ch. prolifer*	*Ch. prolifer*	*Ch. prolifer*
Overwintering conditions	30-32 weeks	Honey water	Honey water	Honey water	Honey water	Honey water	Honey water	Honey water	Honey water
Adult feeding experience	6 weeks	*C. scoparius* flowers	*Ch. prolifer* flowers	*G. monspessulana* flowers	Bee pollen	*C. scoparius* flowers	*Ch. prolifer* flowers	*G. monspessulana* flowers	Bee pollen
Naïve oviposition	2 days (3 reps each)	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*
Adult feeding	1 day	*C. scoparius* flowers	*Ch. prolifer* flowers	*G. monspessulana* flowers	Bee pollen	*C. scoparius* flowers	*Ch. prolifer* flowers	*G. monspessulana* flowers	Bee pollen
Experienced oviposition	2 days (3 reps each)	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*	*C. scoparius* vs *Ch. prolifer*
No. beetle pairs		30	30	30	30	30	30	30	30

5.3.2 The effect of no overwintering on oviposition

Cytisus scoparius pods with remains of egg shell (from eggs already laid by *B. villosus*) on the pod surface were collected from the field when they were near dehiscence in November 2001. The pods were stored in the laboratory in containers until adult beetles emerged. After emergence the beetles were fed pollen and provided with cotton rolls soaked in honey water for five weeks to allow them to build up fat reserves. Following this, six replicates of ten beetles (five male and five female) were placed in Perspex® Prestige® boxes (220 × 130 × 100 mm) each with a flexible push-on lid and four, 25 mm diameter, gauze covered holes for ventilation. A moistened piece of blotting paper was placed on the bottom of each cage, and several pieces of tissue paper were included to absorb beetle excreta. Each box contained a 200 mm long shoot each of *C. scoparius* and *Ch. prolifer* bearing green pods. The boxes were kept at 22-16°C day-night and 14:10 h L:D under approximately 70% humidity for six days during which the numbers of eggs laid were recorded. This was close to a repeat of the conditions beetles were exposed to during collection and shipment from the UK to New Zealand and host specificity tests conducted in 1985 (Syrett and O'Donnell 1987).

5.4 Analysis

A 3-way factorial ANOVA (adult food type (4 levels), larval host (2 levels), experience (2 levels) was carried out to determine the significance of the main effects, adult food source (pollen) and larval rearing host, on number of eggs laid on two host plants by naive and experienced *B. villosus*. The data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. Egg counts for naive *B. villosus* were log transformed before hypothesis testing as the data were not normally distributed (Zar 1984).

To determine if *B. villosus* has an oviposition preference for *C. scoparius* over *Ch. prolifer*, a 'coefficient of preference' was calculated using the formula: CP = (NE*Cs* - NE*Chp*) / (NE*Cs* + NE*Chp*) (Heard 1995a, Withers et al. 2000), where NE*Cs* is the number of eggs laid on *C. scoparius* and NE*Chp* is the number of eggs laid on *Ch. prolifer*. This index varies from –1 (all eggs laid on *Ch. prolifer*) to 0 (when number of eggs laid on both plant species is equal) to +1 (all eggs laid on *C. scoparius*).

Two-sample t-tests (Minitab 2000) were used to test for differences in numbers of eggs laid on *Ch. prolifer* and *C. scoparius* by *B. villosus* beetles which had not overwintered. A Mann-Whitney test was used to compare numbers of eggs laid between host specificity tests conducted in 1985 and 2002. Non-parametric statistics were used because the mean and standard deviation for one group of data was zero.

5.5 Results

5.5.1 Examination of factors

The effect of larval rearing host and adult food type (pollen) on oviposition and choice of oviposition host in naive and experienced *B. villosus* was examined. No significant interactions between the factors were found in the analysis (Table 5.2).

Table 5.2: Significance of factors (larval rearing host and adult food source and their interactions) contributing to oviposition and oviposition host choice in naïve and experienced *Bruchidius villosus* in a two-choice cut shoot test containing pods of *Cytisus scoparius* and *Chamaecytisus prolifer*.

Factors	Naive			Experienced		
	F	df	P	F	df	P
Larval rearing host	1.3	1	0.3	1.1	1	0.3
Adult food source	3.8	3	0.03	1.3	3	0.3
Oviposition host	18.7	1	0.001	17.0	1	0.001
Larval rearing host × adult food source	1.5	3	0.3	0.2	1	0.9
Oviposition host × larval rearing host	0.03	1	0.9	0.1	1	0.7
Oviposition host × adult food source	0.4	3	0.8	0.8	3	0.5
Oviposition host × larval rearing host × adult food source	0.5	3	0.7	0.2	3	0.9

5.5.2 The effect of adult food source and larval rearing host on oviposition

All four pollen types provided during the pretreatment following overwintering in the laboratory successfully induced oviposition in early spring, but different types of food had a significant effect on number of eggs laid ($F = 3.7$, $df = 3$, $P = < 0.05$) by naive beetles (Table 5.3). Naïve *B. villosus* fed bee pollen laid significantly more eggs than *B. villosus* fed *C. scoparius*, *Ch. prolifer* or *G. monspessulana* pollen (Table 5.3). Experienced *B. villosus* were less affected by pollen type where the average number of eggs laid by

beetles fed different pollen types were not significantly different (F = 1.3, df = 3, P > 0.05) although more eggs on average were laid by those beetles fed bee pollen (Table 5.3).

Table 5.3: The effect of four different pollen types on mean number (no.) of eggs laid (± SEM) by naive and experienced *Bruchidius villosus* in a two-choice test cut shoot test contaning *Cytisus scoparius* and *Chamaecytisus prolifer* over 48 hours (n = 240 females, 60 per treatment).

Pollen type	Mean no. log eggs laid over 48 hours by by naive *B. villosus*	Mean no. eggs laid over 48 hours by naive *B. villosus*	Mean no. eggs laid over 48 hours by experienced *B. villosus*
C. scoparius	1.2 ± 0.08 a	3.9 ± 0.4	6.9 ± 0.4
Ch. prolifer	1.4 ± 0.08 a	4.5 ± 0.4	6.7 ± 0.4
G. monspessulana	1.3 ± 0.07 a	3.7 ± 0.3	6.7 ± 0.3
Bee pollen	1.9 ± 0.07 b	7.5 ± 0.4	8.3 ± 0.4

Values with the same letter within the column are not significantly different (5% LSD, 0.5).

An examination of the effect of experience on egg laying showed that significantly more eggs in total were laid on *C. scoparius* than on *Ch. prolifer* by naive (F = 18.7, df = 1, P < 0.001) and experienced *B. villosus* (F = 17.0, df = 1, P < 0.001) (Table 5.4). Naïve *B. villosus* laid 57% more eggs on *C. scoparius* than on *Ch. prolifer* while experienced *B. villosus* laid 71% more eggs on *C. scoparius* than *Ch. prolifer*. Naïve beetles laid an average of 0.86 and 0.76 eggs per seed on pods of *C. scoparius* and *Ch. prolifer* respectively, while experienced beetles laid 1.1 eggs per seed on both hosts.

Table 5.4: Mean number (no.) of eggs laid (± SEM) on *Cytisus scoparius* and *Chamaecytisus prolifer* in a two-choice cut shoot test by naive and experienced *Bruchidius villosus* over 48 hours (n = 240 females, 120 per treatment combination).

Oviposition host	Mean no. log eggs laid per naïve beetle over 48 hours	Mean no. eggs laid per naïve beetle over 48 hours	Mean no. eggs laid per experienced beetle over 48 hours
C. scoparius	1.69 ± 0.05 b	6.2 ± 0.3	4.2 ±0.3 b
Ch. prolifer	1.21 ± 0.05 a	3.6 ± 0.2	3.4 ± 0.2 a

Values with the same letter within columns are not significantly different (naïve - 5% LSD, 0.2, experienced - 5% LSD, 0.6).

Rearing host had no significant effect on the numbers of eggs laid by either naive ($F = 1.3$, $df = 1$, $P > 0.05$) or experienced beetles ($F = 1.3$, $df = 1$, $P > 0.05$) but, over all plant hosts, experienced beetles laid significantly more eggs than naive beetles ($t = 8.1$, $df = 938$, $P < 0.001$) (Table 5.5).

Table 5.5: The effect of larval rearing host (*Cytisus scoparius* and *Chamaecytisus prolifer*) on the mean number (no.) of eggs laid by naive and experienced *Bruchidius villosus* in a two-choice cut shoot test containing *Cytisus scoparius* and *Chamaecytisus prolifer* over 48 hours (n = 240 females, 120 per treatment).

B. villosus	**Larval rearing host**	**Mean no. eggs laid (± SEM) per beetle over 48 hours**
Naïve	*C. scoparius*	4.4 ± 0.3
Experienced		5.3 ± 0.3
Naïve	*Ch. prolifer*	6.8 ± 0.3
Experienced		7.5 ± 0.3

Figure 5.1 and 5.2 reveal the stronger preference both naive and experienced *B. villosus* showed for *C. scoparius* over *Ch. prolifer* as the majority of the coefficients of preference fall on the positive side of the axis. The scatter of the data points (which in some cases are both above and below 0 – neutral preference within treatments) demonstrates a high variation in oviposition preference between treatments. This suggests a high inter-individual variation in preference may be occurring.

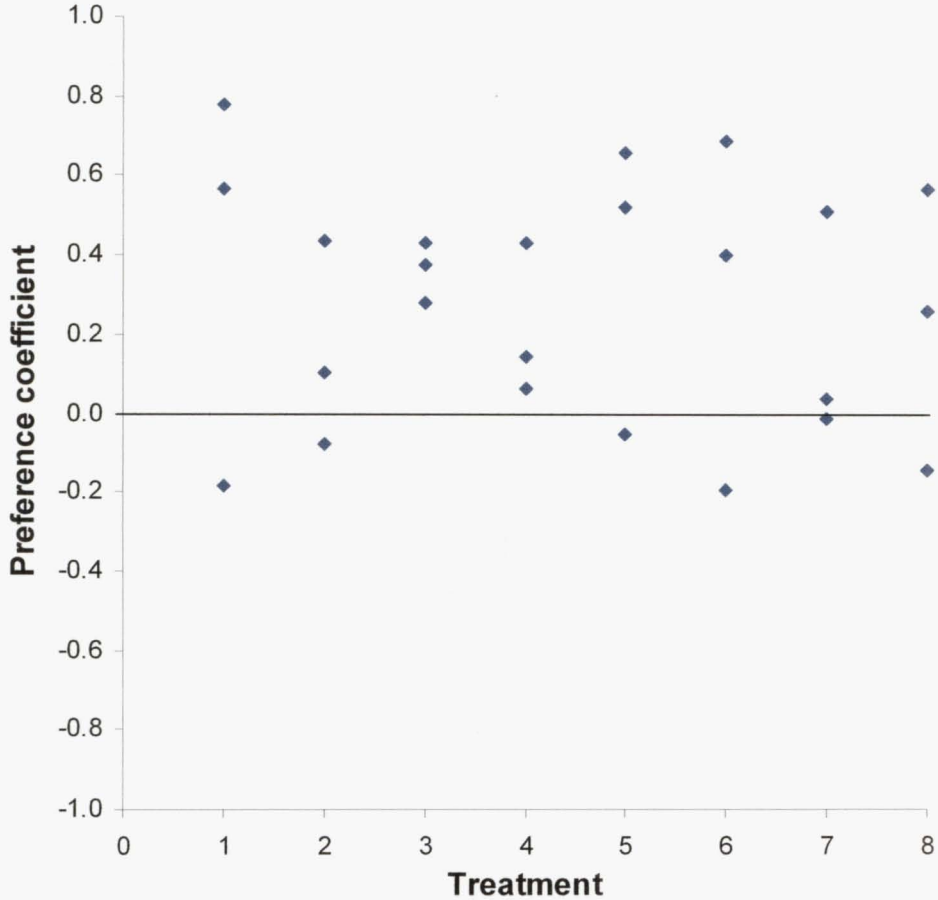


Figure 5.1: The effect of adult food source (pollen type) and larval rearing host (*Cytisus scoparius* and *Chamaecytisus prolifer*) on the coefficient of preference for oviposition (+1 = Preference for *C. scoparius* and -1 = Preference for *Ch. prolifer*) in naïve *Bruchidius villosus*.

Key: Treatment combination 1: rearing host *C. scoparius*, food *C. scoparius*, Treatment combination 2: rearing host *C. scoparius*, food *Ch. prolifer*, Treatment combination 3: rearing host *C. scoparius*, food *Genista monspessulana*, Treatment combination 4: rearing host *C. scoparius*, food bee pollen, Treatment combination 5: rearing host *Ch. prolifer*, food *C. scoparius*, Treatment combination 6: rearing host *Ch. prolifer*, food *Ch. prolifer*, Treatment combination 7: rearing host *Ch. prolifer*, food *G. monspessulana*, Treatment combination 8: rearing host *Ch. prolifer*, food bee pollen.

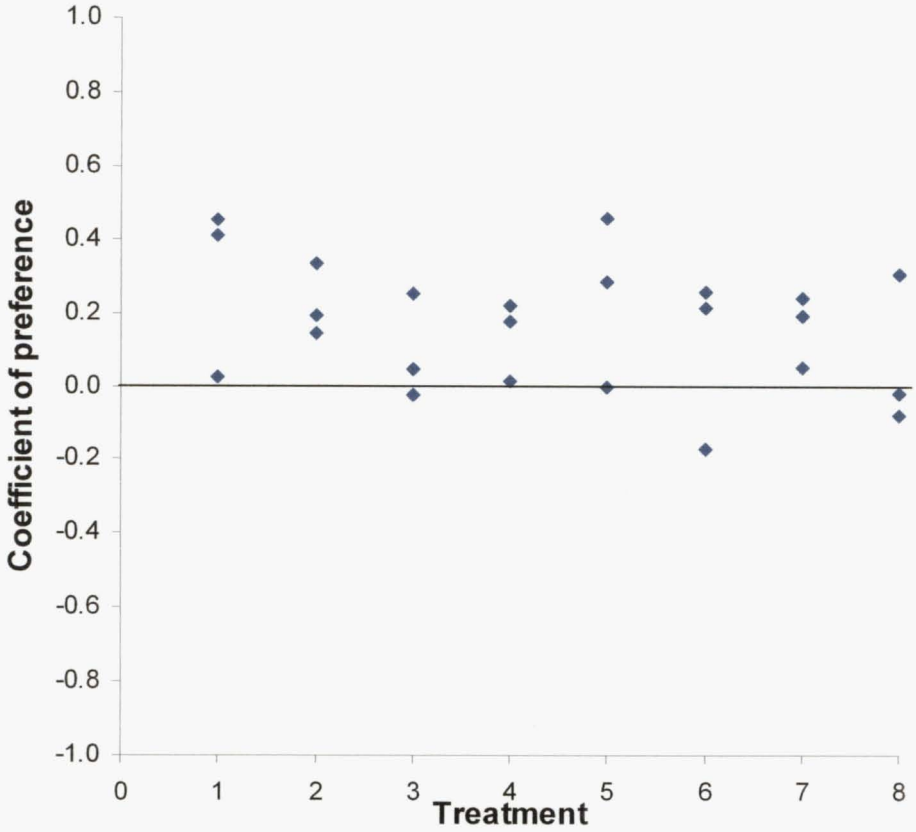


Figure 5.2: The effect of adult food source (pollen type) and larval rearing host (*Cytisus scoparius* and *Chamaecytisus prolifer*) on the coefficient of preference for oviposition (+1 = Preference for *C. scoparius* and -1 = Preference for *Ch. prolifer*) in experienced *Bruchidius villosus*.

Key: Refer to Figure 5.1

In 88% of the treatments naive *B. villosus* showed a stronger mean coefficient of preference for *C. scoparius* than experienced beetles (Figure 5.3). Less variation in response between replicates is demonstrated by experienced beetles (Figure 5.2).

However, 25% of the replicates containing naive beetles laid more eggs on *Ch. prolifer* than on *C. scoparius*, while 17% of the replicates containing experienced beetles laid more eggs on *Ch. prolifer*. Treatment 2 was the only treatment where experienced beetles showed a higher preference for *C. scoparius* than naive beetles (Figure 5.3).

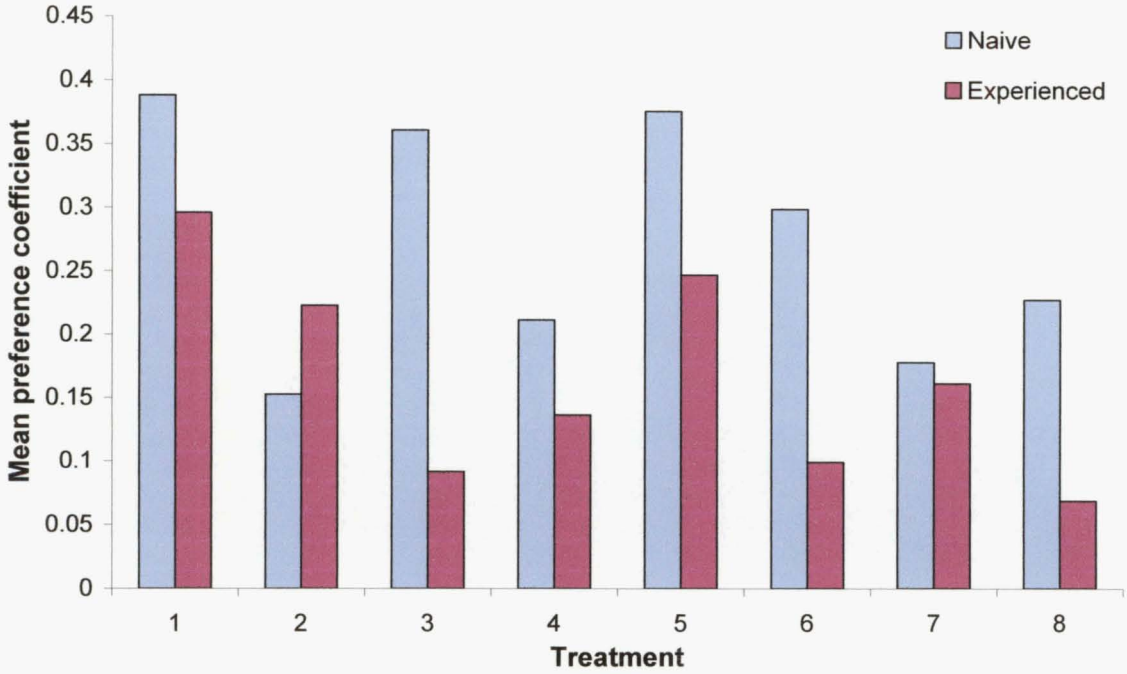


Figure 5.3: Mean coefficient of preference for oviposition of naive and experienced *Bruchidius villosus* in a two-choice cut shoot test containing cut shoots of *Cytisus scoparius* and *Chamaecytisus prolifer* (n = 240 females).

Key: Refer to Figure 5.1

5.5.3 The effect of no overwintering on oviposition

Beetles which had been reared from *C. scoparius* pods and not overwintered but fed on *C. scoparius* flowers and bee pollen laid eggs on *C. scoparius* and *Ch. prolifer* after six days of being provided with pod material. Significantly more eggs ($t = 9.8$, $df = 10$, $P < 0.001$) were laid on *C. scoparius* than on *Ch. prolifer* (Table 5.6). There was no significant difference in mean number of eggs laid by *B. villosus* adults between the 1985 tests and these replicated conditions in 2001 ($W = 24$, $df = 6$, $P > 0.05$) (Table 5.6). However, there was a difference in mean number of eggs laid on *Ch. prolifer* between the two testing years, as eggs were laid on this species in these tests conducted in 2001 while none were laid in 1985.

However, in one of the six replicates no eggs were laid on pods of *Ch. prolifer*, indicating that at least five beetles out of the 30 females used in this 2001 investigation did not lay on *Ch. prolifer*. Eggs laid per female in these tests are presented in Table 5.6.

Table 5.6: Mean number of eggs laid per female (± SEM) by non-overwintered *Bruchidius villosus* in a two-choice cut shoot test containing *Cytisus scoparius* and *Chamaecytisus prolifer*.

Oviposition	1985 (n = 10)		2001 (n = 30)	
	C. scoparius	*Ch. prolifer*	*C. scoparius*	*Ch. prolifer*
Mean number of eggs laid per female	18.7 ± 1.2	0	13.4 ± 0.9	2.26 ± 0.7

Variation in physiology between the two plant species meant that seed number varied significantly. The average number of seeds per pod was significantly higher for *C. scoparius* ($t = 3.0$, $df = 54$, $P = < 0.05$) (Table 5.7) whereas pod length did not differ significantly between species ($t = 1.22$, $df = 69$, $P > 0.05$).

Table 5.7: Mean pod length, mean seeds per pod and mean number of eggs laid per pod by *Bruchidius villosus* (n = 30) in a two-choice cut shoot choice test with *Cytisus scoparius* and *Chamaecytisus prolifer*.

Oviposition and Pod factors	*C. scoparius*	*Ch. prolifer*	P value
Mean pod length (mm) (± SEM)	32.9 ± 1.3	35.3 ± 1.5	> 0.05
Mean seeds per pod (± SEM)	7.4 ± 0.4	6.0 ± 0.2	< 0.05
Mean eggs per pod (± SEM)	11.2 ± 0.8	1.9 ± 0.3	< 0.001

5.6 Discussion

5.6.1 The effect of food source and larval rearing host on oviposition

All adult pre-treatment food sources following overwintering were able to induce oviposition on pods by *B. villosus*. Bee pollen was the food source on which the highest subsequent fecundity was recorded. Two possible explanations for this are, 1) that bee pollen was the easiest pollen to forage and thus less energy was expended by the beetles during foraging improving reproductive fitness, or 2) pollen or nectar (honey water) were somehow limited in the other treatments. Bee pollen was placed in mini Petri dishes at the base of the cages, whilst all other food types required the beetles to collect pollen from within flowers which is a time consuming process. Bee pollen was also probably the food source in most abundant supply and is a mix of pollen from multiple plant species. However, the initial fecundity advantage of the bee pollen food source, was only evident in naïve beetles and not in experienced beetles, suggesting the advantage was only short lived (Table 5.3).

There was no significant difference in fecundity between beetles reared from either *Ch. prolifer* or *C. scoparius*, although there was a slight trend for adult *B. villosus* reared from *Ch. prolifer* to lay more eggs (Table 5.5). This trend is supported in Chapter 9 where it was found that *B. villosus* reared from *Ch. prolifer* were larger and more

fecund than those reared from *C. scoparius*. In this experiment both plant species were apparently equally suitable larval rearing hosts in terms of fecundity.

High inter-replicate variation appears to mask any significant differences among and between treatments (Figure 5.1 and 5.2). This suggests that high individual variations in oviposition behaviour of *B. villosus* should be further investigated (Chapter 8).

The coefficient of preference (Figure 5.3) and the fact the highest overall mean number of eggs were laid on pods of this plant (Table 5.4), shows that *C. scoparius* was the most preferred host for oviposition. This supports findings in other investigations where *C. scoparius* was the most preferred host (Chapter 2 and 4). Naïve *B. villosus* showed a higher mean coefficient of preference for *C. scoparius* than experienced beetles did a day later which indicates there may be a genetic component directing a preference for the ancestral host plant, which can be altered by experience of other suitable hosts in the environment.

Naïve beetles laid on average less than one egg per seed while experienced beetles a day later laid just over one egg per seed. This suggests that the beetles were not limited for oviposition sites in this investigation and that egg laying increases with time over the first five days. *Bruchidius villosus* beetles are known to superparasitise pods heavily when pods are limited (section 6.6.1) and usually lay more than one egg per seed in the field in New Zealand (M. Haines, personal observation). It could be concluded that experience during the first choice assay of 48 hours duration acted to increase female beetles subsequent egg laying on both *C. scoparius* and *Ch. prolifer*, but especially more so on *Ch. prolifer* (Table 5.5). Furthermore maturation may also be a factor and cannot be ruled out.

The order in which eggs were laid was not determined. Because there was no shortage of pod resource it is unlikely that the eggs that were laid on *Ch. prolifer* were a result of the preferred host, *C. scoparius* having already been exploited. In some instances, when resource limitation occurs it is possible that when all available oviposition sites on the preferred host are used first, alternative sites are then utilised on the less preferred host (Barton Browne and Withers 2002). This possibility cannot be totally ruled out, but as mentioned above at approximately one egg per seed laid in tests, pods were probably not superparasitised.

5.6.2 The effect of overwintering on oviposition

The results indicate that a lack of overwintering does not affect the oviposition behaviour expressed by *B. villosus* because eggs were laid on both *C. scoparius* and *Ch. prolifer* in this experiment after continuous rearing. There was no significant difference in the mean number of eggs laid by beetles between the 1985 testing on beetles introduced directly from the UK autumn to the New Zealand spring and those tested in this study. The only difference between the experiments in 1985 and in the current study was increased replication (from two replicates in 1985 to six replicates in this study) (Table 5.6). Therefore these results suggest that the lack of overwintering is not the factor responsible for the failure of the initial testing to predict that *Ch. prolifer* is suitable for oviposition by *B. villosus*, but insufficient replication might be.

Bruchidius villosus either exhibits non-diapause dormancy or facultative diapause. Tauber et al (1986) describe non-diapause dormancy as when insect metabolism is suppressed as a result of unfavourable seasonally recurring environmental conditions. Insects displaying this type of dormancy respond immediately to the conditions of the environment. In contrast, in diapause mediated dormancy physiological and behavioural modifications occur prior to the environment changing. This type of dormancy is persistent, and is not terminated when favourable conditions resume. Diapause can be further described as obligatory or facultative. Obligatory diapause occurs in each member of a population regardless of environmental conditions, whereas facultative diapause can be prevented under certain environmental conditions.

This investigation clearly shows *B. villosus* does not have obligatory diapause. The latter finding is supported by Syrett (1999).

5.7 Conclusion

In conclusion, none of the factors tested here adequately explain why the original host specificity tests failed to identify *Ch. prolifer* as an alternative host for *B. villosus*. *Cytisus scoparius* flowers are not necessary to activate oviposition in *B. villosus*. Larval rearing host and lack of an overwintering period had no significant effect on number of eggs laid. *Cytisus scoparius* was the preferred oviposition host over *Ch. prolifer* regardless of the pollen type fed on by adult females or larval rearing host. These results led to further investigation into aspects of the methodology in order to determine the cause of the false negative result in the original host specificity tests.

Linking page: Chapter 6

Chapter 5 showed that larval host, adult food type (pollen) and lack of overwintering had no significant effect on oviposition preference of naïve or experienced *Bruchidius villosus* for *Cytisus scoparius* versus *Chamaecytisus prolifer*. Therefore further aspects of the methodology of the host testing were investigated. The hypothesis in Chapter 6 was that cage size, position of plant material, or the quantity of resource provided explained the failure of the original choice tests to reveal the acceptability of *Ch. prolifer*.

Chapter 6: Does cage size, the orientation of the test plant material or quantity of resource influence oviposition preference in *Bruchidius villosus?*

6.1 Abstract

Different aspects of two-choice test design layouts were examined to investigate possible causes of false negative results in previous assays. Test plant species and amount of oviposition resource provided significantly affected mean proportion of eggs laid on the control (*Cytisus scoparius*) in two-choice cut shoot tests. There was no significant difference in mean proportion of eggs laid on the control due to either small versus large cage size or orientation of plant material (vertical versus horizontal). Eggs were laid on test plants *Chamaecytisus prolifer*, *Cytisus multiflorus* and *Genista monspessulana*, no eggs were laid on *Sophora microphylla*, *So. prostrata*, *Spartium junceum*, *Lupinus arboreus* and *Laburnum anagyroides*. *Bruchidius villosus* laid a higher proportion of eggs on the test plant in the treatment where control resource was half that of the test plant (30 versus 60 seeds) compared to equal resources (60 seeds each). Eggs were laid on *C. multiflorus* in only one of the treatments where the control number of seeds (30 seeds) was half that available on *C. multiflorus* (60 seeds). None of these factors appear to adequately explain the previous false negative results.

6.2 Introduction

Field host range of an insect used to be considered as a reliable indicator of host specificity of a phytophagous insect. For biological control agents greater certainty of host specificity was required and political and community pressure that arose from concerns about non-target effects led to the development of experiments known as host specificity tests (Dodd 1940). No-choice or sequential host specificity tests were the favoured method until the late 1960s (Harris and Zwölfer 1968). Choice tests became the accepted methodology in the 1970s and 80s for host specificity testing because they were considered less likely to induce false positive results than no-choice tests. A false positive

occurs when test insects accept a plant species within a test, but when they are given a choice or put in a natural environment they do not accept the plant (Marohasy 1998).

Choice testing was thought to be more natural and representative of the field environment (Harley 1969, Winder et al. 1984, Cullen 1990, Heard 1997). The major drawback now understood about choice tests is that the presence of the normal host plant may influence the behaviour of the insect toward the test plant (Heard 1997). Choice tests have been particularly criticised for their inability to pick up the acceptance of lower ranked alternative host species (Marohasy 1998, Edwards 1999, Hill 1999, Heard 2000, Purcell et al. 2000, Barton Browne and Withers 2002). In many circumstances, the presence of the normal host plant may result in the production of a false negative result.

A false negative occurs when an insect accepts only the normal host in the test but when it is released into the field it accepts a wider range of hosts than were predicted by the test (Marohasy 1998). One of the purported reasons for this occurrence is that the normal host plant is ranked significantly higher than the test plant, and in a choice test feeding or oviposition preferences result in the highest ranked plant receiving all the feeding damage or egg laying. However in the absence of the highest ranked plants (such as in a no-choice test), time dependent effects such as deprivation come into play. These can induce a lower ranked test plant to be accepted if the duration of the test is sufficiently long (Withers and Barton Browne 1998, Withers et al. 2000). Depending on the circumstances that the insect will face in the field, this result (acceptance in no-choice, but no acceptance in choice) may in fact be the real one, and indicative of what will occur in the field.

Another problem with host specificity tests is that they must often be conducted in a caged environment or in the laboratory, particularly if they are undertaken outside the country of origin of the insect. This is necessary for quarantine purposes and also to artificially increase the density of insects per plant (Cullen 1990). Even if testing is conducted in the country of origin, insect densities in the field can be too low to obtain meaningful results (Briese 1999). Low densities may be the result of natural enemies, which regulate the population in its native range, but, once the agent is released into a new environment free of its natural enemies, densities often increase and as a consequence so does damage to the host plant and the risk of transfer to a non-target host plant. It is important during testing to ensure populations of the prospective biological control agent are large enough to indicate potential damage and to determine the

likelihood of non-target plants being attacked when populations of the target weed decline (McFadyen 1998). There is evidence in situations of intense competition and/or starvation that lower ranked hosts can become temporarily acceptable (Blossey et al. 1994, Blossey 1995, Withers 1997).

Some of the latest recommendations on host specificity testing suggest utilising combinations of the aforementioned strategies (surveys of field host range, choice and no-choice tests and open-field testing) to reduce the likelihood of tests producing false negative or false positive results (Marohasy 1998, Edwards 1999, Sheppard 1999). In addition, consideration of the biology of the agent is necessary to ensure the most appropriate testing regime is selected (Sheppard 1999).

In host specificity testing it is inevitable that there are some restrictions on the insect's host finding behaviour, however these should be kept to a minimum. Test conditions should be kept as natural as possible and studies on host finding cues of the insect require consideration (Cullen 1990). In work done by Cullen (1989) clear differences in *Pachycerus cordiger* behaviour were observed between natural and standard laboratory conditions. Sometimes cage size is known to disrupt insect behaviour (Marohasy 1998, Sands and Van Driesche 2000). Inability to provide cages large enough for unrestricted flight for insects which require it can make results difficult to interpret (Syrett and Emberson 1997). For instance, a difference in specificity was predicted in the beetles *Leptinotarsa texana* (Schaeffer) and *L. defecta* (Stål), when testing was conducted in cages of two different size dimensions (Olckers et al. 1995). To minimise the effect of cage size, Wan et al (1996) suggest using large test arenas to incorporate as many steps as possible in the natural host selection process of the insect.

In choice tests, it is necessary to give the insects a 'choice' of plant material on which to oviposit or feed but at the same time apply realistic pressure on the insect so that information from the tests can be accurately related to a potential field situation. Tests that combine the advantages of both choice and no-choice tests are also under development. One approach is to conduct choice tests but include a limited amount of host plant material or resource, so all available oviposition sites are used throughout the duration of the trial. Insects are then forced to look elsewhere and thus the test becomes similar to a no-choice test (Heard 1997).

Some non-insect factors which affect the outcome of choice tests in the laboratory have been examined, for example, the effect of whole plants versus cut shoots (Palmer 1999), as well as cage size and limited host plant oviposition resource. However, a factor which has not been considered widely is the orientation of test and control plant material and whether it could affect oviposition decisions in insects.

Bruchidius villosus was introduced to New Zealand as a biological control agent for *Cytisus scoparius* after choice oviposition tests on cut shoots conducted in 1985 indicated that it was host specific to *C. scoparius*. The host specificity tests failed to predict that *Chamaecytisus prolifer* would be an appropriate alternative host for *B. villosus*. This study investigates the possibility that the failure of the host specificity tests that led to the release of *B. villosus* in New Zealand as a biological control agent of *C. scoparius*, occurred as a result of test methodology. In this investigation a series of changes were made to the original host specificity choice tests done in New Zealand to try to understand why a false negative result was produced. The new methods included increasing the level of replication, changing cage sizes and the orientation of plant material within each cage, and altering the quantity of pod resource available to *B. villosus* in the tests.

If eggs are laid in treatments where the original methodology has been changed, then it is likely that the change of one or more of the above factors in the original tests is to blame for the false negative result. If eggs are laid in the treatments that replicate the original tests as closely as possible, then it is likely that low replication in relation to the level of variation in behaviour among *B. villosus* or subtle differences in methodology that are unable to be controlled for, may be at fault.

6.3 Methods

A randomised 8 (plant species) × 2 (resource levels) × 2 (cage sizes) × 2 (orientation levels) factorial was used for this experiment. For each of the eight test plant species, the effect of two levels of each of the following three factors on beetle oviposition were investigated: quantity of oviposition resource (equal or unequal number of seeds), cage size (small (Prestige® box) or large (Perspex® cage)) and orientation of plant material (horizontal or vertical).

It is not known whether beetles may have accepted lower ranked pod species in the original tests if the quantity of preferred test plant material had been lower. So to

examine this all test plant pod material selected bore approximately 60 seeds. This was based on an estimate made from photos taken of the original experimental set-up of the quantity of pod material presented by Syrett and O'Donnell (1987), in which test plant and control plant material each provided approximately 60 seeds. In one of the treatments in this experiment *C. scoparius* (control) pod material bore approximately 30 seeds and the test plant bore approximately 60 seeds. This allowed a comparison to be made between treatments where test and control plant material was equal (as in the original tests) and where control plant material was half that of the test plant.

For the cage size factor, Perspex® boxes made by Prestige® (220 × 130 × 100 mm) with push-on lids, and four 25 mm diameter, gauze covered holes for ventilation (used in the original tests) were compared to Perspex® cages (500 × 500 × 500 mm) with sponge doors (365 × 360 × 50 mm). A moistened piece of blotting paper was placed at the bottom of each cage, and several pieces of tissue paper were included to absorb beetle excreta. Two sprigs, one each of *C. scoparius* and the test plant bearing young green pods, were placed in vials of water in each test cage. A disc of plastizote, 6 mm thick, with a twig pushed through its centre acted as a stopper to each vial to ensure that the shoot remained in water. The pods containing seeds of both plant species were chosen to be at an equivalent developmental stage.

In the field, pod material from Fabaeae nearly always hangs vertically, yet in the original tests the smaller Prestige® boxes prevented this natural orientation from being presented to the beetles. In this investigation plant material was either presented horizontally as in the original tests or vertically, as occurs in the field.

Tests were run independently each with ten adult beetles (five male and five female) field collected from *C. scoparius*. Each test species was replicated four times for each of the eight treatment combinations (Figure 6.1). Test plant material was collected from different individual plants within the species population. Replicating the same procedure as was carried out in 1985, 2000 adults were maintained in Perspex® cages with ample bee pollen and honey water, followed by *C. scoparius* flowers, under a 22:16°C (day:night) temperature regime with a day length of 14:10 h L:D until testing resumed.

Beetles were held in each test cage for six days (as in the original tests) at 22-16°C day-night temperatures with a 14:10 h L:D regime during testing. Relative humidity was

held at approximately 70% and beetles were fed pollen and provided with cotton dental rolls soaked in a honey water solution. After the test period, the beetles were removed and all plant material and cages were carefully examined for eggs. The numbers of eggs found on the pods of *C. scoparius* and the test plant species were recorded. Because each part of the experiment was conducted when each of the test plant species had pods at the appropriate stage of development, not all plant species were tested at the same time. This also replicates how tests were conducted in 1985 (Syrett and O'Donnell 1987). In this way, testing was repeated for four key plant species of the nine plant species originally tested in the 1985 host specificity tests. Plants tested in this investigation were *Chamaecytisus prolifer*, *Sophora microphylla*, *So. prostrata*, *Cytisus multiflorus*, *Genista monspessulana*, *Laburnum anagyroides*, *Lupinus arboreus*, *Spartium junceum*. Plants tested in 1985 and repeated in this experiment were *Ch. prolifer*, *So. microphylla*, *C. multiflorus* and *G. monspessulana*.

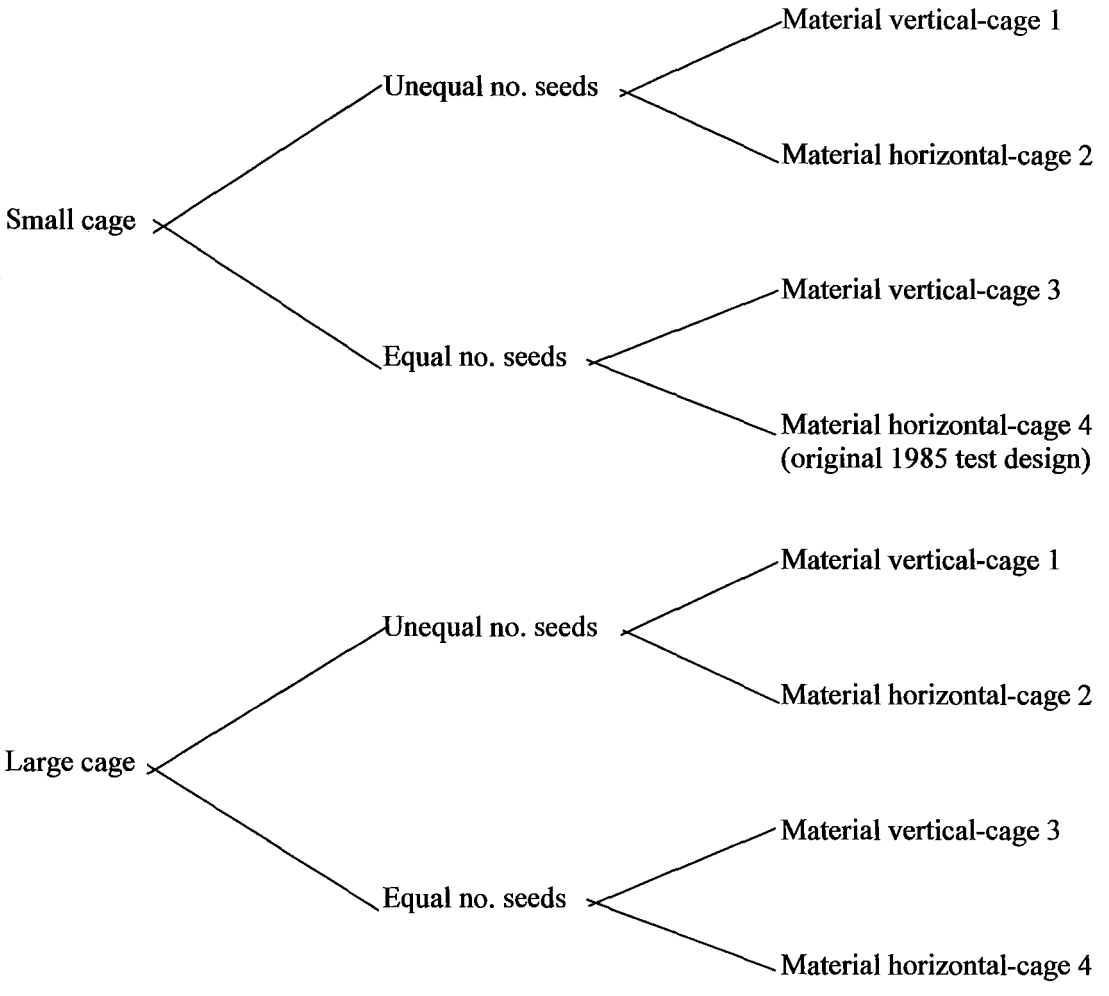


Figure 6.1: Experimental design to test factors of the methodology: cage size, quantity of oviposition resource and orientation of pod material. Each treatment combination (following each branch from start to finish) was replicated 4 times for 8 plant species.

6.4 Analysis

The data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. An ANOVA was used to determine any significant differences in the number of eggs laid by *B. villosus* on comparative control plants. Fishers LSD was used to compare individual treatment means. To determine the effect of test plant species, quantity of oviposition resource, cage size and orientation of plant material on the proportion of eggs laid on the control plant (*C. scoparius*) by *B. villosus*, a four factor ANOVA was used. Fishers LSD was again used to compare among individual treatment means. All statistical analysis was done using GenStat (2002).

6.5 Results

6.5.1 Examination of factors

The effect of test plant species, quantity of oviposition resource provided, cage size and orientation of plant material on *B. villosus* oviposition were factors tested in this investigation. There were no significant interactions between any of the factors (Table 6.1).

Table 6.1: The significance of factors (test plant species, cage size, plant orientation, resource quantity and their interactions) contributing to oviposition by *Bruchidius villosus* in two-choice cut shoot tests on *Cytisus scoparius* (control) and 8 plant species

Factors	F	df	P
Test plant species	19.0	2	<0.001
Cage size	0.5	1	0.5
Plant orientation	0.06	1	0.8
Resource quantity	4.2	1	0.05
Plant species × cage size	0.3	2	0.8
Plant species × plant orientation	0.05	2	1.0
Cage size× plant orientation	1.8	1	0.2
Plant species × resource quantity	1.3	2	0.3
Cage size× resource quantity	0.5	1	0.5
Plant orientation × resource quantity	0.06	1	0.8
Plant species × cage size × resource quantity	1.1	2	0.3
Plant species × cage size × plant orientation	1.2	2	0.3
Plant species × plant orientation × resource quantity	0.04	2	1.0
Cage size × plant orientation × resource quantity	1.8	1	0.2
Plant species × cage size × plant orientation × resource quantity	1.1	2	0.3

6.5.1.1 Species

Mean numbers of eggs laid by *B. villosus* on *C. scoparius* (the control) throughout the investigation were not significantly different over the time the test species were tested ($F = 0.9$, $df = 7$, $P > 0.05$).

There were significant differences in the mean proportion of eggs laid on the three test plant species that were laid on (Table 6.2). The mean number of eggs laid on *Ch. prolifer* was significantly higher than that laid on *C. multiflorus* and *G. monspessulana* (Table 6.2).

Table 6.2: Mean number (no.) of eggs laid by *Bruchidius villosus* (n = 1280) on both the control (*Cytisus scoparius*) and 8 test plants in two-choice cut shoot tests.

Test plant species	Mean no. eggs laid on test plants	Mean no. eggs laid on control (*C. scoparius*)
**Chamaecytisus prolifer*	11.3 b	102.9 a
**Sophora microphylla*	0	98.3 a
Sophora prostrata	0	107.0 a
**Cytisus multiflorus*	1.3 a	104.8 a
**Genista monspessulana*[1]	0.2 a	113.7 a
Laburnum anagyroides	0	115.9 a
Lupinus arboreus	0	113.5 a
Spartium junceum	0	108.7 a

Values with the same letter within columns are not significantly different (control - 5% LSD, 18.3, test plant - 5% LSD, 3.8%, df = 72)

* - Plants tested in 1985 host specificity tests - cut shoot.

[1] – A total of five eggs were laid on one *G. monspessulana* pod. All of the eggs were in the same cluster attached to the surface hairs of the pod. This pod was touching the surface of a *C. scoparius* pod, such that it was unclear exactly on which pod oviposition was intended. For this reason this finding is not discussed in detail.

The overall mean number of eggs laid per female per replicate in these 2001 tests by New Zealand field collected beetles was 22.3, which is more than double the mean eggs laid in the original tests conducted in 1985 of 9.1 (Syrett and O'Donnell 1987).

6.5.1.2 Quantity of oviposition resource

In the *Ch. prolifer* treatment, the proportion of eggs laid on the control (*C. scoparius*) was significantly different between the two levels of the resource factor. A larger proportion of eggs was laid on *C. scoparius* when equal quantities (60 seeds each) of the control and test plant material were presented (Table 6.3). This means that an increased proportion of eggs (from 7.7% to 14.2%) was laid on *Ch. prolifer* when unequal quantities (30 versus 60 seeds) of the control and test plant material were presented (Table 6.3).

When oviposition resource was halved the number of eggs laid on *C. scoparius* decreased by 25% and numbers laid on *Ch. prolifer* increased by 43% (calculated using values in Table 6.3). The mean number of eggs laid per seed on *C. scoparius* increased by almost 1 egg per seed (from 1.9 to 2.9 (calculated using values in Table 6.3)) when the oviposition resource was halved. The difference in mean number of eggs laid per female between the resource treatments was 4.6 (calculated using values in Table 6.3), with more eggs being laid in the equal resource treatment where 120 seeds in total were presented compared to 90 in the unequal resource treatment.

Table 6.3: Mean number of eggs laid (± SEM) on *Cytisus scoparius* and *Chamaecytisus prolifer* in a two-choice cut shoot test with two levels of oviposition resource.

Host	*C. scoparius*	*Ch. prolifer*	
Resource quantity	**Mean no. eggs laid per female**	**Mean no. eggs laid per female**	**% eggs laid on control**
Equal (60 seeds control, 60 seeds test plant)	23.5 ± 2.4	1.6 ± 0.4	92.3 a
Half (30 seeds control, 60 seeds test plant)	17.6 ± 2.9	2.9 ± 0.6	85.8 b

Values with the same letter within columns are not significantly different (5% LSD, 5.4%).

In the *C. multiflorus* treatment, the proportion of eggs laid on the control (*C. scoparius*) was not significantly different between the two levels of the resource factor (5% LSD, 5.4%) (Table 6.4). However, an increased proportion of eggs (from 0 to 2.7%) was laid on *C. multiflorus* when unequal quantities (30 versus 60 seeds) of the control and test plant material were presented. In other words, in the treatment where oviposition resource for *C. scoparius* and *C. multiflorus* was equal, all eggs were laid on the control (Table 6.4).

When oviposition resource was halved eggs laid on *C. scoparius* increased by 9% (calculated using values in Table 6.4) and considerably more eggs were laid on *C. multiflorus*. The average number of eggs laid per seed on *C. scoparius* more than doubled from 1.7 to 3.7 (calculated using values in Table 6.4) when the resource was halved (beetles laid more eggs in total on half the resource). The difference in mean number of eggs laid per female between resource levels was 2.4 (calculated using values in Table 6.4), with more eggs being laid in the unequal resource treatment where 90 seeds in total were presented compared to 120 in the equal resource treatment.

Table 6.4: Mean number of eggs laid (± SEM) on *Cytisus scoparius* and *C. multiflorus* in a two-choice cut shoot test with two levels of oviposition resource.

Host	*C. scoparius*	*C. multiflorus*	
Resource quantity	**Mean no. eggs laid per female**	**Mean no. eggs laid per female**	**% eggs laid on control**
Equal (60 seeds control, 60 seeds test plant)	20 ± 2.2	0	100
Half (30 seeds control, 60 seeds test plant)	21.9 ± 2.3	0.5 ± 0.09	97.3

6.5.1.3 Cage size

There was no significant difference in overall mean number of eggs laid by *B. villosus* in large cages (109.6) compared with small cages (106.6) (5% LSD, 9.2), nor where they were laid.

6.5.1.4 Plant orientation

There was no significant difference in overall mean number of eggs laid by *B. villosus* on horizontal cut shoots (109.6) compared with vertical cut shoots (106.6) (5% LSD, 9.2), nor where they were laid.

6.5.1.5 Replication

Four replicates within this experiment exactly replicated the original host specificity tests conducted in 1985 (Prestige® boxes, plant material horizontal and equal oviposition resource, Figure 6.1). The mean number of eggs laid per *B. villosus* female on *C. scoparius* in 1985 in the same treatment containing *C. scoparius* and *Ch. prolifer* was 18.7 (in two replicates) (Syrett and O'Donnell 1987) compared to 25.5 in the current test. However the critical result is that a mean of 1.0 egg per female was laid on the test plant *Ch. prolifer* in this experiment, whereas in 1985 no eggs were laid on any test plant. The range of number of eggs laid on *Ch. prolifer* was between 0.2 and 2.6 per female and in each of the four replicates at least 1 egg was laid.

In 6 out of the 32 replicates no eggs were recorded on *Ch. prolifer*. This means that at least 30 out of the 160 females tested did not lay on *Ch. prolifer*.

6.6 Discussion

6.6.1 Quantity of oviposition resource

It appears that the quantity of oviposition resource presented to *B. villosus* had an impact upon the results of choice tests. Despite that the proportion of eggs laid on *C. scoparius*, in the test with *C. multiflorus*, was not statistically different between the two resource treatment levels, the difference between the proportion of eggs laid in the two treatments was 2.7% (Table 6.4) and does have important implications, because eggs were laid by *B. villosus* only on *C. multiflorus* in treatments in which the control plant oviposition resource was half that of the *C. multiflorus* resource. A consequence of the usual host plant material being halved was that the egg-load per seed more than doubled compared with treatments in which the control (host plant) resource was equal to that of the test plant.

This indicative result with the *C. multiflorus* treatment was strengthened by the significant trend in the results of the choice trials between *C. scoparius* and *Ch. prolifer*.

The proportion of eggs laid on *C. scoparius* was significantly different between the two resource treatments (Table 6.3) with more eggs laid on *Ch. prolifer* when only half the control resource was available. Eggs were laid on *C. scoparius* in all replicates while eggs were laid on *Ch. prolifer* in 26 out of 32 replicates. Again, in treatments where the control plant resource was halved, egg-load per seed increased by almost one egg per seed compared to treatments where resource was equal.

6.6.2 Host ranking

The findings in this investigation suggest that *C. scoparius*, *Ch. prolifer* and *C. multiflorus* are more highly ranked than the other test species because these species were oviposited on, while no eggs were laid on *L. anagyroides*, *Lu. arboreus*, *S. junceum*, *So. microphylla, So. prostrata. Genista monspessulana* had five eggs laid on one pod, but because the pod was touching a *C. scoparius* pod the finding was discounted. *Bruchidius villosus* showed no significant variation in fecundity over the entire testing period, meaning the beetles did not become less fecund as the tests proceeded.

Egg-load per seed increased when the amount of control resource decreased in treatments containing *Ch. prolifer* and *C. multiflorus* (section 6.5.1.2), which may indicate that until a certain threshold (where beetles are deterred from laying on the higher ranked host) is reached, beetles will continue to lay eggs on the target host (the highest ranked plant) even if pods become over-loaded. Available oviposition sites on the target host decrease and the pressure for the insect to utilise a lower ranked host increases. Courtney et al (1989) predicted that relative ranking of preference for species is fixed and less favoured species are only oviposited on when the more preferred species are rare, absent or fully utilised. West and Cunningham (2002) in their general model for host plant selection predict that as females become more host limited they will oviposit on less favourable hosts, whilst still ovipositing on more favourable hosts. The switch to ovipositing on less favourable hosts shows that a threshold for accepting lower ranked hosts exists.

Pods are a limited resource and only one larva per seed can develop. In a situation where the number of eggs laid begins to exceed the carrying capacity of the pods, and as a consequence subsequent larval mortality will increase, the lower ranked host may be utilised to increase the chance of survival. If this is true, the maximum threshold for number of eggs per seed for the test which included *Ch. prolifer* would be equal to a

mean of 2.0 (calculated using values in Table 6.3), and the threshold for *C. multiflorus* would be a mean of 3.7 (calculated using values in Table 6.4). This assumes no further eggs were laid on the control once ovipositing resumed on the test plant, however, this is not known because eggs were not counted at time intervals during the experiment, only at the conclusion.

The threshold is calculated by determining the point where the pods became acceptable, which for *Ch. prolifer* was in the treatment where control and test plant oviposition resource was equal, and mean number of eggs per seed on the control was 2.0. Eggs were laid on *C. multiflorus* when the control resource was half that of the test plant and mean number of eggs per seed on the control was 3.7. It is likely that threshold levels would vary between higher and lower ranked hosts. The lower the ranking of the host, the higher the threshold of acceptance. In this investigation *Ch. prolifer* is ranked higher than *C. multiflorus* (while *C. scoparius* ranks higher than them both) based on the higher number of eggs that were laid on this species. Therefore *Ch. prolifer* may have a lower threshold of acceptance for oviposition and be laid on earlier than the lower ranked host *C. multiflorus* (Courtney et al. 1989). This was not tested, but another series of choice tests comparing the oviposition preference of *B. villosus* between these two plants could confirm this ranking.

Such information could have implications for field tests since resource factors such as the relative abundance of the test plant species and the likelihood of an encounter by the insect will influence test results. It is unknown how many beetles were responsible for the eggs laid on *Ch. prolifer* as individual variation is masked by testing in groups (Singer 1986, Thompson and Pellmyr 1991, Jallow and Zalucki 1996b, Schoonhoven et al. 1998). No time series data is available nor is it known how oviposition decisions are made by *B. villosus*.

6.6.3 Egg-loading

The overall mean number of eggs laid per female per replicate by the New Zealand field collected beetles tested in this experiment was more than twice (22.3) that of 1985 UK imported beetles (9.1 eggs) (section 7.5), which suggests these beetles had a higher egg-load than their imported counterparts. Egg-load could explain why the original 1985 host specificity tests were not indicative of field host range. Papaj & Rausher (1983), Jaenike (1990), Odendaal & Rausher (1990) and Prokopy et al (1994) report that egg-load

is a factor that influences an insect's oviposition behaviour. Courtney et al (1989) in a hierarchy threshold model predicted that diet breadth would increase with increasing egg-load.

6.6.4 Methodology

6.6.4.1 Replication

In this investigation, eggs were laid on *Ch. prolifer* in the treatment that closely replicated the design of the original host specificity tests conducted in 1985 in which no eggs were laid. There were some small differences in methodology, other than increased replication, which may also have contributed to the outcomes. Beetles were field collected from *C. scoparius* in New Zealand whereas the beetles used in the original tests were collected upon emergence from *C. scoparius* and imported from the UK to quarantine in New Zealand. Despite the fact imported beetles laid less eggs on average than the New Zealand field collected beetles (section 6.5) this is not likely to explain the differences (section 5.6 and 7.6).

In this investigation four replicates were identical to the original host specificity tests conducted in 1985 (section 1.7.2.1). In 1985 no eggs were laid on *Ch. prolifer* in two replicates (Syrett and O'Donnell 1987), however by doubling the initial level of replication, a total of 20 eggs were laid on this test plant. One explanation for this finding is that by increasing the level of replication (Ruxton and Colegrave 2003), variation in oviposition preference within the *B. villosus* population was detected. Overall 18.8% of the replicates laid no eggs on *Ch. prolifer*, which is suggestive of just such an effect. This factor of individual variation will be examined in more detail in Chapter 8.

6.6.4.2 Sequential testing

Approximately 15 generations separates the two groups of beetles, that are otherwise believed to be of the same genotype, given that they were collected from the same plants (*C. scoparius*) from the same part of the UK (Silwood Park, Ascot). The possibility that a lack of overwintering could have been responsible for aberrant behaviour of *B. villosus* tested in 1985 was investigated in Chapter 5 and discounted. Another difference in methodology was that independent groups of beetles were used in these tests whereas the same beetles were used sequentially on all test plants in 1985. It was initially thought that the sequential nature of the experiments may have altered the preference of *B. villosus* by

increasing the level of experience toward *C. scoparius* pods over time, in turn reducing the acceptability of the other plant species. This is unlikely to be a significant factor because *Ch. prolifer* was the first plant species to be tested in the original tests, as well as in these tests in 2001, so the beetle behaviour would not have been affected in this way for this plant.

Due to differences in phenology, the test plant species bore pods at different times so that not all of the plants could be tested at the same time. Despite the fact that tests ran over ten weeks no significant difference in the mean number of eggs laid on *C. scoparius* was observed, which suggests that reproductive maturity of *B. villosus* was similar throughout all tests (Table 6.2). This result differs from the experience in 1985. In 1985 the same beetles were used sequentially (section 1.7.2.1) for all test plant species, suggesting that the reduced egg laying rate on the control over time may have been due to the declining fecundity of individuals. In this study, independent groups of beetles were used for each test plant in the sequence and prior to use in the experiment were held in cages without access to pods so oviposition did not occur beforehand. This was done to try and minmise the effects of past experience.

The orientation of plant material (horizontal versus vertical) (section 6.5.1.4) and cage-size (small versus large) (section 6.5.1.3) did not significantly affect oviposition in this investigation of two-choice tests, but oviposition resource quantity did affect oviposition behaviour (section 6.5.1.2). These findings suggest *B. villosus* will oviposit in a wide range of conditions regardless of factors such as cage size and orientation of resource. Cage size has been known to induce or inhibit oviposition in host range testing of a number of other insects (Marohasy 1998, Sands and Van Driesche 2000).

6.6.4.3 Problems

In replicates where 60 seeds of the test plant and 60 seeds of the control were required, difficulty was experienced keeping the plant material sufficiently separate in the small Prestige® boxes to ascertain true oviposition preferences (footnote Table 6.1). This was especially the case with *C. multiflorus* where an average mean number of 60 pods was required to get 60 seeds and also with *G. monspessulana*, where on average 15.7 pods were used. In comparison, for the control *C. scoparius*, an average of only 7.6 pods was needed to reach the required seed resource.

6.7 Conclusion

In conclusion, it seems that none of the factors tested here adequately explain why the original host specificity tests failed to identify *Ch. prolifer* as an alternative host for *B. villosus*. The most pertinent observation appears to be that more eggs were laid on a low ranked plant, *Ch. prolifer*, in the choice tests when lower proportions of the preferred oviposition resource *C. scoparius* were available. In addition, it appears that variation in behaviour of groups of *B. villosus* in these choice tests further complicated results, with 81.3% of replicates showing egg laying on *Ch. prolifer*, suggesting that if greater replication (involving more beetles) than the two replicates (ten beetles) used in 1985 had been done, the acceptability of *Ch. prolifer* to *B. villosus* may have been detected. These results led to further investigations into increased sample size and individual variation in behaviour (Chapter 7 and 8) to help identify the reason for the previous false negative result. In this study, oviposition resource quantity was found to be a significant factor affecting use of lower ranked hosts, highlighting the importance of taking account of results of both choice and no-choice tests for predicting host range.

Linking page: Chapter 7

In Chapter 6 it was found that oviposition on *Chamaecytisus prolifer* was not significantly affected by cage size or the orientation of test plant material provided. However, the quantity of test plant material presented significantly affected the number of eggs laid on the control treatment (*Cytisus scoparius*). When the control treatment resource was halved in relation to that of the test plants *Ch. prolifer* and *C. multiflorus*, more eggs were laid on the test plant. Beetles laid on *C. scoparius* and *Ch. prolifer* in at least one replicate of all treatment combinations. No other significant factors associated with the testing procedure were identified in Chapter 5 or 6 so a decision was made to import *Bruchidius villosus* from the original source population in the UK. The hypothesis for Chapter 7 was that if the imported beetles refused to oviposit on *Ch. prolifer* in tests carried out in the same way as the original tests then *B. villosus* has undergone a host range expansion since its introduction to New Zealand.

Chapter 7: Has a host range expansion occurred to explain the unpredicted attack of *Chamaecytisus prolifer* by *Bruchidius villosus*?*

7.1 Abstract

In 1999 *Bruchidius villosus* was discovered developing in the seeds of an unpredicted host, *Chamaecytisus prolifer*. Although the original choice tests failed to predict acceptance of *Ch. prolifer* by ovipositing females, the current population in New Zealand clearly finds this species an acceptable host (Chapter 2, 4, 5 and 6). One possibility is that a host range expansion might have occurred in this weed biological control agent in the years between release and 1999. To test this, beetles from the original population (Silwood Park, UK) were imported and the original handling and host choice tests were replicated. Despite showing a strong preference for *Cytisus scoparius* the UK beetles tested in this study did accept *Ch. prolifer* for oviposition. These results allow us to discount the possibility that a host range expansion has occurred in New Zealand.

7.2 Introduction

Host specificity testing of *Bruchidius villosus* in the UK began in 1985 and comprised no-choice oviposition tests in which adults were confined to either whole potted plants or to single branches of larger plants inside cotton mesh sleeve cages (Syrett and O'Donnell 1987). Seven species of potted non-target plants were tested together with *Cytisus scoparius* in a choice test within a field cage in the UK (Syrett and O'Donnell 1987). Cut shoots of nine plant species (including *Chamaecytisus prolifer*) were tested in two-choice tests with *C. scoparius* in the laboratory in New Zealand. In all these assays, eggs were laid only on *Cytisus* species (*C. scoparius* and *C. praecox* cv. Allgold) (section 1.7.1.2 and 1.7.2.1).

In 1999, however, *B. villosus* was found emerging from *Ch. prolifer* seeds in New Zealand, and further studies showed that this plant was a suitable and commonly used alternative host (Syrett, 1999). At the time *Ch. prolifer* had only been tested in choice

* The material in this Chapter was presented as a conference paper and has been published in the conference proceedings (see Appendix I).

tests with *C. scoparius* as a control, in quarantine in both New Zealand and Australia (Sheppard and Hodge, 1994).

All previous experiments have failed to definitely explain this false negative result, although a lack of replication coupled with high variability in oviposition behaviour may explain the result.

It also seemed plausible that a host range expansion (Dennill et al. 1993) (otherwise referred to as a host shift, (Howarth 1991)) had occurred in the population of established beetles in New Zealand some time in the 14 years since its introduction (Syrett 1999). Recent literature suggests that significant changes in host use can evolve rapidly within 10s or 100s of generations (Thompson 1998). Many purported host range expansions, defined by Marohasy (1996) as 'feeding by biological control agents on plant species other than those on which they were known to feed prior to their release', have been reported in weed biological control. Marohasy (1996) argued that these were generally caused by other phenomena, such as preadaptation (established behavioural concepts), threshold change as a result of host deprivation, or effects of experience (learning).

This study aimed to test whether a host range expansion may have occurred in *B. villosus*. Oviposition acceptance behaviour of the current New Zealand population of *B. villosus* was compared with beetles collected from Silwood Park, the same field site from which the original beetles were collected for shipment to New Zealand in the 1980s. The hypothesis was if British beetles still refused to accept *Ch. prolifer* for oviposition, using similar testing methods but with higher levels of replication, while their New Zealand progeny now accepted it, then a host range expansion would be a possible explanation.

7.3 Methods

Adult *B. villosus* beetles were beaten from *C. scoparius* at Silwood Park, U.K. in summer 2002. These beetles were placed into 1 m diameter by 2 m long, 1 mm mesh sleeve cages on branches of *C. scoparius* bearing young pods. When pods turned black they were picked from the sleeves and held in a glasshouse in mesh bags until beetles emerged. The emerged adult beetles were imported into quarantine in New Zealand. Replicating the procedure used in 1985 as closely as possible, 150 adults were maintained in Perspex® cages (500 × 500 × 500 mm) with sponge doors (365 × 360 × 50 mm), with ample bee

pollen and honey water, followed by *C. scoparius* flowers, under a 22:16°C (day:night) temperature regime with a day length of 14:10 h L:D.

Host specificity tests undertaken in the original study in 1985 were replicated as far as possible in 2002 using UK beetles. The procedures recorded in the original quarantine laboratory books were followed as closely as possible, although minor differences were necessary with regard to timing and experimental design.

Perspex® Prestige® cages (220 × 130 × 100 mm), with flexible push-on lids and four, 25 mm diameter gauze-covered holes for ventilation, were used as test cages. Moistened blotting paper was placed at the bottom of the cage, and several pieces of tissue paper were included to absorb excreta. *Cytisus scoparius* twigs (approximately 200 mm long) bearing young green pods were placed in vials of water in each test cage. A disc of plastizote, 6 mm thick, with the twigs pushed through its centre acted as a stopper for the vial, which was supported on an angle to ensure the shoot remained in the water. Twigs of each test plant were selected that had approximately equal amounts of pod material and pods judged to be at an equivalent developmental stage to the *C. scoparius* pods. Test material of the different plant species, prepared in the same way as the *C. scoparius*, was placed in each cage with an equivalent amount of *C. scoparius*, to constitute paired choice tests comprising *C. scoparius* and a test plant (Syrett and O'Donnell 1987).

Test plant material was collected from at least three different individual plants for each species. Beetles were held in each test cage for six days at 22:16°C day-night temperatures with a 14:10 h L:D regime during the tests. Relative humidity was approximately 70%. Beetles were fed pollen and provided with cotton dental rolls soaked in a honey water solution. After the beetles were removed all plant material and cages were carefully examined for eggs. The numbers of eggs found on the pods of *C. scoparius* and each of the test species were recorded. Each phase of the experiment was conducted when each of the test plant species had pods available at the appropriate stage of development (Table 7.1) so not all plant species were tested at the same time. Every attempt was made to ensure laboratory conditions, cage type used, number and sex ratio of beetles, bee pollen source, twig size, approximate number of pods presented, the presentation of pod material, duration of assays, and approximate timing of presentation of various host plants, were the same as in the previous experiments.

In the original choice tests conducted in 1985 only one or two replicates were used for each test plant species, whereas ten replicates for each test plant were used in 2002. Each replicate contained five male and five female beetles as in 1985, and tests were run sequentially (the same groups of beetles were reused on each test plant species).

Table 7.1: Timing of two-choice cut shoot oviposition tests for *Bruchidius villosus* imported from the UK. The tests included the target weed *Cytisus scoparius* and the following plant species; *Carmichaelia australis, Carmichaelia petriei, Carmichaelia stevensonii, Carmichaelia williamsii, Chamaecytisus prolifer, Clianthus puniceus, Cytisus multiflorus, Genista monspessulana, Laburnum anagyroides* and *Sophora microphylla*

Weeks	1985 UK import (1 or 2 reps)	2002 UK import (10 reps)
1–5. (2nd week Sept – 3rd week Oct)	*C. scoparius* flowers and bee pollen	*C. scoparius* + *Ch. prolifer* flowers and bee pollen
6. (4th week Oct)	*C. scoparius* flowers, green pods and bee pollen	*C. scoparius* vs *Ch. prolifer* No eggs laid
7. (1st week Nov)	*C. scoparius* flowers, green pods and bee pollen. First eggs laid	*C. scoparius* vs *Ch. prolifer* No eggs laid
8. (2nd week Nov)	*C. scoparius vs Ch. prolifer*	*C. scoparius* vs *Ch. prolifer* First eggs laid
9. (3rd week Nov)	*C. scoparius* vs *So. microphylla*	*C. scoparius* vs *So. microphylla*
10. (4th week Nov)	-	*C. scoparius* vs *Ca. australis*
11. (1st week Dec)	*C. scoparius* vs *C. australis*	*C. scoparius* vs *Ca. petriei*
12. (2nd week Dec)	*C. scoparius* vs *Ca. petriei, Ca. williamsii, G. monspessulana, Cl. puniceus* & *C. multiflorus*	*C. scoparius* vs *Ca. williamsii*
13. (3rd week Dec)	Repeated *C. scoparius* vs *C. multiflorus*	*C. scoparius* vs *Cl. puniceus*
14. (4th week Dec)	-	*C. scoparius* vs *C. multiflorus*
15. (1st week Jan)	-	*C. scoparius* vs *G. monspessulana*
16. (2nd week Jan)	-	*C. scoparius* vs *Ca. stevensonii*
17. (3rd week Jan)	*C. scoparius* vs *Ca. stevensonii*	*C. scoparius* vs *L. anagyroides*

Note: in Syrett and O'Donnell (1987) *Ch. prolifer* was referred to as *C. palmensis* (Christ) Bisby & Nicholls, *C. australis* as *C. ovata* G.Simpson, and *C. stevensonii* as *Chordospartium stevensonii*

7.4 Analysis

A two-sample t-test (Minitab 2000) was used to test for differences in mean numbers of eggs laid by *B. villosus* on test and control plants in the 1985 and 2002 experiments.

7.5 Results

In 1985 female *B. villosus* laid a mean of between 4.2 and 18.4 eggs per replicate on *C. scoparius* and 0 eggs on the test plants. In 2002 tests *B. villosus* laid a mean of between 3.0 and 12.3 eggs per replicate on *C. scoparius*, and 0.7 eggs on the test plant *Ch. prolifer*. The range of number of eggs laid by an individual female on *Ch. prolifer* in 2002 was between 0 and 2.6. In six of the ten replicates with *Ch. prolifer* female beetles laid no eggs on the test plant (Table 7.2).

Table 7.2: Mean number of eggs laid per *Bruchidius villosus* female in a two-choice cut shoot test run sequentially over time. The tests included the host plant *C. scoparius* and the test plant species. (In addition to the following plants a further three were tested but the results are not presented here).

	1985 UK import		2002 UK import (10 replicates)	
Plant material	**test plant**	***C. scoparius***	**test plant**	***C. scoparius***
Chamaecytisus prolifer	0**	18.7	0.7	12.3
Sophora microphylla	0**	6.2	0	4.8
Carmichaelia australis	0	12.2	0	7.4
Carmichaelia petriei	0**	7.0	0	9.2
Carmichaelia williamsii	0	4.2	0	8.5
Genista monspessulana	0	10.0	0	9.5
Clianthus puniceus	0	7.6	0	4.3
Cytisus multiflorus	0	12.2	0	9.0
Carmichaelia stevensonii	0	4.2	0	5.3
Laburnum anagyroides	-	-	0	3.0

Key ** plant species replicated twice, all others in 1985 were replicated once

In the ten replicates in 2002 with UK imported beetles, beetles laid on only four of the replicates of *Ch. prolifer*. The overall mean number of eggs per female per replicate was 9.1 and 7.9, for beetles in 1985 and in 2002, respectively (excluding *L. anagyroides* which was an extra plant in the 2002 sequence). There was no significant difference in the overall mean number of eggs laid per female per replicate on *C. scoparius* between the sequential choice tests conducted in 1985 and 2002 with beetles imported from the UK ($t = 0.7$, $df = 12$, $P > 0.05$).

7.6 Discussion

For an expansion in fundamental host range to occur in phytophagous insects, so that an insect can move from one host plant to another, a 'host race' must first develop. To be

classified as a host race (as defined in Marohasy (1996)) populations must first fulfill the following criteria: 1. be non-interbreeding and sympatric, 2. differ in biological characteristics but not (or only marginally) in morphology, and finally 3. be prevented from interbreeding as a result either of preference for different host plant species, or as a consequence of physiological adaptation to different host plant species.

So which of the above criteria have either been fulfilled or have the potential to be fulfilled in New Zealand, with *B. villosus*? Firstly, there is no indication at this stage that *B. villosus* adults emerging from both *C. scoparius* and *Ch. prolifer* are not interbreeding. Beetles emerging from both species of pods at similar times have been observed mating (M. Haines, personal observation). Furthermore both plant species frequently grow in the same area, and within the same habitats in New Zealand (no geographical isolation). *Bruchidius villosus* show high mobility (L. Hayes, unpublished data) and therefore it appears that beetles continue to interbreed after emerging from different host pods. Seasonal asynchrony is, however, a possible mechanism that could also lead to sympatric separation. Certainly *Ch. prolifer* flowers earlier than *C. scoparius* in spring and is the first pollen source available to *B. villosus* when it emerges from its overwintering period (Fowler et al. 2000). However, *Ch. prolifer* flowers for a longer period and simultaneously with *C. scoparius* over summer, suggesting seasonal asynchrony in New Zealand may be insufficient to lead to sympatric separation or to prevent interbreeding.

Secondly, laboratory studies will be used to investigate whether or not lines of *B. villosus* reared from different host plant pods retain oviposition preferences for the species of pod in which they spent their larval development (Chapter 9).

Thirdly, it would be necessary to establish that *B. villosus* is in the process of being prevented from interbreeding as a result of a preference developing for the new host plant species. *Cytisus scoparius* remains the preferred host over *Ch. prolifer* in all choice tests to date (Chapter 2, 4, 5, 6, 8 and 9), suggesting that no preference has yet developed for *Ch. prolifer.* Test results confirm this (section 6.5.1.5), as beetles randomly collected from the field laid on average 25 times as many eggs on *C. scoparius* as on *Ch. prolifer.*

So it appears that the attributes that would indicate a host race has developed, or is in the early stages of developing in *B. villosus,* are not present. The fact that some imported UK beetles accepted *Ch. prolifer* does not indicate host range expansion has

occurred in New Zealand, but instead that for some reason the 1985 tests failed to elicit oviposition on *Ch. prolifer.*

There are at least two possible explanations for the discrepancy in laboratory testing results between 1985 (no eggs were laid on *Ch. prolifer*) and 2002 (some eggs were laid on *Ch. prolifer*). There were differences in the number of *B. villosus* tested (smaller sample sizes in 1985), and beetles may have been treated subtly differently between tests despite best attempts to replicate conditions (Table 7.1). For example, the 1985 beetles were held for two weeks prior to testing with very small pods and flowers of *C. scoparius*, which may have caused greater motivation towards *C. scoparius* in 1985. In 2002 beetles were held prior to testing with pods of both *C. scoparius* and *Ch. prolifer* at the same time, to check for onset of oviposition. In both cases all beetles imported from the UK had never experienced *Ch. prolifer* pods prior to being imported into New Zealand quarantine. All testing was conducted sequentially, in both 1985 and 2002 the same groups of beetles were reused for each test plant.

Having ruled out a host range expansion, why did the original choice tests not indicate some acceptability of *Ch. prolifer* pods? The hierarchy-threshold model of host selection (Courtney et al. 1989) hypothesises that insects rank hosts in a hierarchical fashion and that selection of diet by individual insects is determined by the hosts 'acceptability'. One prediction of the model is that female oviposition behaviour is influenced by female egg-load, such that when egg-load is high, so is the tendency for a wider range of hosts to become acceptable (Courtney et al. 1989). So could egg-load explain why the original host tests were not indicative of field host range? Eggs laid per female in the 2002 imported beetles were approximately a third lower than that of the 1985 population (Table 7.2). Yet the lower-ranking host *Ch. prolifer* was still accepted for oviposition at an equivalent rate despite the reduced egg laying. So it appears unlikely that egg-load is responsible for the discrepancy in the 1985 test results (suggested as a possibility in section 6.6).

From the 2002 test results which indicate that *B. villosus* laid approximately 18 times as many eggs on *C. scoparius* as on *Ch. prolifer* (Table 7.2) we might have predicted that its non-target impact in the field would be minor, however this does not take into consideration the population levels of the beetles. The level of seed attack by the beetle in New Zealand is in fact substantial with up to 40% of seeds destroyed in the field (M. Haines, unpublished data). On a more positive note, despite the non-target

attack on the exotic plant *Ch. prolifer*, *B. villosus* remains a useful agent against *C. scoparius* in New Zealand as all the test results consistently predict that no native Fabaceae are under any risk of attack (Chapter 2, 4 and 6), and no field attack has been observed on these plants.

7.7 Conclusion

A host range expansion in *B. villosus* is ruled out. The implication for biological control releases from these results, is that we cannot assume non-target impacts will be insignificant on the grounds that results of choice tests indicate a strong preference for the target plant.

Linking page: Chapter 8

In Chapter 7 *Bruchidius villosus* oviposited on *Chamaecytisus prolifer* in some but not all replicates ruling out the possibility of a host range expansion having occurred in New Zealand. However, in the choice tests between *Cytisus scoparius* and *Ch. prolifer*, eggs were laid on *Ch. prolifer* in only 40% of the replicates indicating variable oviposition behaviour in the population. The hypothesis for Chapter 8 was that the original host specificity tests failed to predict *Ch. prolifer* was an acceptable host because of individual variation in oviposition behaviour and the small sample size of beetles tested.

Chapter 8: Can individual variation in host preference by *Bruchidius villosus* explain the unpredicted use of *Chamaecytisus prolifer* by *Bruchidius villosus*?

8.1 Abstract

It is common practice to use groups of insects in host specificity testing and individual variation in oviposition behaviour is rarely studied. Two-choice oviposition tests, using a number of test plant species were carried out to determine variation in host plant preference of individual female *Bruchidius villosus* from two populations from New Zealand and the UK. The results were compared with the results of a study in which groups of *B. villosus* females were used to determine oviposition preferences between host plants. Results showed a high level of variation in oviposition preference between individual *B. villosus* for the less preferred host *Chamaecytisus prolifer*. New Zealand field collected *B. villosus* all laid eggs on *Cytisus scoparius*, while 59% also laid eggs on *Ch. prolifer*. Similarly, all the beetles in the UK sample laid eggs on *C. scoparius* but only 40% also laid on *Ch. prolifer*. The preference for *C. scoparius* in both populations declined over time as the choice tests were repeated. Because a significant proportion of both populations found *Ch. prolifer* acceptable it is proposed that large sample sizes are needed to have a high chance of eliciting an accurate oviposition response. Small population samples in choice tests increase the likelihood of a false negative result (or Type II errors) as presumably occurred with the original tests. This study highlights the importance of assessing the level of behavioural variability within insect populations to improve the reliability of prediction of host range tests.

8.2 Introduction

Many cases of variation in oviposition preference within and between populations of insects among host plant species have been reported (Tabashnik et al. 1981, Wiklund 1981, Rausher and Papaj 1983, Ng 1988, Fox 1993, Janz et al. 1994, Jallow and Zalucki 1996a, 1996b, Schoonhoven et al. 1998, Singer and Lee 2000). Singer (2000) defines oviposition preference as a behavioural trait of an insect that can be defined

quantitatively, and therefore measured experimentally, and compared among individual insects.

Host choice by individual ovipositing females can be influenced by many different factors. Courtney and Kibota (1990) (sensu Tinbergen 1963, Charnov and Skinner 1985) categorise these factors into four groups; 1) proximate causes such as the interaction of physiology with sensory cues, 2) ontogenetic causes that includes all developmental changes such as learning, 3) ultimate causes that reflect the action of natural selection and 4) phylogenetic causes that are determined by evolutionary history.

Proximate causes that influence whether a female oviposits on a host, include factors such as female egg-load (Papaj and Rausher 1983, Jaenike 1990, Odendaal and Rausher 1990, Prokopy et al. 1994), age (Gossard and Jones 1977), number of matings (Watanabe 1988), environmental factors (Horner and Abrahamson 1999, Cronin et al. 2001), host plant quality (Leather 1994, Awmack and Leather 2002, Leather 2002), time since last oviposition experience (Singer 1982, Horton and Krysan 1991, Withers et al. 2000), stage of host development (Messina 1984, Heard 1995b), host density (Diaz-Fleischer and Aluja 2003), host egg-load (Messina and Renwick 1985, Heard 1995a), and intra-specific variation in host acceptability (Singer and Parmesan 1993, Singer and Lee 2000). Ontogenetic causes affecting oviposition are, for example, prior adult female experience (Rausher 1983a, b, Papaj and Prokopy 1988, Szentesi and Jermy 1990, Bernays 1995) and larval experience (Jermy et al. 1968), while phylogenetic causes such as genetic variation in preference among individual insects have also been shown to be influential (Tabashnik et al. 1981, Rausher 1983a, Futuyma and Peterson 1985, Jaenike 1988, Singer et al. 1988, Via 1990, Jallow and Zalucki 1996b).

Oviposition preference for different hosts is an important trait to estimate in the testing of potential biological control agents. In host specificity testing it is necessary to ensure that the above factors are standardised as much as possible within the groups of test insects and plants to minimise variation. Greater precision allows results to better predict the host range of the insect species when it is released in the field. Some influential variables are not easy to control in the laboratory, and this, along with an often inadequate range of alternative hosts examined (due to restrictions on time, money and availability of insects) can greatly over-simplify the complex system that exists in the field. Such simplification can result in poor decisions concerning biological control importations.

Choice tests and no-choice tests are commonly used in host specificity testing (Sheppard 1999). The pros and cons of these methods are well discussed in the literature (Cullen 1990, Withers 1997, Marohasy 1998, Hill 1999, Withers et al. 1999, Heard 2000, Barton Browne and Withers 2002). One common feature of both these types of assays is that groups of insects are invariably used (Heard 1997), but clearly the level of intraspecific behavioural variability in potential biocontrol agents needs to be more carefully investigated. Most important, the number of individual insects used in any test must be high enough to detect rare behavioural traits, and/or to overcome the problems associated with a potentially high degree of behavioural variability.

A number of authors point out the shortfalls of using groups for host specificity testing Schoonhoven et al. (1998) state that "the significance of individuals with host choices deviating from normal is often underestimated because of an emphasis on the average amounting almost to a fetish". Singer (1986) suggests that the mean preference of a group of insects has much less predictive value than the proportions of insects with specific preferences, and Jallow & Zalucki (1996b) and Thompson & Pellmyr (1991) show that testing in groups can mask the true level of variation within the population. More generally, Bolnick (2003) suggests that describing a species as the sum or average of its parts can vastly over simplify both empirical data collection and theoretical models. To fully understand the level of behavioural variability within a population of test insects and how it affects the interpretation of host specificity test results, the behaviour of individual insects needs to be studied.

This study aims to compare the results of choice oviposition tests that use and measure the behaviour of individual insects with a previous study carried out by Syrett and O'Donnell (1987) that used groups of insects.

Syrett and O'Donnell (1987) carried out two-choice oviposition tests on the biocontrol agent *Bruchidius villosus* (a seed-feeder), its host plant *Cytisus scoparius* and the test plant *Chamaecytisus prolifer* under quarantine conditions in New Zealand. In these tests, groups of insects were used and the results suggested that *Ch. prolifer* was an unsuitable host for *B. villosus* as no eggs were laid on this host. However, *B. villosus* now clearly finds *Ch. prolifer* a suitable host in the field in New Zealand, 16 years after its introduction. The possibility of host range expansion (Chapter 7) and species level genetic variation within the two test populations of *B. villosus* (Chapter 3) has been ruled out in previous chapters. The hypothesis tested in this Chapter was that if individual

variability in acceptance for a less preferred host (*Ch. prolifer*) is high in the *B. villosus* population, then a small sample of insects used in choice tests is more likely to deliver a false negative result. An additional objective was to calculate the probability of selecting a sample of beetles which would lay no eggs on *Ch. prolifer*.

8.3 Methods

The original source of the field collected New Zealand *B. villosus* was Silwood Park, UK. Therefore the two populations described in the methodology below share the same ancestry, but the New Zealand beetles had been geographically separated from the source population for a period of 15 generations when these experiments were conducted.

8.3.1 New Zealand field collected beetles

In October 2001, 40 terminal branches from both *C. scoparius* and *Ch. prolifer* bearing flowers and small green pods were sleeved, to prevent oviposition by *B. villosus*, in field plots at Lincoln, New Zealand. The individual plants chosen to represent both species were those that in previous seasons had had abundant numbers of eggs laid on their pods by *B. villosus* suggesting that the plant phenotype was acceptable to at least some of the beetle population.

One hundred *B. villosus* were caught from the field (from *C. scoparius*) and placed in a Perspex® cage (500 × 500 × 500 mm) with a sponge door (365 × 360 × 50 mm), and cotton rolls soaked in honey water, *C. scoparius* flowers, and bee pollen, for three weeks. From this cage 30 females were collected, and each female was placed into a separate Perspex® Prestige® box (220 × 130 × 100 mm) with a flexible push-on lid and four, 25 mm diameter gauze-covered holes for ventilation. Moistened blotting paper was placed at the bottom of the cage, and several pieces of tissue paper were included to absorb excreta. One shoot each of *C. scoparius* and *Ch. prolifer* (approximately 200 mm long, collected from within the sleeves in the field) bearing equivalent numbers of young green pods (the part of the plant on which *B. villosus* oviposits), at an equivalent stage of development, were placed in vials of water in each test cage. A disc of 6 mm thick plastizote, with the base of the shoot pushed through the centre acted as a stopper for each vial. The shoots were supported on an angle to ensure they remained in the water. The two vials containing the plant material were placed at opposite ends of the test cages, so that when the plant material was placed horizontally on the base of the cage the two host

species were parallel to each other. The two plant species were arranged randomly to eliminate potential position effects within the cages. This general methodology is identical to that used by Syrett and O'Donnell (1987).

Test plant material was collected from three different plants from within each species (this effect is tested in 8.5.5). Beetles were held in each test cage for six days at 22:16°C day-night temperatures with a 14:10 h L: D regime during the tests. Relative humidity was approximately 70%. Beetles were fed pollen and provided with cotton dental rolls soaked in a honey water solution. Every two days, for six days, the number of eggs laid on each of the two plant species was counted. Every attempt was made to ensure laboratory conditions, cage type used, bee pollen source, shoot size, approximate number of pods presented, the presentation of pod material, and duration of assays were the same as in the original experiment conducted by Syrett and O'Donnell (1987).

8.3.2 Recently imported UK beetles

In June 2002, adult *B. villosus* were beaten from *C. scoparius* at Silwood Park, U.K. These beetles were placed into 1m diameter by 2 m long, 1 mm mesh sleeve cages on branches of *C. scoparius* bearing young pods. In July, infested pods were picked from the sleeves and held in a glasshouse in mesh bags until emergence. The emerged adult beetles were imported into quarantine in New Zealand in August 2002 (section 7.3). Replicating the same procedure as carried out in the study by Syrett and O'Donnell (1987), 15 females were maintained in Perspex® cages with bee pollen, *C. scoparius* flowers and honey water, under a 22:16°C (day:night) temperature regime with a day length of 14:10 h L:D for six weeks. In October 2002, twenty terminal branches from both *C. scoparius* and *Ch. prolifer* bearing flowers and small green pods were sleeved, to prevent oviposition by *B. villosus*, in field plots at Lincoln, New Zealand. Test plant material was collected from two different plants within each species. From the cage containing 15 females, ten were collected, and each individual female was placed into a Perspex® cage as described for the New Zealand field collected beetles.

8.4 Analysis

The data were tested for normality and homogeneity of variances using the Kolmogorov-Smirnov test and Levene's test, respectively. Differences in egg laying among New Zealand and UK beetles were analysed using two-sample t-tests (Minitab

2000). Because the data did not fit a normal distribution it was log transformed before analysis (Zar 1984). To determine the level of variation in the data coefficients of variation were calculated.

To explore the influence of small or inadequate sample sizes on the outcome of a host specificity test, the observed data were used to estimate the probability of an individual female laying no eggs. To do this, an appropriate theoretical distribution was fitted to each data set in accordance to how the data was dispersed (random or clumped). The theoretical probability distribution was then used to estimate the zero probability (of laying no eggs) for each data set. A negative binomial distribution was fitted to the New Zealand data, and a Poisson distribution to the UK data. Variance-mean ratios were calculated to further confirm the nature of the dispersion of the data in each set. The estimated frequency of non-laying females from the combined data was then used in a binomial distribution function to estimate the probability of selecting samples of varying composition (Sheskin 2000) (GenStat 2002). It is important to note here, however, that because the data were not independent of each other this analysis must be considered as exploratory only.

To test for a significant difference in species preferences between host plants a 'coefficient of preference' (Heard 1995a, Withers et al. 2000) was calculated using the formula stated in 5.4. Differences in preference over time were calculated using a sign-test (Minitab 2000).

A one-way ANOVA (Minitab 2000) was used to test for differences in numbers of eggs laid between conspecific plants of the same species in tests which included New Zealand beetles. Because the data for UK beetles could not be normalised, a nonparametric sign-test (Minitab 2000) on the median number of eggs was used to analyse differences in the number of eggs laid by UK beetles between the two conspecific plants used to represent each species.

8.5 Results

8.5.1 Oviposition by New Zealand field collected beetles

The overall mean number of eggs laid per beetle on *C scoparius* was 8.6 ± 1.6 SEM and 6.5 ±1.8 SEM on *Ch. prolifer* (log mean number of eggs presented in Table 8.1). The

CV% was high 97.0% for *C. scoparius* and 148.2% for *Ch. prolifer* indicating considerable variability in the oviposition rates of individuals.

Table 8.1: Log mean number (no.) of eggs laid by field collected New Zealand *Bruchidius villosus* (n = 29) on *Cytisus scoparius* and *Chamaecytisus prolifer* in a two-choice cut shoot test.

Oviposition and Pod factors	*C. scoparius*	*Ch. prolifer*
Log mean no. eggs laid per pod ± SEM	0.9 ± 0.05	0.5 ± 0.1
Mean pod length (mm) ± SEM	35.5 ± 0.3	41.3 ± 0.3
Mean seeds per pod ± SEM	6.8 ± 0.1	6.2 ± 0.1

The high CV% shows that there was greater variability in mean log number of eggs laid on *Ch. prolifer* than *C. scoparius*. The range in total number of eggs laid on both hosts by an individual female was 2-53.

There was a significant difference between the log number of eggs laid on each plant species ($t = 3.0$, $df = 42$, $P < 0.05$). The mean numbers of eggs laid on *C. scoparius* were significantly higher compared with the mean laid on *Ch. prolifer* indicating a preference for *C. scoparius* (Table 8.1).

Plant morphology and phenology varied between the two plant species which meant that despite attempts to present equivalent plant material to each female, pod length ($t = 13.3$, $df = 277$, $P < 0.001$) and seed number ($t = 3.1$, $df = 286$, $P < 0.05$) varied significantly between the two host plant species (Table 8.1).

Only one individual out of the original 30 females failed to lay eggs on either plant species. This beetle died three days into the experiment so was removed from the analysis. It was considered damaged in some way so was removed from the analysis as an outlier. All beetles in the trial (excluding the individual that died) laid eggs on *C. scoparius* and 17 out of the 29 females (59%) laid eggs on *Ch. prolifer* as well. Nine of the 29 females showed a higher preference for *Ch. prolifer* than the generally higher-ranked *C. scoparius* (Figure 8.1).

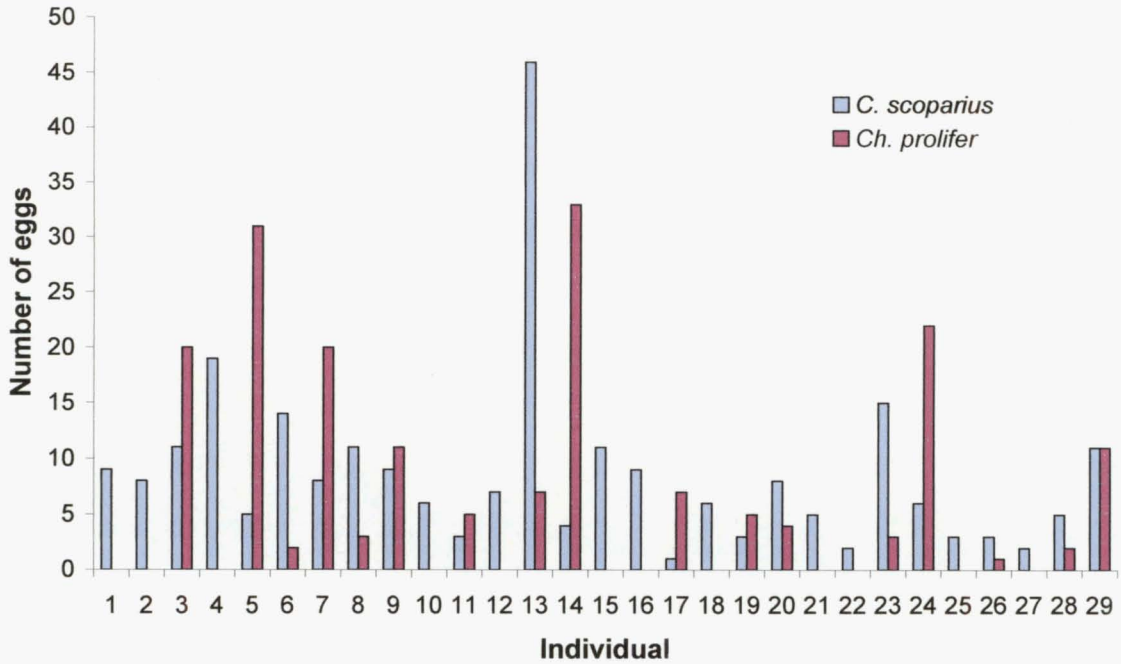


Figure 8.1: Number of eggs laid on *Cytisus scoparius* and *Chamaecytisus prolifer* by New Zealand field collected *Bruchidius villosus* in a two-choice cut shoot test.

By day two of the trial, 23 out of the 29 (79%) of the females had already laid eggs on *C. scoparius*, whereas only 3 (10%) had laid eggs on *Ch. prolifer*. By day six, 100% of the females had oviposited on *C. scoparius* but 12 (41%) still found *Ch. prolifer* unacceptable (Table 8.2).

Table 8.2: Pattern of oviposition of New Zealand field collected *Bruchidius villosus* in a two-choice cut shoot test with *Cytisus scoparius* and *Chamaecytisus prolifer*.

Day	Percentage of *B. villosus* ovipositing on *C. scoparius*	Percentage of *B. villosus* ovipositing on *Ch. prolifer*
2	79	10
4	86	41
6	100	59

If a variance to mean ratio is much larger than one it indicates aggregation (Davis 1994). The variance-mean ratio for number of eggs laid per female was 2.33 (n = 29) for *C. scoparius* which suggests that the distribution of egg laying among females is more clumped than random. The variance-mean ratio for *Ch. prolifer* however was much higher 6.7 (n = 29), indicating greater variability and aggregation than for *C. scoparius*. A negative binomial distribution fitted the individual New Zealand beetle oviposition data well (*C. scoparius*: Deviance = 11.8, df = 10, *Ch. prolifer*: Deviance = 12.7, df = 10). Using the negative binomial distribution, the probability of a female laying no eggs on *C. scoparius* was estimated as 0.001 and much higher for beetles laying on *Ch. prolifer* for which the probability was estimated as 0.4.

There was no significant difference in total number of eggs laid per beetle over the two-day intervals ($F = 0.1$, $df = 2$, $P > 0.1$).

8.5.2 Oviposition by UK *Bruchidius villosus*

A total of 166 eggs were laid by ten females and the mean number of eggs laid per beetle was 16.6 (12.7 ± 2.0 SEM on *C. scoparius* and 3.9 ± 2.0 SEM on *Ch. prolifer*) (Table 8.3). The CV% for *Ch. prolifer* was high (166.0%), suggesting that there was considerable variability within the oviposition rates of individuals, whereas the CV% for *C. scoparius* was low (50.4 %), indicating much less variation between the oviposition rates of individuals.

Table 8.3: Number of eggs laid by *Bruchidius villosus* (n = 10) imported from the UK in a two-choice cut shoot choice test containing *Cytisus scoparius* and *Chamaecytisus prolifer*.

Oviposition and Pod factors	*C. scoparius*	*Ch. prolifer*	P value
Total eggs laid	127	39	
Mean pod length (mm) ± SEM	35.1 ± 0.6	38.0 ± 0.53	< 0.001
Mean seeds per pod ± SEM	8.0 ± 0.3	6.5 ± 0.27	< 0.001
Log mean eggs laid ± SEM	1.0 ± 0.1	0.4 ± 0.17	<0.05

Because the data for *Ch. prolifer* was not normally distributed egg counts were log transformed before hypothesis testing. The range of total eggs laid per female was between 7 and 42. Significantly more eggs were laid on *C. scoparius* ($t = 3.2$, $df = 16$, $P < 0.01$), suggesting a preference for that species.

As for the New Zealand beetles, variation in plant morphology and phenology, meant that despite attempts to ensure the presentation of equivalent material, pod length and seed number differed significantly between the two plant species (Table 8.3)

Only one beetle out of ten failed to lay eggs on either plant species, despite that it was found to be fertile when dissected. All other beetles in the trial laid eggs on *C. scoparius*, while only four out of ten laid eggs on both *C. scoparius* and *Ch. prolifer*, and those that did lay on both plant species laid more on *C. scoparius* in every case (Figure 8.2).

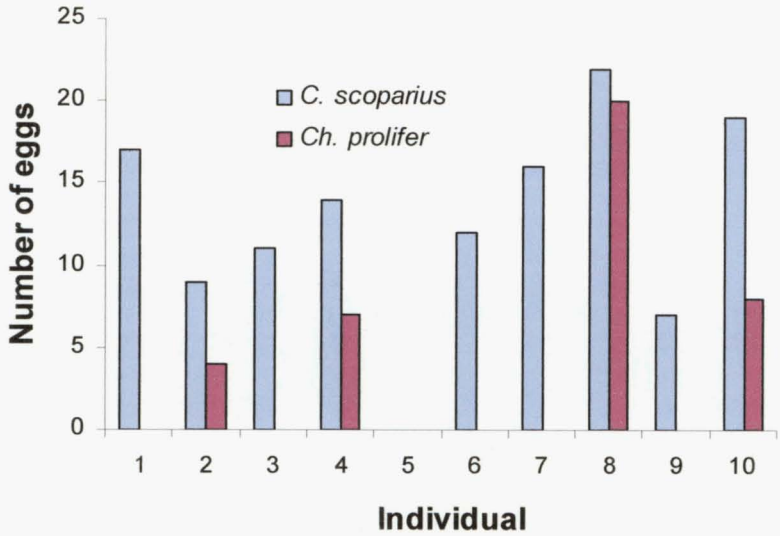


Figure 8.2: Number of eggs laid on *Cytisus scoparius* and *Chamaecytisus prolifer* by *Bruchidius villosus* imported from the UK in a two-choice cut shoot test.

On day two of the trial six out of the ten (60%) of the females had laid on *C. scoparius* whereas none had laid eggs on *Ch. prolifer*. By day six, only one had not oviposited on *C. scoparius*, whereas 60% had failed to lay eggs on *Ch. prolifer* (Table 8.4).

Table 8.4: Pattern of oviposition of imported UK *Bruchidius villosus* in a two-choice cut shoot test with *Cytisus scoparius* and *Chamaecytisus prolifer.*

Day	Percentage of *B. villosus* ovipositing on *C. scoparius*	Percentage of *B. villosus* ovipositing on *Ch. prolifer*
2	60	0
4	90	10
6	90	40

The variance-mean ratio of number of eggs laid per female was 2.0 (n = 10) for *C. scoparius* suggesting a random dispersion of egg laying. The very high variance-mean ratio of 7.0 for *Ch. prolifer* (n = 10) again confirms the greater variability of egg laying by individuals on that species than on *C. scoparius*. A Poisson and negative binomial distribution was fitted to the *C. scoparius* individual oviposition data but neither fitted the data well. A negative binomial distribution gave a better fit to the *Ch. prolifer* data (Deviance = 48.1, df = 11, P > 0.2) and the probability of an individual female laying zero eggs on *Ch. prolifer*, estimated from that distribution, was again much higher at 0.57 and higher than for New Zealand beetles. However, because of the small sample size this interpretation is cautionary.

A negative binomial distribution fitted the combined data sets (New Zealand plus UK) giving a pooled sample size of 39 adult females (*C. scoparius*: Deviance 11.5, df = 10, P > 0.2, *Ch. prolifer*: Deviance 14.7, df = 10, P > 0.2). The probability of selecting an individual beetle that would lay zero eggs was estimated as 0.002 for *C. scoparius* and 0.4 for *Ch. prolifer*.

8.5.3 Oviposition preference

Over the whole six day testing period New Zealand field collected beetles preferred (laid more eggs on) *C. scoparius* in 19 out of 29 (65.5%) cases, with 9 (31.0%) individuals exhibiting a preference for *Ch. prolifer* and 1 (3.4%) showing equal preference. Of the UK imported beetles 90% of the individuals showed a preference for *C. scoparius*. On day two of the investigation New Zealand field collected *B. villosus* had a high positive median preference coefficient that was at its maximum at 1. The positive direction of the coefficient of preference values as measured by a sign-test test indicated a signifcant preference for *C. scoparius* (P < 0.05). Any preference disappeared on days four and six where the median preference coefficient was zero indicating no preference for either *C. scoparius* or *Ch. prolifer* (Table 8.5).

Table 8.5: Percentage of New Zealand *Bruchidius villosus* individuals within specified coefficients of preference (CP) categories for oviposition over time.

Day	Number of *B. villosus*	CP < 0	CP = 0	CP > 0	Median	P value
Day 2	29	10	35	55	1	< 0.05
Day 4	29	31	38	31	0	1
Day 6	29	41	28	31	0	> 0.05

On day two and four the UK beetles showed a significant preference for *C. scoparius* (P < 0.05) as measured by a sign-test test. On day six, the median coefficient had declined to 0.59 (Table 8.6).

Table 8.6: Percentage of UK *Bruchidius villosus* individuals within specified coefficient of preference (CP) categories for oviposition over time.

Day	Number of *B. villosus*	CP < 0	CP = 0	CP > 0	Median	P value
Day 2	10	0	40	60	1	< 0.05
Day 4	10	0	10	90	1	< 0.001
Day 6	10	20	10	70	0.59	> 0.05

8.5.4 The probability of sample composition

To explore the effect of small sample size on the outcome of host specificity tests for the UK beetles in particular, the probability of selecting ten beetles that would lay zero eggs

on *Ch. prolifer* was estimated. To get the best estimate the data from the UK beetles used in this study and the data from the original importation in 1985 was combined (Table 8.7).

Table 8.7: Number (No.) of eggs laid by *Bruchidius villosus* imported to New Zealand from the UK in two-choice tests with *Cytisus scoparius* and *Chamaecytisus prolifer* in 1985 and 2002.

Year	No. of *B. villosus*	No. of eggs laid on *C. scoparius*	No. of eggs laid on *Ch. prolifer*	No. of *B. villosus* that laid zero eggs
1985	10	111 + 76	0	10
2002	10	127	39	6

Using the combined data from 1985 and 2002 a total of 16 out of 20 female *B. villosus* did not lay eggs on *Ch. prolifer*. If the true proportion of females in the population that may not accept *Ch. prolifer* as an oviposition host is 16/20 = 0.8 then using a Table of the binomial distribution of individual probabilities (Sheskin 2000), the probability of selecting ten female *B. villosus* that will lay no eggs on *Ch. prolifer* is $0.8^{10} = 0.107$. In other words there is approximately a 11% chance of choosing a sample where none of the females lay eggs on *Ch. prolifer*. However using the estimated proportion of females laying no eggs estimated from the negative binomial distribution fitted to the combined data, the probability of selecting ten female *B. villosus* that may not lay eggs on *Ch. prolifer* is $0.43^{10} = 0.0002$.

8.5.5 Variation in plant material

Because the condition or genetic make up of the plant material used in this study could strongly influence the outcome of oviposition tests the variability of egg laying on the different plants used in these experiments was investigated.

8.5.5.1 New Zealand beetles

No significant difference in egg laying was found between the three different plants used to collect foliage and represent *C. scoparius* ($F = 0.2$, $df = 2$, $P > 0.05$) and *Ch. prolifer* ($F = 0.4$, $df = 2$, $P > 0.05$) in the tests, which indicates each plant (within each species) is equally acceptable for oviposition.

8.5.5.2 UK beetles

No significant difference in egg laying was found between the two different plants used to collect foliage and represent *C. scoparius* ($P > 0.05$, 95% CI) and *Ch. prolifer* ($P > 0.05$, 95% CI), which indicates both plants (within each species) are equally acceptable for oviposition.

8.6 Discussion

8.6.1 Host preference

Host preference describes the probability that particular test plants will be accepted. When testing for oviposition preference, if an encounter with plant A is more likely to result in oviposition than an encounter with plant B then the insect prefers A over B (Singer 1986). Early in the experiments carried out in this Chapter both New Zealand and UK individuals of *B. villosus* preferred *C. scoparius* over *Ch. prolifer.* Later, the behavioural threshold for both populations appeared to change, where an encounter with either host was equally likely to result in oviposition by the New Zealand beetles but the preference for *C. scoparius* was more strongly maintained for UK beetles (Table 8.5 and 8.6).

Such variation has been shown in other studies with other species. Singer and Lee (2000) examined individual oviposition preferences of the butterfly *Melitaea cinxia* L. for two plant species, *Plantago lanceolata* L. and *Veronica spicata* L. They found that some individuals repeatedly preferred one plant species over the other, while others preferred a mixture of both plant types. In this investigation the same trend was noted in both the New Zealand and imported UK populations where most individuals preferred *C. scoparius* over *Ch. prolifer* on which to oviposit for the duration of the experiment while others oviposited on both species (Figure 8.1 and 8.2).

Variation in the rate of acceptability of the lower ranked host *Ch. prolifer* in this study was found, with some beetles never ovipositing on this species, while others oviposited on the plants after the second day of the experiment (Table 8.2, 8.4, 8.5 and 8.6).

8.6.2 Host ranking and acceptability

In this study *C. scoparius* is clearly the higher ranked host based on the significantly higher number of individual adults preferring this species (Figure 8.1 and 8.2). Because all beetles were individually tested, exactly how many females were responsible for the eggs laid on each species is now known. Furthermore, the order of egg laying is known at least across days. Singer (1986) discusses the importance of noting the egg laying sequence in the determination of preference. If eggs are allowed to accumulate in assays, in other words if the counting interval is too long, there is a risk that results can be

confounded by factors such as presence of conspecific eggs that can either attract or deter insects with potential to oviposit. Some individuals have equal preferences for two plant species, as is known to occur in butterflies (Thomas and Singer 1999). When using groups in host specificity testing such an important level of detail can be lost (Singer 1986).

Initially, for both beetle populations, *C. scoparius* was the preferred host but over time *Ch. prolifer* became more acceptable for oviposition (Table 8.5 and 8.6). West and Cunningham (2002) in their general model show that as female insects become more host-limited they oviposit on less favourable hosts whilst still ovipositing on more favoured hosts. A threshold may govern the switch from favourable to less favourable hosts. The hierarchy threshold model (Courtney et al. 1989) also predicts that diet breadth should increase with factors that increase egg-loads.

Pods are a limited food resource in that they can only accommodate one larva per seed, and larvae are relatively immobile which means they cannot move between pods. In pods that become overloaded with eggs, it is likely that high levels of larval competition will occur, resulting in high levels of larval mortality. In this situation there is pressure on females to be more selective about oviposition sites. As available oviposition sites on *C. scoparius* declined as more eggs were laid the motivation for females to lay on the lower ranked host, *Ch. prolifer* where oviposition spaces were available, may have increased. When the highest-ranked host is limiting, females may reach a point where they have to choose between either laying no eggs, laying additional eggs on the limited host that may be already at or above carrying capacity (further increasing larval mortality) or utilising the lower ranked host (possibly increasing the chance of offspring survival). In the study of the New Zealand beetles laying on *C. scoparius,* the mean number of eggs laid per seed per individual was 0.25 (calculated using data from Table 8.1) and only in one case did the number of eggs laid exceed the number of seeds available. Therefore resource limitation by itself, may not be an adequate explanation for these findings.

8.6.3 New host association

Chamaecytisus prolifer (a native of the Canary Islands) does not grow naturally in the UK as the climate is too cold for it to set flowers and pods (S. Fowler, personal communication). Despite never having been exposed to this plant species, 40% of the

UK beetles in this study oviposited on it suggesting this species possesses some attributes such that it should be included in the host range of *B. villosus*. The New Zealand beetles, that have been exposed to *Ch. prolifer* in the field in New Zealand for 15 generations and gained experience on this plant, appear to find it more acceptable than UK beetles do (Table 8.2 and 8.5 compared with 8.4 and 8.6).

There are several possible explanations for the New Zealand beetles having a higher preference for *Ch. prolifer* than the UK beetles tested here. If a change in preference is evolving, it needs to be determined if the variation in oviposition preference exhibited is under genetic control (Tabashnik et al. 1981). Individual variation in oviposition preference has been demonstrated in the cowpea weevil *Callosobruchus maculatus* by Fox (1993), and genetic variation in oviposition preference has been found within populations (Wasserman and Futuyma 1981, Wasserman 1986). Ng (1988) showed that a single population of insects may consist of both generalist and specialist genotypes. Schneider and Roush (1986) investigated the genetics of host preference by using genetic crosses on two populations of *Heliothis virescens* (F.) that varied in their preference for two plant species. Hybrid offspring were produced that showed intermediary oviposition preferences.

If intermediary oviposition preferences are the case, then to avoid misrepresentative results it is necessary to establish whether selective forces are responsible for maintaining this level of variation within the population and if so what are these forces. Such a selective force could be a temporal change in host quality or phenology (Tabashnik et al. 1981). *Chamaecytisus prolifer* flowers earlier than *C. scoparius* in spring and is the first available pollen source (Fowler et al. 2000) for the first *B. villosus* emerging from their overwintering period in New Zealand. When beetles emerge from overwintering they feed on pollen from flowers of a number of different species before *C. scoparius* flowers appear (M. Haines, personal observation).

If *C. scoparius* and *Ch. prolifer* are equally suitable as larval rearing host plants for *B. villosus* which is supported by findings in other experimental work (Chapter 5 and 9, but not supported in Chapter 4) then this may explain why the variation in acceptability of *Ch. prolifer* in the *B. villosus* population in New Zealand has been maintained and is possibly increasing. There may be no strong selection which favours preference for either plant if reproductive performance on both is equivalent (Tabashnik et al. 1981). Lack of selection pressure may also explain the more neutral preference shown by the

New Zealand beetles later in the experiment (Table 8.5). Perhaps there is little or no selective pressure to discriminate between the two hosts.

Beetles may have been attracted to *C. scoparius* the ancestral host initially, as a result of a short-term bias toward the plant to which the beetles were most recently exposed. Both populations of beetles were collected from *C. scoparius* (assumed to be the larval rearing host) and were exposed to flowers and pollen from this species prior to the experiment. This exposure to *C. scoparius* as part of larval experience (Courtney et al. 1989, Courtney and Kibota 1990), may result in induced preference (Szentesi and Jermy 1990), food imprinting (Jermy 1987) or conditioning (Firempong and Zalucki 1991) via Hopkin's host selection principle (Jermy et al. 1968).

Adult conditioning (Jaenike 1982, 1983) or learning (Papaj and Prokopy 1989) did not appear to have any long-term effect on consequent oviposition, as no overall bias was shown toward *C. scoparius* in this investigation or in other experimental work that examined the effect of larval rearing host and food type (section 5.5.2). That is not to say however, that in the short-term the exposure to *C. scoparius* did not influence the initial responses of the beetles, and that they were attracted to the chemically and physiologically familiar *C. scoparius* before recognising *Ch. prolifer* as also suitable for oviposition. Most beetles in the population may retain a preference for their ancestral host (*C. scoparius*) while a minority of individuals that accept *Ch. prolifer* do well on this 'new association' so that, over time, a 'strain' may evolve that uses *Ch. prolifer* preferentially. Clearly there is risk involved in ignoring a low average oviposition level that may reflect a few individuals accepting the alternative host as readily as their 'normal' host.

8.6.4 Implications for host testing methodology

8.6.4.1 Sample size

The small sample size of UK beetles reduced the power of the statistical methods used in this study (section 8.5.4). A higher level of replication would have improved the level of precision of the estimates measured in the choice experiments. Despite the small sample size the data suggest that for the UK beetles *C. scoparius* is the preferred host. Using the number of females that did not lay eggs on that species in the combined tests as an exploratory estimate the chance of selecting ten female UK *B. villosus* that would lay no eggs on *Ch. prolifer* was estimated at 11%. However, using the estimated probability

from the negative binomial distribution it was much lower at 0.02%. In combining the data it was assumed that there was little change in beetle behaviour over the 17 years between 1985 and 2002. There is good reason to believe this is the case, as the beetles were collected from the same location and from the same plants and the handling procedures were replicated as closely as possible.

If the number of females had been doubled from ten to 20 in the 1985 tests, and using the higher probability of non-egg laying, (0.8) then based on the binomial distribution function, the probability of selecting ten non-layers on *Ch. prolifer* would drop from 0.11 (about one in ten samples) to 0.002 (2 in 1000 samples). Furthermore, the probability of selecting 20 non-layers in a sample of 20 would be 0.012. Using the lower probability of non-egg laying estimated from the negative binomial distribution (0.43) the probability of selecting 20 non-egg layers in a sample of 20 is extremely small (4.67×10^{-8}). Clearly even a modest increase in sample size makes a false negative result much more unlikely. The examples here highlight the risk of using small sample sizes and that researchers in the field of host specificity testing should consult standard equations (Karandinos 1976, Sedcole 1977) and statistical distribution tables or equations that are commonly available to determine adequate sample sizes.

In the host specificity tests conducted in 1985 the fact that ten *B. villosus* used in the host specificity tests did not lay eggs on *Ch. prolifer* (section 1.7.2.1) appeared to have occurred by chance. The decision to release *B. villosus* into New Zealand was based on the results of these tests. No eggs were laid on *Ch. prolifer* or any other test plant in the investigation apart from the known host *C. scoparius*. If a larger sample size had been used, the relative preference of some *B. villosus* for *Ch. prolifer* in a choice test might have been revealed. *Chamaecytisus prolifer* is now recognised as a host in New Zealand and the initial choice tests failed to predict that it would be, constituting a false negative result.

Marohasy (1998) states that choice tests are prone to producing false negative results as they often only indicate a strong preference for the target plant. This study confirms that choice tests with inadequate sample sizes could fail to predict the acceptability of less preferred hosts, particularly because of high inter-female variability. No-choice tests are considered less likely to produce false negatives (Cullen 1990, Heard 1997). However, no-choice tests are known to overestimate field host range due to the effects of deprivation and experience, so choice tests are often used to reveal what are

believed to be more realistic host plant preferences (Marohasy 1998, Withers and Barton Browne 1998, Edwards 1999, Withers et al. 2000).

It may be beneficial in host specificity testing to concentrate on higher levels of replication on fewer test plant species, rather than low levels of replication on a large number, in order to gain more accurate results from the available resources. In order for this to be implemented however, regulatory authorities need to recognise that it is not necessary to test very large numbers of plants to determine host range, and that it can in fact be counterproductive.

The duration of host specificity testing is clearly also very important in the outcome of tests. In choice tests, a test duration of four days would have resulted in very few eggs having been laid on the non-target host. For the UK beetles nine eggs were laid by one individual (Table 8.4) and could possibly have been discounted as a random or unusual occurrence resulting in an inaccurate conclusion about host range. Only when monitored for longer periods of time is a degree of confidence in the result obtained. This is particularly so when oviposition preferences are significantly different between two plants.

8.7 Conclusion

In conclusion, had a larger sample size (Ruxton and Colegrave 2003) in the choice tests, been used in 1985, the acceptability of *Ch. prolifer* for oviposition may well have been revealed. The false negative result was therefore a combination of the small sample sizes of the beetles used in the original host specificity tests, exacerbated by high inter-beetle variation in oviposition preference. No-choice tests may have also revealed the relative acceptability of *Ch. prolifer* (Chapter 2 and 4). However, at the time this testing was done, choice tests were favoured (as they were considered to simulate more natural conditions than no-choice tests) (Harley 1969), and were often the only type of test used (Winder et al. 1984).

This study shows clearly that UK beetles will accept *Ch. prolifer* as an oviposition host despite that this species does not occur in its native range. There are indications that New Zealand beetles show higher levels of acceptance of *Ch. prolifer* than UK beetles suggesting that New Zealand beetles may have changed their host preference in the 15 generations since their introduction.

Linking page: Chapter 9

Significant variation in individual oviposition behaviour was found in Chapter 8 and in previous chapters. The probability that the ten beetles used in the original tests would be non-layers on *Chamaecytisus prolifer* was estimated as 11%, indicating that inadequate sample size is a plausible explanation for the false negative result. Observations in the field showed that beetles reared from *Ch. prolifer* display phenotypic plasticity (Chapter 3). So an investigation comparing the fitness characteristics of *Bruchidius villosus* reared from *Ch. prolifer* and *Cytisus scoparius* was undertaken. The hypothesis for Chapter 9 was that the phenotypic plasticity is an indicator of improved fitness, which could be a precursor for host race formation, in beetles reared from *Ch. prolifer*.

Chapter 9: A sibling experiment to investigate the potential for host race development in *Bruchidius villosus*.

9.1 Abstract

Phenotypic plasticity in insects is widespread and can sometimes indicate selection toward host race formation. In this investigation the larval rearing host effects of two pod-bearing plant species (*Chamaecytisus prolifer* and *Cytisus scoparius*) on the seed feeding beetle, *Bruchidius villosus* were examined. Using two-choice oviposition tests, preferences and fitness parameters of *B. villosus* reared from the two hosts were measured in order to determine whether a host race could form. Whilst *B. villosus* reared from both hosts preferred to oviposit on *C. scoparius*, there was a significant difference in the strength of the preference shown toward *C. scoparius* depending on larval rearing host. There was some evidence that maternal effects may influence body size and oviposition preference in *B. villosus*. Adult *B. villosus* reared on *Ch. prolifer* were larger, more fecund and showed higher overwintering survival than those reared on *C. scoparius* but development time from egg to adult was longer on this host. Longevity of *B. villosus* reared from both hosts was similar. In conclusion, the phenotypic plasticity observed between *B. villosus* reared from *C. scoparius* and *Ch. prolifer* may be indicative of differences in fitness. *Bruchidius villosus* reared from *Ch. prolifer* were fitter than those from *C. scoparius* so an increase in the use of *Ch. prolifer* in the field may be a consequence of this.

9.2 Introduction

Phenotypic plasticity (sometimes referred to as phenotypic variation (Fox 1997)), or polyphenism (Diehl and Bush 1984) can be considered as an adaptation by species to cope with a variable environment (Gotthard and Nylin 1995). Phenotypic plasticity is when a single genotype can produce alternative phenotypes, depending on the environment, during ontogeny (Nylin and Gotthard 1998). Phenotypic plasticity may allow members of an insect population to move from an existing host and adapt to an alternative host. If the relationship between the insect species and the alternative host is

maintained over time, gradually the adaptation can result in specialisation on this host, at which point the insect may be unable to utilise the original host. This process may result in the formation of a new host race (Rausher 1988) and in expansion of the host range of this species.

A host race is defined by Diehl and Bush (1984) "as a population of a species that is partially reproductively isolated from other conspecific populations as a direct consequence of adaptation to a specific host". Marohasy (1996) states that populations must fulfil the following criteria to be considered distinct host races: 1) be non-interbreeding and sympatric, 2) differ in biological characteristics but not (or only marginally) in morphology, and finally 3) be prevented from interbreeding as a result either of preference for different host plant species, or as a consequence of physiological adaptation to different host plant species.

Variation in biological performance can expose critical features of insect-plant relationships such as the evolution of host shifts (Nylin et al. 1996). Through the examination of life-history traits that are biological characteristics and determinants of fitness of any species it is possible to evaluate the likelihood that a host race will evolve. The major life history traits of insects that are determinants of fitness are age and size at maturity (body size), reproductive investment (fecundity), reproductive lifespan (survival) and aging (which affects survival and fecundity) (Stearns 2000).

Body size has been reported in many studies as a key component of an organism's ecological and physiological properties (Clutton-Brock 1988, Klingenberg and Spence 1997, Nylin and Gotthard 1998, Fox and Czesak 2000) and is often influenced by diet. In particular for phytophagous insects plant host quality (Price 1997, Nilsson et al. 2002) as well as quantity (Mitchell 1975, McClure et al. 1998, Mousseau and Fox 1998, Koo et al. 2003) have been reported to be influential factors in the determination of body size. For example, larval diet alters horn length-body size allometry in the beetles *Onthophagus acuminatus* (Emlen 1994), and body weight of the seed-feeding bruchid, *Stator limbatus* is affected by the size of the seed from which individuals emerge, where larger seeds produce larger beetles than smaller ones (Fox 1997). Host size can affect offspring survival and body size, particularly in species that oviposit on discrete hosts, such as parasitoids, seed beetles and leafminers (Mousseau and Fox 1998). Host size is especially important in species that oviposit on discrete hosts within which their offspring must complete development, because the quantity of food available influences offspring

survival and determines offspring body size (Mitchell 1975, McClure et al. 1998, Koo et al. 2003).

Many studies have shown a strong, positive relationship between female body size and fecundity (Varley et al. 1973, Credland et al. 1986, Clutton-Brock 1988, Garcia-Barros 2000, Smith et al. 2002, Tammaru et al. 2002). Furthermore body size has been shown to account for much of the variation in potential and realised fecundity in insects. Because fecundity generally increases with increased body size (Honek 1993, Sopow and Quiring 1998) it is considered an indicator of fitness (Clutton-Brock 1988, Nylin and Gotthard 1998).

Body size (mass) and longevity (life-span or survival) are also well correlated in some insects (Møller et al. 1989, Tatar and Carey 1995, Messina and Fry 2003). However Leather (1994) found no relationship between weight and longevity in *Panolis flammea* Denis & Schiffermüller. Larger individuals may also survive better during overwintering when food sources are limited (Hokkanen 1993, Ohgushi 1996), as body size is often correlated with the presence of good nutrient reserves (Palmer 1985, Nylin and Gotthard 1998).

As stated above, Marohasy (1996) suggests preference for different host plant species within a population of insects can be a critical step in the development of a host race. Many different factors can influence host choice by ovipositing females (Courtney and Kibota 1990). For example, adult experience (Tabashnik et al. 1981, Jaenike 1982, Papaj and Rausher 1983, Diehl and Bush 1984, Futuyma and Peterson 1985, Prokopy et al. 1988, Via 1991) and genetic variation may affect oviposition preference (Tabashnik et al. 1981, Rausher 1983a, Futuyma and Peterson 1985, Jaenike 1988, Singer et al. 1988, Via 1990, Jallow and Zalucki 1996b).

Field observations (M. Haines, unpublished data, Wittenberg and Thomann 2001) suggest phenotypic variation in body size and colour of *Bruchidius villosus* (a seed-feeder) depends on the host plants in which they develop. Female *B. villosus* lay eggs on the outside of immature green pods and larvae subsequently bore through the pod wall and develop inside a single seed. Adults emerging from seeds of *Chamaecytisus prolifer* (the alternative host) appeared larger with a browner colouration compared to those emerging from the usual host *Cytisus scoparius*, which were smaller

and blacker in colour. It is unknown whether this phenotypic plasticity is correlated to differences in performance or suitability of genotypes according to host plant.

To ascertain this, the influence of two larval rearing host species, *C. scoparius* and *Ch. prolifer* was determined by measuring their effect on the body size, lifetime fecundity, longevity and oviposition preference of siblings, from a limited number of female *B. villosus* collected from *C. scoparius* in the field. The study was specifically designed to determine whether the development of different biological characteristics (body weight, overwintering survival, longevity, fecundity and host preference) in response to different larval rearing hosts could be a precursor for host race formation.

9.3 Methods

Critical to this experiment was the requirement to minimise genetic variation on life history responses of the insects, so experiments were conducted with beetles that were siblings. A controlled rearing procedure was used to obtain the experimental individuals. In October 2001, fifteen female *B. villosus* were caught from the field and placed in individual vials with bee pollen only, thus depriving them of oviposition sites for 24 hours. Fifteen branches each of *Ch. prolifer* and *C. scoparius* bearing fertilised flowers and immature pods were sleeved in the field, to ensure there was no contamination by the natural field population of *B. villosus*. Two weeks later the sleeves were checked to ensure no eggs had been laid on the pods. Following this, each female was secured in one of each of the 15 sleeve cages on *C. scoparius* and left to oviposit for seven days. On the eighth day the females were removed from *C. scoparius* and deprived of oviposition sites for a further 24 hours before being secured in the sleeve cages on *Ch. prolifer* for a further seven days. Each sleeve cage was clearly labelled to ensure individual females could be identified. After the beetles were removed from the sleeve cages they were resealed to prevent contamination from other *B. villosus* beetles.

When the pods matured (but prior to dehiscence) they were harvested and stored in individual plastic containers, and once again were clearly labelled to avoid cross-contamination. When sufficient numbers of adult *B. villosus* had emerged, the six containers containing the highest number of adults were kept for use in the experiment.

9.3.1 Sibling weight – body size

As each adult beetle emerged it was weighed and its sex was recorded before it was placed in one of 12 Perspex® cages (500 × 500 × 500 mm) with sponge doors (365 × 360 × 50 mm), according to its mother (3 levels) individual larval rearing host plant (2 levels) and sex (2 levels).

9.3.2 Sibling overwintering survival

The beetles held in the laboratory cages were supplied with cotton rolls soaked in honey water and bee pollen and maintained for 10-13 weeks at 22-16°C day-night temperatures with a 14:10 h L:D regime. The temperature was gradually lowered to 8-3°C day-night with a 10:14 h L:D regime for 20 weeks to replicate the seasonal changes in the field. The beetles were gradually exposed to rising temperatures representative of spring temperatures and were then maintained at 22-16°C day-night temperatures with a 14:10 h L:D regime. The beetles were given access to *C. scoparius* flowers for six weeks before further experimentation resumed. Humidity levels were kept at approximately 70% throughout. Numbers of adult beetles alive and dead at the conclusion of this period was recorded.

9.3.3 Sibling longevity, fecundity and preference

In September 2002, a subsample of seven female siblings was chosen at random from each container and were mated for two weeks with 14 male siblings from the same host plant and same mother. The beetles were supplied with pollen and cotton rolls soaked in honey water. After two weeks the six most active-looking females (those which were moving around the cage most quickly) were placed individually into Perspex® cages, along with a pair of males (selected using the same criteria) to ensure that mating had occurred. Within each cage beetles were given continual access to cut shoots of *C. scoparius* and *Ch. prolifer* (identical to the design in Chapter 8) each bearing five pods at the same phenological stage, in order for oviposition preference to be measured. Beetles were also given access to pollen and cotton rolls soaked in honey water.

In total there were 36 cages for siblings from three mothers and two host plants replicated 6 times. Every ten days the cut shoots were replaced with fresh ones and the number of eggs laid on each were recorded to measure fecundity. Every day individual beetle survival or mortality was recorded in order to calculate longevity. The experiment

continued until each female died, or 90 days whichever was shortest. In this way lifetime fecundity could be measured.

9.3.4 Analysis

The data set was tested for normality using the Kolmogorov-Smirnov test and homogeneity of variances using Levene's test before hypothesis testing. To determine the effect of larval rearing host plant on sibling weight (body size) the data was analysed using REML (residual maximum likelihood) variance components analysis. The Wald statistic was used to assess the contributions of individual terms in the fixed model (GenStat 2002). The effect of larval rearing host on overwintering survival was analysed using binomial GLM (generalised linear model) and a nested design.

The effect of larval rearing host and mother on longevity and fecundity was analysed using ANOVA. A repeated measures ANOVA (GenStat 2002) was used to compare mean number of eggs laid per female on test plants over each ten day period.

To determine the effect of larval rearing host and mother on oviposition preference of *B. villosus* a 'coefficient of preference' (Heard 1995a, Withers 1999) (previously used in Chapter 5 and 8) for oviposition on *C. scoparius* over *Ch. prolifer* was calculated. Significant main effects were identified using ANOVA (GenStat 2002). Means for weight, fecundity and preference were compared using Fishers LSD.

9.4 Results

9.4.1 Examination of factors

The effect of larval rearing host, mother and sex and their interactions on *B. villosus* body weight (Table 9.1) and overwintering survival were examined (Table 9.2). The effect of larval rearing host and mother and the interaction between these factors on female longevity (Table 9.3) and oviposition host preference were also examined (Table 9.5).

9.4.1.1 Sibling weight - body size

Larval rearing host and sex had a significant effect on sibling body weight (Table 9.1). Mean weights of *B. villosus* reared from *C. scoparius* and *Ch. prolifer* were 2.0 mg ± 0.03 SEM and 2.5 mg ± 0.03 SEM respectively. Offspring reared from *Ch. prolifer* were significantly heavier than those from *C. scoparius* and females were significantly heavier

than males in every case (significance was tested using an approximate 5% LSD, 0.2) (Figure 9.1).

The interactions between larval rearing host and mother, larval rearing host and sex, mother and sex and larval rearing host, mother and sex were also significant factors (Table 9.1). The last three significant interaction terms reflect that only female siblings reared from *Ch. prolifer* showed a significant difference according to mother (Figure 9.1).

Table 9.1: Significance of factors (larval rearing host, mother and sex and their interactions) contributing to *Bruchidius villosus* body weight.

Factors	W	df	P
Larval rearing host	491.7	1	<0.001
Mother	9.28	2	0.010
Sex	196.9	2	<0.001
Larval rearing host × mother	9.4	2	0.009
Larval rearing host × sex	31.9	1	<0.001
Mother × sex	12.1	2	0.002
Larval rearing host × mother × sex	11.7	2	0.003

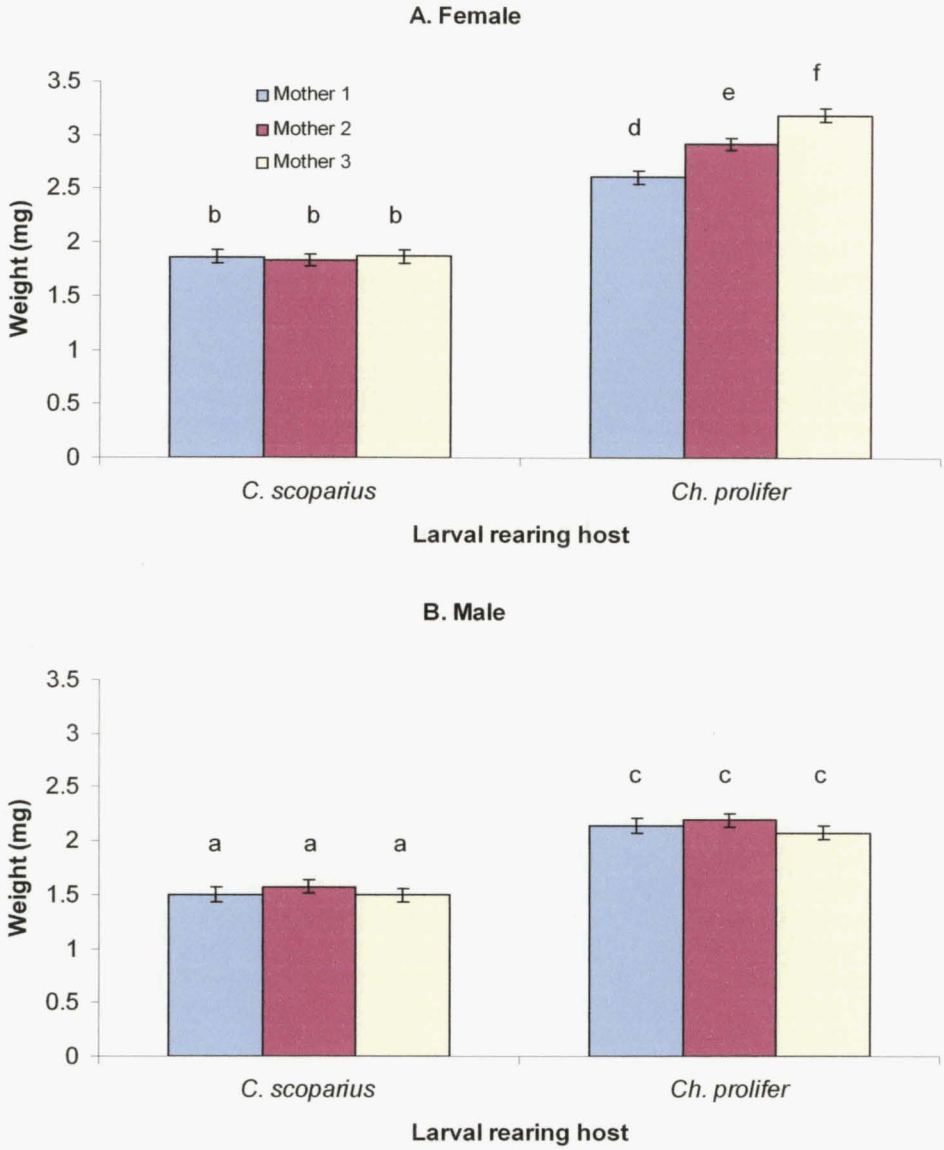


Figure 9.1: Mean weights (mg) of newly emergent female (A) and male (B) *Bruchidius villosus* offspring reared from two different hosts (*Cytisus scoparius* and *Chamaecytisus prolifer*) and three mothers. Means with the same letter are not significantly different.

9.4.1.2 Sibling overwintering survival

A highly significant difference in overwintering survival as a result of larval rearing host plant (*C. scoparius* and *Ch. prolifer*) was observed (Table 9.2). The overwintering survival of beetles was highest for those reared from *Ch. prolifer* regardless of mother and sex. Mother had no significant effect on the overwintering survival of offspring (Table 9.2). Male overwintering survival was significantly higher than that of female beetles (Figure 9.2). The interaction between host and sex was not significant (Table 9.2).

Table 9.2: Significance of factors (larval rearing host, mother and sex and their interactions) contributing to *Bruchidius villosus* overwintering survival.

Factors	Deviance	df	P
Mother	0.9	2	0.64
Larval rearing host	36.4	1	<0.001
Sex	4.8	1	0.028
Larval rearing host × sex	0.4	1	0.54

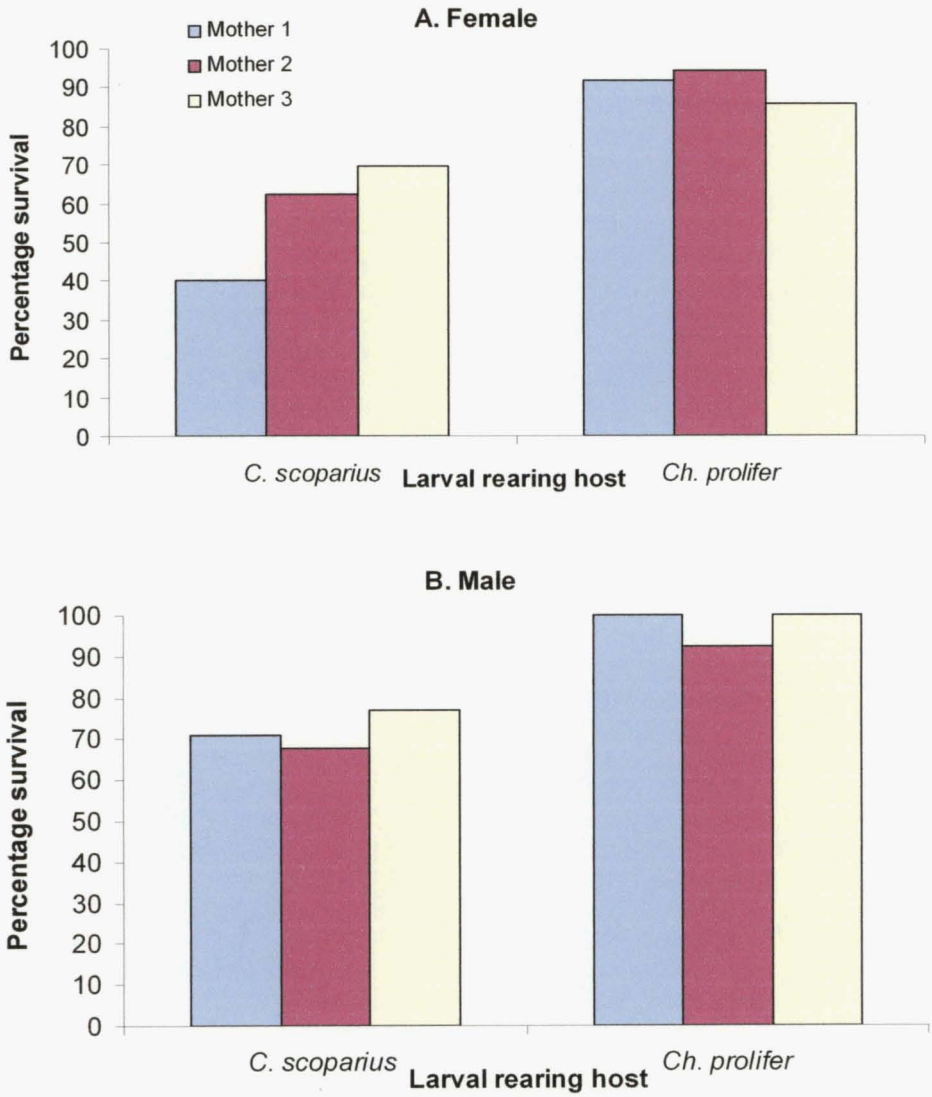


Figure 9.2: Percentage survival of overwintered females (A) and males (B) *Bruchidius villosus* offspring reared on *Cytisus scoparius* and *Chamaecytisus prolifer* from three mothers (Mothers 1, 2 and 3).

9.4.1.3 Sibling longevity

The host on which beetles were reared had a significant effect on mean longevity of *B. villosus* (Table 9.3). Beetles reared on *Ch. prolifer* had significantly greater mean longevity than those reared on *C. scoparius.* Mean longevity was 43.4 days for beetles

reared from *C. scoparius* and 58.8 days for those reared from *Ch. prolifer* (5% LSD, 13.9). There were no significant maternal effects on mean longevity nor a significant interaction between mother and host (Table 9.3).

Table 9.3: Significance of factors (larval rearing host, mother and sex and their interactions) contributing to female *Bruchidius villosus* longevity.

Factors	F	df	P
Larval rearing host	5.2	1	0.03
Mother	1.2	2	0.32
Larval rearing host × mother	0.05	1	0.95

The survival of *B. villosus* reared from both hosts after overwintering was similar for the first 30 days of the choice trial. After 30 days mortality rate increased more rapidly for *B. villosus* reared on *C. scoparius* than *Ch. prolifer*. The biggest difference in survival was observed on day 60, when 72% of *B. villosus* from *Ch. prolifer* were alive but only 33% from *C. scoparius* remained (Figure 9.3).

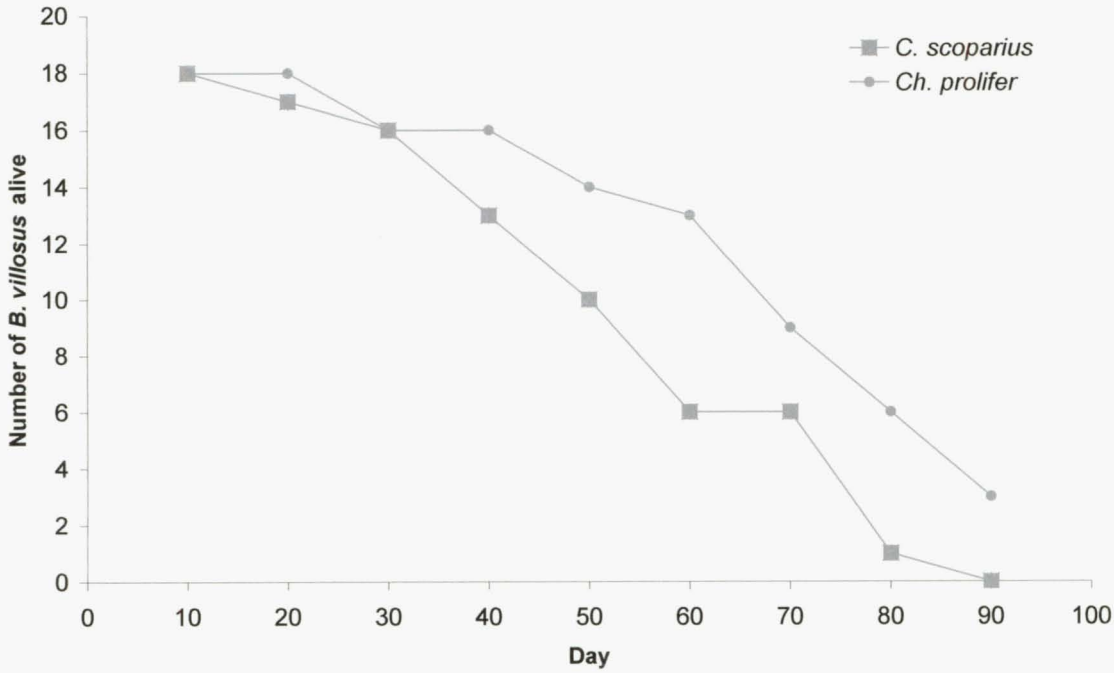


Figure 9.3: Survival of *Bruchidius villosus* offspring reared from two different hosts *Cytisus scoparius* (■) and *Chamaecytisus prolifer* (●) in a two-choice cut shoot test containing *C. scoparius* and *Ch. prolifer*. Data are averages of 18 female *B. villosus* reared from each host.

Beetles reared from *Ch. prolifer* lived longer than those reared on *C. scoparius* by an average of 15.4 days. Offspring of Mother 1 lived for the shortest time when reared from either *C. scoparius* (mean 34.7 days) or *Ch. prolifer* (mean 52.8 days) (Figure 9.4 A, D), while offspring of Mother 2 lived longest independent of which host that they were

reared from *C. scoparius* (mean 48.0 days) or *Ch. prolifer* (mean 63.3 days) (Figure 9.4 B, E) but these differences were not significant (5% LSD, 24.0).

At the conclusion of the experiment after 90 days, only three *B. villosus* (all reared from *Ch. prolifer*) were still alive (Figure 9.4 E & F). The longest lived individual reared from *C. scoparius* survived for 77 days (Figure 9.4 C).

See section 9.5.3 for further information on the results in this section.

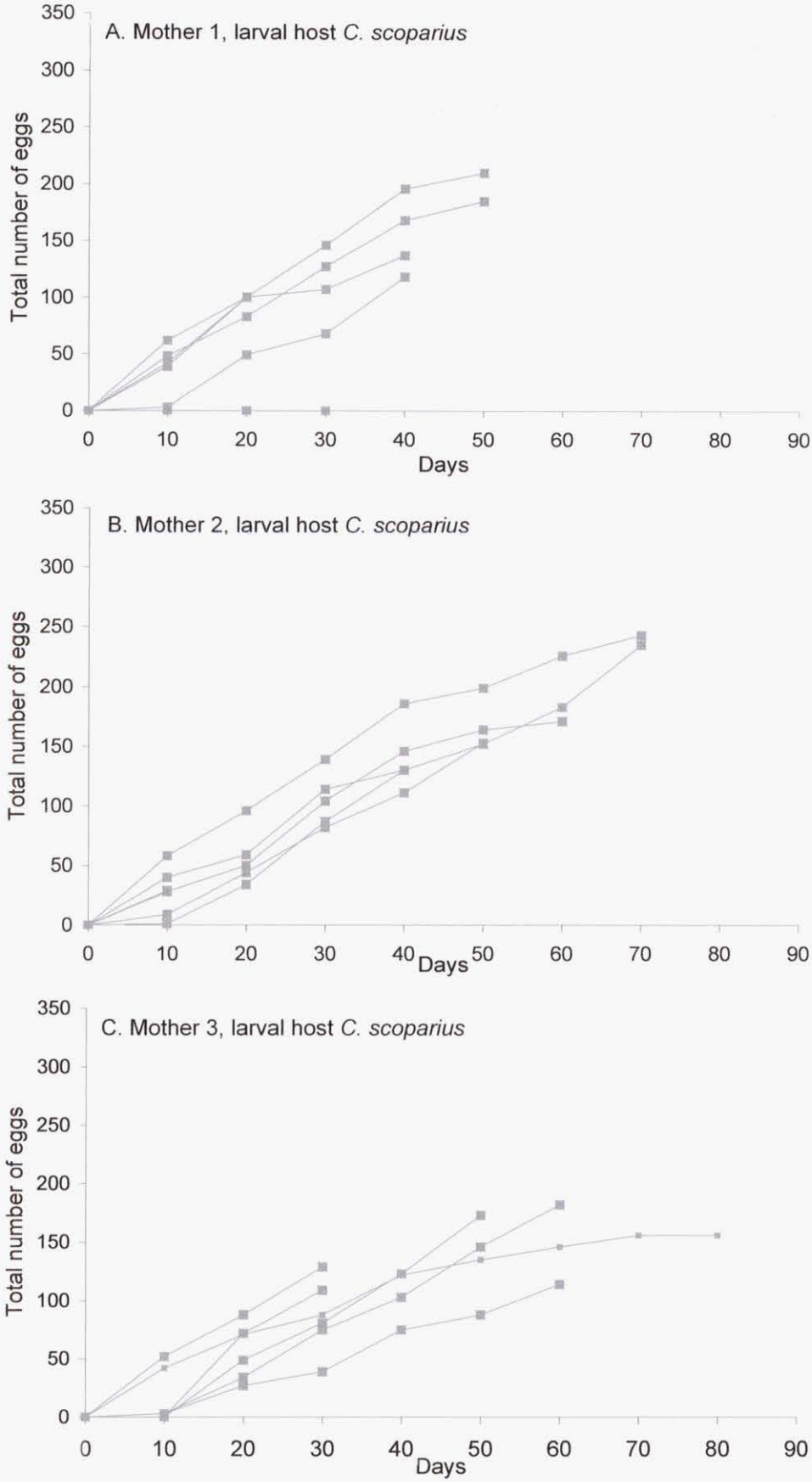
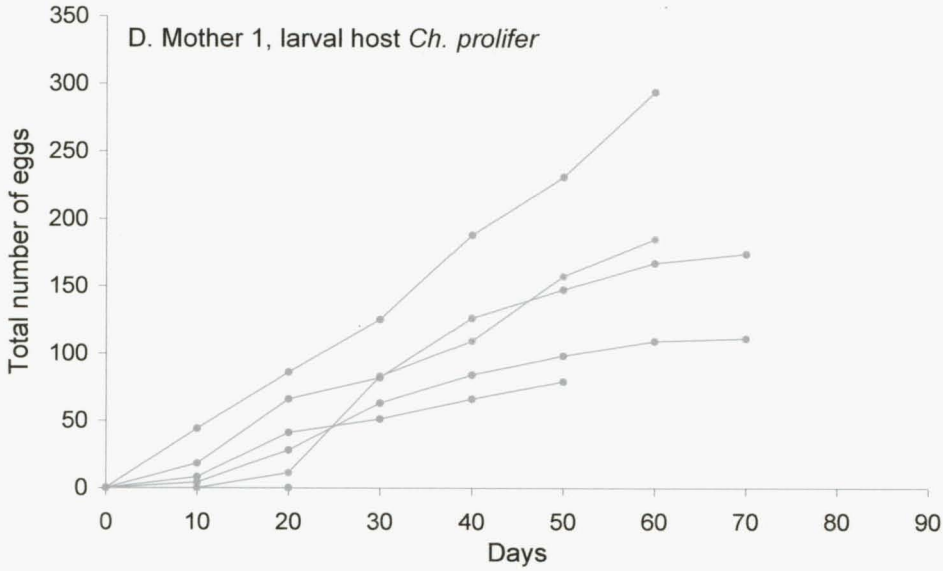
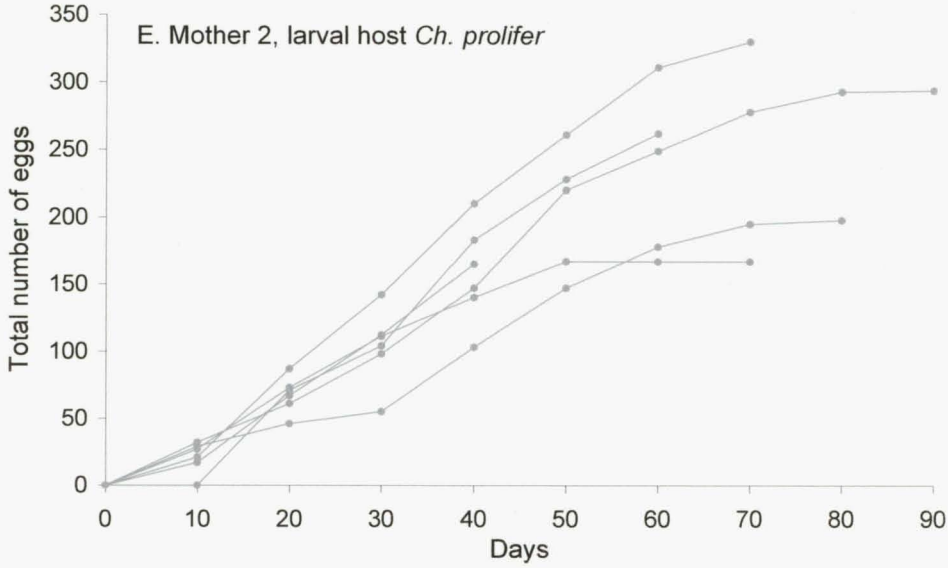
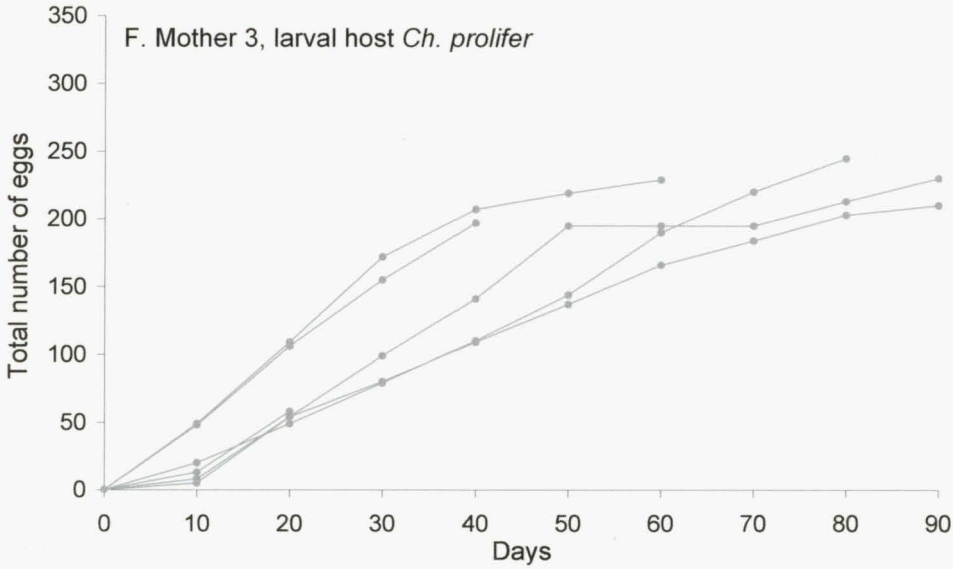


Figure 9.4: Total cumulative eggs laid by individual female *Bruchidius villosus* offspring over their lifetime on *Cytisus scoparius* and *Chamaecytisus prolifer* (rounded to the nearest 10 day period). A, B, C, D, E and F, six offspring each of Mothers 1, 2 and 3 reared on *C. scoparius* (■) and *Ch. prolifer* (•).


D. Mother 1, larval host *Ch. prolifer*
Total number of eggs
Days
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70
80
90


E. Mother 2, larval host *Ch. prolifer*
Total number of eggs
Days
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70
80
90


F. Mother 3, larval host *Ch. prolifer*
Total number of eggs
Days
0
50
100
150
200
250
300
350
0
10
20
30
40
50
60
70
80
90

9.4.1.4 Sibling fecundity

Offspring of Mother 1, 2 and 3 laid 1593, 2377 and 2032 eggs respectively (Figure 9.5). Two *B. villosus* females laid no eggs throughout the trial (one from each of the larval rearing hosts), although upon dissection both were found to be reproductively mature (Figure 9.4 A & D).

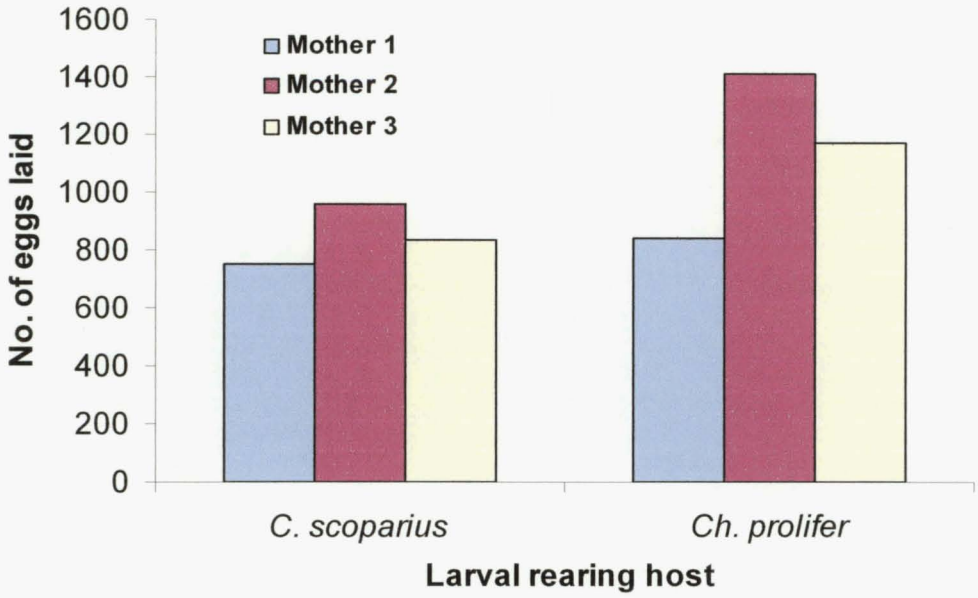


Figure 9.5: Total number (No.) of eggs laid by *Bruchidius villosus* offspring grouped by mother (Mothers 1, 2, and 3) and larval rearing host (*Cytisus scoparius* and *Chamaecytisus prolifer*) in a two-choice cut shoot test containing *C. scoparius* and *Ch. prolifer*.

Larval rearing host had a significant effect on the number of eggs laid ($F = 4.0$, $df = 1$, $P < 0.05$). *Bruchidius villosus* reared from *C. scoparius* laid an average of 143 eggs each over the duration of the experiment while those reared from *Ch. prolifer* laid 190.5 (Table 9.5, Figure 9.6).

Cumulative average number of eggs laid was approximately linear over time until becoming asymptotic on approximately day 40 for *B. villosus* reared from *C. scoparius,* and day 60 for *B. villosus* reared from *Ch. prolifer* (Figure 9.6).

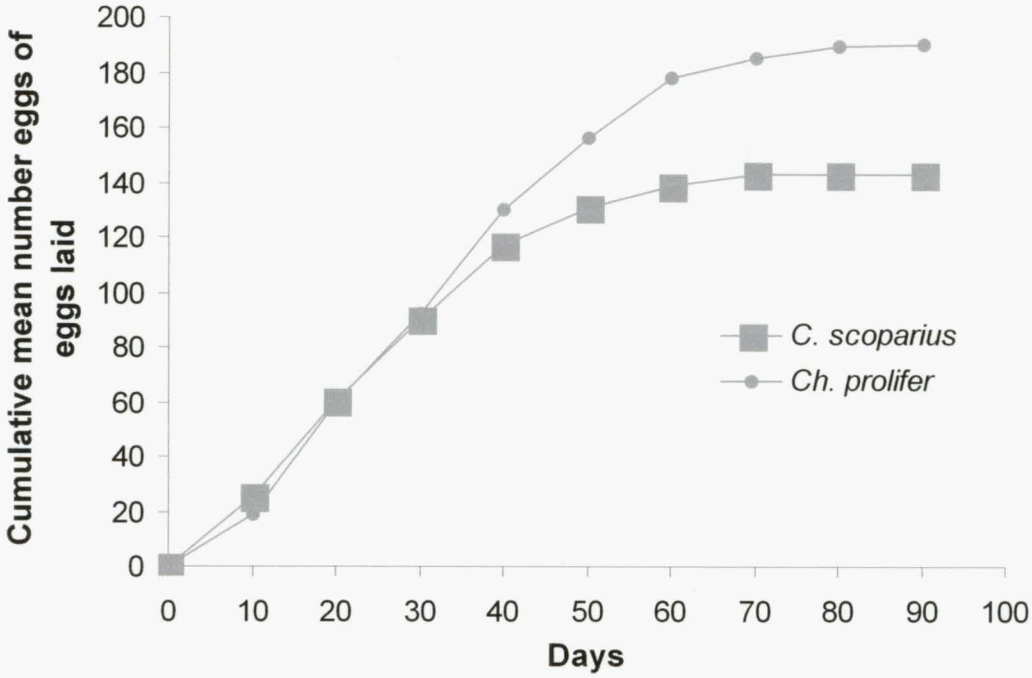


Figure 9.6: Cumulative mean number of eggs laid on *Cytisus scoparius* and *Chamaecytisus prolifer* by *Bruchidius villosus* female offspring reared on two hosts (*C. scoparius* and *Ch. prolifer*) over time. Each mean was calculated from the oviposition data of 18 female *B. villosus*.

9.4.1.5 Sibling preference

Larval rearing host significantly affected oviposition preference of female *B. villosus* (Table 9.4). There was no significant difference in the mean number of eggs laid on *C. scoparius* by beetles reared from either host. However beetles reared from *Ch. prolifer* laid significantly more eggs on this host over the duration of the experiment than their counterparts reared from *C. scoparius* (Table 9.5). Overall 75% of total eggs were laid on *C. scoparius* (Table 9.5).

Table 9.4: Significance of factors (larval rearing host, mother and sex and their interactions) contributing to *Bruchidius villosus* host preference.

Factors	F	df	P
Larval rearing host	6.0	1	0.02
Mother	3.3	2	0.05
Larval rearing host × mother	1.52	2	0.24

Table 9.5: Mean number (no.) and percentage of eggs laid by *Bruchidius villosus* offspring on *Cytisus scoparius* and *Chamaecytisus prolifer* according to larval rearing host (*C. scoparius* and *Ch. prolifer*) in a two-choice cut shoot test.

Larval rearing host	Mean no. eggs laid on *C. scoparius*	Mean no. eggs laid on *Ch. prolifer*	Mean no. eggs laid on both hosts combined
C. scoparius	128.4 (89.8%) a	14.6 (10.2%) a	143.0 a
Ch. prolifer	121.9 (64.0%) a	68.6 (36.0%) b	190.5 b
Hosts combined	125.2 (75.1%)	41.6 (24.9%)	166.8

Values with the same letter and within the same column are not significantly different (5% LSD, 23.8).

Overall mean coefficients of preference for *B. villosus* reared on *C. scoparius* and *Ch. prolifer* were 0.68 (outlier included) and 0.32 respectively, showing that offspring reared on *C. scoparius* had a significantly stronger preference for this host than those reared on *Ch. prolifer* (5% LSD, 0.3) (Table 9.6)

Table 9.6: Mean coefficient of preference for oviposition (+1 = Preference for *Cytisus scoparius* and -1 = Preference for *Chamaecytisus prolifer*) by *Bruchidius villosus* offspring reared from three mothers and two hosts (*C. scoparius* and *Ch. prolifer*).

Mother	Larval rearing host	Mean preference	Mean preference (with outlier excluded)
1	*C. scoparius*	0.77 a	0.77 a
	Ch. prolifer	0.70 a	0.70 a
2	*C. scoparius*	0.43 ab	0.71 a
	Ch. prolifer	0.12 b	0.12 b
3	*C. scoparius*	0.83 a	0.83 a
	Ch. prolifer	0.14 b	0.14 b

Values with the same letter (within columns) are not significantly different (5% LSD, 0.52).

Mother did not significantly affect offspring oviposition preference (Table 9.5) but a tendency toward variation in preference between offspring of different mothers was shown where offspring of Mother 1 from *Ch. prolifer* showed a stronger preference toward *C. scoparius* (0.70) (Table 9.6) than siblings from other mothers reared on this host.

By excluding one outlier (one offspring of Mother 2 which was reared on *C. scoparius* and oviposited solely on *Ch. prolifer*) and repeating the analysis on a subset of the data, the overall results changed. This made the effect of larval rearing host on offspring oviposition preference more significant ($P < 0.001$) and the effect of mother less significant ($P > 0.05$).

All offspring of Mother 1 reared on either *C. scoparius* or *Ch. prolifer* strongly preferred *C. scoparius* over *Ch. prolifer* for oviposition except two individuals (one reared from each host) which had an equal preference for both hosts (Figure 9.7 A).

Five of the six offspring of Mother 2 reared from *C. scoparius* preferred *C. scoparius* for oviposition while one individual strongly preferred *Ch. prolifer* and laid all eggs on this host. One offspring reared from *Ch. prolifer* strongly preferred *C. scoparius* while all others showed an equal preference for both hosts or a slight preference for *Ch. prolifer* (Figure 9.7 B).

All offspring of Mother 3 reared on *C. scoparius* preferred *C. scoparius* for oviposition. Four of the six offspring reared on *Ch. prolifer* had a preference for *C. scoparius* while two showed a slight preference for *Ch. prolifer* (Figure 9.7 C).

No significant interaction between larval rearing host and mother was found (Table 9.5). Overall, all siblings regardless of larval rearing host and mother exhibited a preference for *C. scoparius* indicated by the positive preference indices in all cases. Mothers reared on *Ch. prolifer* showed a more variable mean coefficient of preference for oviposition.

The mean number of eggs laid per pod on *C. scoparius* was 4.6 (95% CI: 4.2, 5.0) while mean seeds available for oviposition was 9.4 (95% CI: 9.2, 9.6). Mean number of eggs laid per pod on *Ch. prolifer* was 1.5 (95% CI: 1.3, 1.7) while the mean seeds available for oviposition was 6.6 (95% CI: 6.4, 6.7).

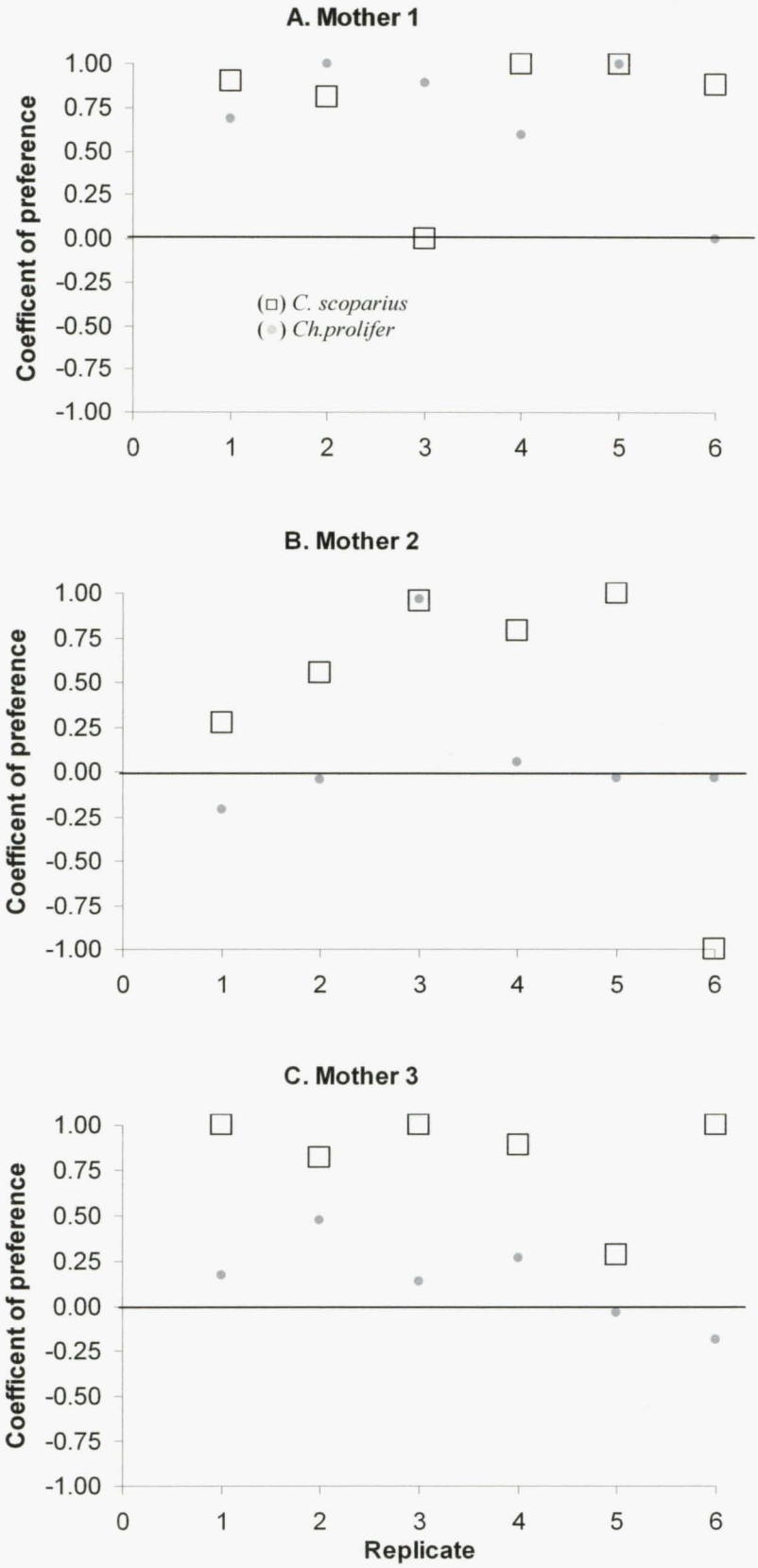


Figure 9.7: A, B and C: The effect of Mother (1, 2, and 3) and larval rearing host (*Cytisus scoparius* (□) and *Chamaecytisus prolifer* (●) on the coefficient of preference (+1 = preference for *C. scoparius* and -1 = preference for *Ch. prolifer*) for oviposition of *Bruchidius villosus* offspring (36) in a two-choice cut shoot test containing *C. scoparius* and *Ch. prolifer*.

9.5 Discussion

9.5.1 Sibling weight – body size

Bruchidius villosus females determine the fitness of offspring by selecting the host plant on which their progeny must develop. Oviposition choice by maternal females is important as *B. villosus* larvae are relatively immobile and therefore must burrow into the pod upon which they are laid.

In this investigation *B. villosus* reared from *Ch. prolifer* were significantly heavier than those reared from *C. scoparius* (Figure 9.1). This finding supports other studies that have shown that host size is a major determinant of body size (weight) in species that oviposit on discrete resources such as seeds, within which offspring must complete development. Host size is a limiting factor for such insects, because the amount of food available for development is the contents of a single host seed (Mitchell 1975, McClure et al. 1998). *Stator limbatus* beetles are affected by the size of the seed from which they emerge (larger seeds produce larger beetles than smaller ones) (Fox 1997). Similarly, larvae and newly emerged adults of the weevil *Mechoris ursulus* Roelofs were heavier when reared from larger acorns (Koo et al. 2003). This trend has been observed for *B. villosus* in this study (M. Haines, personal observation).

Mother and sex (and their interaction) significantly affected offspring body size (Table 9.1). It is unknown whether this was genetically inherited or via a maternal effect. Maternal effects are non-genetic influences of maternal phenotype or environment on progeny phenotype. This means that the progeny in a new generation are affected by the environment experienced by their mother in the previous generation (Mousseau and Dingle 1991, Fox 2000). They can also be considered as non-mendelian parental effects, which can include both maternal and paternal contributions (Lacey 1998, Hunter 2002). Maternal size generally affects egg size and/or composition, which in turn can affect progeny growth and development (Mousseau and Dingle 1991). Females in this study were significantly heavier than males which appears to be the trend in most insects (Nylin and Gotthard 1998).

9.5.2 Sibling overwintering survival

Both female and male adult *B. villosus* reared from *Ch. prolifer* had a higher overwintering survival than those reared on *C. scoparius* (Figure 9.2). Females and males

reared from *Ch. prolifer* also had larger body sizes which may have contributed to the increased rate of survival. Large adult insects are thought to store more lipid or glycogen reserves than small ones (Ohgushi 1996), improving overwintering success. Of note however, is that if body size was the only factor affecting overwintering survival, females would be expected to live longer than males because they are significantly heavier (Figure 9.1). However male overwintering survival was significantly greater than that of females in this investigation. This suggests that body size is not the only factor governing overwintering survival.

Large females of the beetle *Labidomera clivicollis* Kirby had significantly higher overwintering survival than small females and large males in the population had significantly lower overwintering survival than small males (Palmer 1985). Large adults of the herbivorous ladybird, *Epilachna niponica* (Lewis), showed higher rates of survival during hibernation than their smaller counterparts (Ohgushi 1996). The main factor explaining variation in overwintering survival of *Meligethes aeneus* (F.) was body weight, only the heaviest beetles survived (Hokkanen 1993). So it is unclear why the lightweight males in this study lived longer than their heavier female counterparts.

9.5.3 Sibling longevity

Bruchidius villosus reared on *Ch. prolifer* lived significantly longer than those reared on *C. scoparius* by 15.4 days (Figure 9.3). However, this result could be confounded by the methodology, because the beetles reared from *Ch. prolifer* were younger than those from *C. scoparius* (by two-three weeks) when the experiment commenced. This occurred firstly because the beetles were sleeved on *Ch. prolifer* one week after *C. scoparius* (due to differences in plant phenology), and secondly because beetles on *Ch. prolifer* take approximately two weeks longer to develop to maturity on *Ch. prolifer* (Table 4.2).

For the purposes of this investigation longevity was measured as the period of time from adult emergence to death, not from egg to adult as is usual, and is considered to be equal between *B. villosus* reared from both hosts. However, if it is argued that the increased time spent as a larvae or pupae was a trade-off against time as an adult then greater longevity exhibited by offspring reared on *Ch. prolifer* may well be described as a host effect.

In a study by O'Donnell and Manfield (1986) the *B. villosus* oviposition period lasted 68 days from the start of oviposition, whereas on average beetles in this

investigation died between 43.4 and 58.8 days after commencing oviposition (section 9.4.1.2). This suggests that the laboratory conditions provided may not have been ideal throughout the oviposition period.

Cumulative mean egg laying rate for beetles reared on *C. scoparius* began to level off after 40 days and at this point 13 out of the initial 18 females were still alive. In the case of beetles reared on *Ch. prolifer*, 13 beetles also were still alive at 60 days when the egg laying rate began to level off (Figure 9.4).

Longevity often shows a positive correlation with fecundity in insects (Cheng 1972, Leather 1984, Møller et al. 1989, Messina and Slade 1999, Messina and Fry 2003). However, Martin and Hosken (2004) showed that longevity is negatively affected by egg laying and other authors suggest a trade-off between the two, as females deprived of seeds and mates laid fewer eggs and lived longer (Møller et al. 1989, Tatar et al. 1993). Messina and Slade (1999) and Tatar and Carey (1995) reported the weakness of body mass as a predictor of longevity (life span).

9.5.4 Sibling fecundity

Lifetime fecundity in this study was estimated from the total number of eggs laid. Beetles were dissected after death in order to determine potential fecundity from unlaid eggs, but no unlaid eggs were found. *Bruchidius villosus* reared from *Ch. prolifer* were bigger (Figure 9.1) and laid more eggs than those reared from *C. scoparius* (Figure 9.5). Cumulative mean number of eggs laid by *B. villosus* in this investigation ranged from 143 (larval rearing host *C. scoparius*) to 191 eggs (larval rearing host *C. prolifer*) (Figure 9.6). This is lower than estimates of mean fecundity made by Parnell (1966) who predicted an average range of between 185–235 eggs per female in the field.

Lifetime fecundity shows a strong positive relationship to female body-size (Nylin and Gotthard 1998, Sopow and Quiring 1998, Garcia-Barros 2000, Smith et al. 2002) and oviposition rate in many insects (Smith et al. 2002). Kawecki (1994) also found that fecundity increased with body size in *Callosobruchus maculatus*, another seed feeding beetle. Fox and Czesak (2000) state in their extensive review on progeny size that larger individuals generally lay both more and larger eggs.

Male genotype can affect all aspects of female reproduction, through its effects on female longevity, total offspring production, reproductive rate, mating rate and fertility (Nilsson et al. 2002). Clearly knowledge of paternal influence(s) (referred to as paternal

effects (Mousseau and Fox 1998)) may account for some of the unknown variation among siblings. Many females throughout various taxa are known to copulate multiply with more than one mate (multiple mating) or repeatedly with the same mate (repeated mating) (Page 1986, Eady 1995, Choe 1997, Birkhead and Møller 1998, Yasui 1998, Arnqvist and Nilsson 2000, Jennions and Petrie 2000). After completing this investigation it was discovered that it is likely that *B. villosus* females mate multiple times with different males, as this is common in other bruchids (Eady 1995). The mothers in this investigation were collected pre-mated from the field so it is not known if all offspring from each mother were fathered by the same male, or whether siblings are full or half.

Mean daily oviposition rate in *B. villosus* correlated well with survival, declining as mortality increased (Figure 9.3 and Figure 9.6). Variability in the number of eggs laid over the 90 day duration of the experiment was as expected: the mean numbers of eggs laid were low at the beginning and end of the investigation, with higher numbers laid in between. This data supports the pattern observed for egg laying in the field (M. Haines, unpublished data).

9.5.5 Sibling preference

Larval rearing host had a small but significant effect on oviposition preference (Table 9.5, Table 9.6 and Figure 9.7) which may suggest there is some type of conditioning occurring between larvae and host which affects adult behaviour (Hopkin's selection principle) (Thorpe 1939, Jermy et al. 1968). No effect of larval rearing host was found on oviposition in Chapter 4 where groups of insects were tested. Many studies have shown host experience often influences oviposition preference of female insects (Rausher 1983a, b, Papaj and Prokopy 1988, Szentesi and Jermy 1990, Bernays 1995) and if host experience also influences larvae on these hosts it could lead to rapid speciation particularly in systems where maternal oviposition choices and offspring performance determine fitness (Mousseau and Fox 1998). However, there is little evidence that adult behaviour is affected by larval experience (Tabashnik et al. 1981, Jaenike 1982, Papaj and Rausher 1983, Diehl and Bush 1984, Futuyma and Peterson 1985, Prokopy et al. 1988, Via 1991).

The influence of larval experience has now been largely discounted in favour of early adult experience, which may in fact be of the larval rearing host (Courtney and

Kibota 1990 and references therein). Prokopy et al (1988) propose that fidelity to a certain host as a result of larval or adult experience may give rise to and assist in maintaining host races.

All offspring in this investigation preferred *C. scoparius* but there were varying degrees of preference (Table 9.6). Two replicates reared from *Ch. prolifer* had a significantly lower preference for *C. scoparius* and found their larval rearing host to be almost equally suitable for oviposition as *C. scoparius*, whereas the third replicate found *C. scoparius* as acceptable as those which were reared from it. On the other hand, while two replicates reared on *C. scoparius* significantly preferred their larval rearing host the third replicate (without the outlier excluded) displayed a preference that was not significantly different from either group and fitted in between the strong and weak preference for *C. scoparius* (Figure 9.7). Maternal, paternal and genetic effects may be useful tools in explaining the variation in preferences. As with many other traits, there would be a selective advantage in having a small frequency of individuals that display a wider host range within a population, that would act as insurance against the disappearance of the normal host.

Mother did not significantly affect host preference, but the low P-value indicates (Table 9.4) that there is a tendency for offspring of different mothers to behave differently. Mousseau and Fox (1998) suggest that if maternal or paternal larval rearing host is predictive of local or future host availability it is advantageous for parents to produce offspring that are acclimated to that host, and if this could be demonstrated for any insect, it would have profound implications for the understanding of host-race formation.

However, the result obtained here could be a sampling related effect because only three mothers were used which may not be representative of the population as a whole. For statistical purposes one outlying value (an offspring which was reared on *C. scoparius* and laid all her eggs on *Ch. prolifer*) was removed from the data set (Table 9.6) and it significantly altered the overall preference. Care must be taken when interpreting outliers, they should not be discarded for statistical convenience as they can be important in a biological capacity (Barnett and Lewis 1994). It is uncertain as to how this beetle should best have been treated.

Substantial larval rearing host effects have been reported in *S. limbatus* by Fox et al. (1995). Their study showed that progeny performed best with regard to size, survival and development time on the host from which their mother was reared. This is a good example of a host plant mediated maternal effect (Fox 2000).

It is possible that offspring may be able to condition to hosts from which they are reared via non-genetic maternal effects. Researchers have begun to recognise that maternal effects can affect offspring phenotypes and patterns of host suitability (Via 1991, Rossiter 1996). However, Via (1991) demonstrated that maternal host species had no effect on the relative performance of clones of aphids reared on two different hosts. Maternal larval rearing host may have major effects on offspring phenotype (Fox et al. 1995). It is also possible that the preference patterns exhibited are under genetic control (section 8.6.3).

Oviposition preference was not influenced by resource limitation in this study. Mean number of eggs laid per *C. scoparius* pod was 4.6 (95% CI: 4.2, 5.0) while mean seeds available for oviposition was 9.4 (95% CI: 9.2, 9.6) (section 9.4.4). Over the duration of the experiment pod material available was variable in quality, particularly at the end of the season when green pods on both species became rare. However, every attempt was made to ensure pods at an equivalent stage of development were presented together, in order to minimise pod quality as a source of error.

9.5.6 Host race development

The criteria that populations must fulfil to be considered distinct host races (Marohasy 1996) have been discussed in the introduction to this Chapter.

The contributions of Criteria 1 and 3 toward host race development have been discussed in Chapter 7 and there was no indication that populations reared from *C. scoparius* and *Ch. prolifer* fulfilled these criteria. There was no evidence from experimental work at that time to suggest that beetles from the two hosts were not interbreeding or that a preference was developing for *Ch. prolifer*. In this investigation biological characteristics of beetles reared from both host species were examined to answer the question of the final criterion (2). All relevant findings in relation to host race development are summarised in Table 9.7.

Table 9.7: Is there selective pressure for a host race to develop in New Zealand?

Criteria for host race development	Answer
Can an extra generation per year be completed on *Chamaecytisus prolifer*?	Possibly (M. Haines, unpublished data)
Are beetles reared on *Ch. prolifer* bigger than those reared on *Cytisus scoparius*?	Yes (section 9.4.1.1)
Are offspring reared on *Ch. prolifer* more fecund than those reared on *C. scoparius*?	Yes (section 9.4.1.4)
Is overwintering survival greater on *Ch. prolifer*?	Yes (section 9.4.1.2)
Is the total number of offspring which survive higher on *Ch. prolifer* than *C. scoparius*?	No (Chapter 4)
Are the adults reared from *Ch. prolifer* longer lived?	No – assumed equal, possibly shorter (section 9.4.1.3 and 9.5.3)
Is developmental time faster on *Ch. prolifer*?	No – constrained by host (Chapter 4)
Do beetles reared on *Ch. prolifer* host show a preference for this host?	Not completely – possibly some influence of larval rearing host (section 9.4.1.5)

With respect to most criteria tested in this investigation *B. villosus* appear to do better on *Ch. prolifer*. There is significant variation depending on mother (Table 9.1 and a tendency towards it in 9.4 and section 9.4.1.5) which indicates that fitness and survival are influenced by genetics and the environment (host). Body-size, overwintering survival and fecundity were greater when larvae were reared on *Ch. prolifer* (section 9.4.1.1, 9.4.1.2 and 9.4.1.4) but *C. scoparius* supported higher survival and shorter development time (section 4.5.2.2). Longevity was greater on *Ch. prolifer* but this result is not conclusive (section 9.4.1.3). It is possible that in some seasons *B. villosus* may be able to complete a second generation (M. Haines, unpublished data). Overall preference in all experiments to date support *C. scoparius* remaining as the most preferred host (section 9.4.1.5 and Chapter 2, 4, 5, 6, 7 and 8). However, some individuals showed almost equal preferences for both hosts or a preference for *Ch prolifer* (Figure 9.7A and 9.7B and section 8.5.3). The existence of populations that have shifted onto a new host but still show preference for the ancestral host is not unique (Futuyma et al. 1984, Robert 1985, Prokopy et al. 1988).

Host preference is an obvious precursor for host-race formation. Induction of preference for an alternative host and physiological adaptation thereafter may result in higher performance on this host, which may contribute to the early stages of host race formation. Despite the fact that beetles reared on *Ch. prolifer* take longer to develop and that survival on this host during development is not as high as on *C. scoparius* (section

4.5.2.2 and Figure 4.4), those surviving the larval stage show better overwintering survival (Figure 9.2) and they are more fecund (Figure 9.5) which may counteract this.

Persistence of environmental variation across generations has been reported in another bruchid, *C. maculatus* (Fox 2000). Maternal effects influence responses to natural selection and can respond to natural selection, providing an important mechanism for adaptation to a variable environment or variation among hosts in suitability (Fox 2000).

The results in this study are in accordance with those of Prokopy et al (1988) who compared host acceptance behaviour of *Rhagoletis pomonella* (Walsh) from populations reared on the native host, *Crataegus oxyacantha* L. (hawthorn) and from the introduced host, *Malus domestica* Borkh. (apple). Generally, hawthorn fruit were accepted for oviposition nearly equally by hawthorn and apple origin females, whereas apples were accepted more frequently by apple origin females compared with hawthorn origin females. Irrespective of origin, females always responded more positively to hawthorn than apples. Larval survival was higher in hawthorn than apples. However, Prokopy et al (1988) found no effect of preimaginal conditioning as a result of larval rearing host, and they concluded that the phenotypic differences in host acceptance behaviour was possibly genetic.

What factors caused *B. villosus* to include *Ch. prolifer* in its host range? Perhaps emigration to *Ch. prolifer* by a few individuals was generated by pods of *C. scoparius* becoming overloaded with eggs. Approximately 90% of seed at the study site is being destroyed by *B. villosus* (M. Haines unpublished data). However, *B. villosus* is known to heavily superparasitise pods and deposit many more eggs on the surface of a pod than seeds available within it when resources are limited. Despite the high density of eggs being laid in the field some pods still have no eggs deposited on them, so this may not be an adequate explanation for the colonisation of *Ch. prolifer* by *B. villosus.*

Variation in preference among individuals within the *B. villosus* population has been demonstrated (Figure 8.1 and 8.2), but the factors responsible for this variation have not yet been examined. There is a possibility that there is a genetic basis for the differences in oviposition preference shown by individuals, which may allow them to accept some hosts more readily than others. Genetic differences in host plant preferences

between conspecific populations may contribute to host race maintenance or formation (Prokopy et al. 1988).

Both *C. scoparius* and *Ch. prolifer* are in the tribe Genisteae, and the two genera are so closely related that some authorities place them in the same genus, so it is likely that they share some similar chemical and morphological attributes which may stimulate an oviposition response in some members of the beetle population. Pods of *Ch. prolifer* contain bigger seeds (Table 4.3) and produce larger (Figure 9.1), more fecund adults (Figure 9.5) which may constitute a selective advantage despite lower survival and longer development time, especially if a second generation per season can be produced on this host. It is possible that host race formation and maintenance can be affected by differences among hosts in factors that may affect survival (Prokopy et al. 1988).

When *C. scoparius* ceases flowering at the end of the season, *B. villosus* have been observed feeding on pollen of other flowering plants (M. Haines, personal observation). If individuals migrated to *Ch. prolifer* (which generally exhibits a longer flowering period than *C. scoparius*) at the end of the season to feed on pollen, they may overwinter and resume feeding on this plant when it flowers earlier than *C. scoparius* in the following spring. Sometimes *Ch. prolifer* is already bearing its first green pods as flowering commences in *C. scoparius*, so that mating on *Ch. prolifer* may have already occurred and oviposition on this species will proceed in the absence of *C. scoparius* pods. Hence, differences in phenology between the two plant species may contribute to host race formation. This idea is supported by Prokopy et al (1988) who refer to the potential influence of temporal differences in host race formation and maintenance.

Adaptation to *Ch. prolifer* (the alternative host) may lead to the evolution of reproductive isolation among populations if it produces assortative mating (non-random mating in which individuals mate preferentially according to phenotype) (Bush and Diehl 1982). This may be further exemplified if preferences for larval rearing host are apparent in males. Further exploration of mating behaviour is necessary before any further inferences can be made.

If an insect can recognise and survive on a new host plant then the host range will expand to include the new host. If mutation and selection which would improve fitness on the new host do not occur, it is unlikely that the population will diverge greatly. However, if they do occur, host-race formation could evolve (Bush and Diehl 1982).

Host race formation has occurred in *Jadera haematoloma* (Herrich-Schaeffer), where large differences in beak size have occurred as a result of host fruit size. This adaptation has occurred over a period of 20-50 years which equates to 40-150 generations (Carroll and Boyd 1992). *Bruchidius villosus* has only been present in New Zealand for 17 years, so it may be that it is currently in a transition phase toward host race formation. If more than one generation per year can be produced on *Ch. prolifer* evolution may proceed faster as a result of the longer flowering period of this plant.

Further experimentation is required to validate these findings before any conclusion can be made about the likelihood that a host race is forming. Firstly a larger sample size is necessary to provide a more representative sample of the field population. In future investigations, knowledge of paternity and paternal influences would be useful, as would data on egg size and effects of age. The examination of offspring reared from females originating from both hosts is critical if the influence of maternal effects is to be determined. Quantitative genetic analyses will be necessary to measure genetic based components and estimate heritability.

9.6 Conclusion

To date interbreeding is assumed to occur between the two potential populations and both of these populations prefer the same host, *C. scoparius*, meaning that two of the three host race criteria proposed by Marohasy (1996) are not yet fulfilled. This investigation was designed to establish whether the remaining criterion is met, ie. whether the beetles reared from different hosts differ in biology and not (or marginally) in morphology. Beetles do differ marginally in morphology: they exhibit phenotypic plasticity as a result of larval rearing host. Biologically, those reared from *C. scoparius* develop faster and show higher levels of survival than those reared on *Ch. prolifer*. However, *Bruchidius villosus* reared from *Ch. prolifer* are bigger, more fecund and show greater overwintering survival than their counterparts reared on *C. scoparius*. There is a possibility that a second generation of beetles may be produced in some seasons from *Ch. prolifer* due to flowering phenology and that preference may be influenced by larval rearing host. Hence it appears that the second criterion is adequately fulfilled. That this criterion is fulfilled indicates that *B. villosus* may be in the early stages of host-race formation on *Ch. prolifer*, but further investigations on oviposition preference are necessary. This may be monitored by observing the phenotypic response to the host over time, for example at 3-5 year intervals.

Because *B. villosus* is highly mobile and both hosts are commonly available in New Zealand complete separation of populations reared from each host is unlikely.

9.6.1 Future work

To test if the differences in body size and preference observed in this investigation are maternally inherited, performing reciprocal crosses between the two *B. villosus* populations may be useful. This was done by Savalli et al. (2000) with *C. maculatus*. Four types of crosses could be set up: two sets of intra-population crosses (female *C. scoparius* (*Cs*) × male *Cs* and female *Ch. prolifer* (*Chp*) × male *Chp*) and two sets of inter-population crosses (female *Cs* × male *Chp* and female *Chp* × male *Cs*). If inheritance was autosomal, the interpopulation crosses would resemble each other and be intermediate between the two intrapopulation crosses. If inheritance was maternal (either because of sex-linkage or maternal effects), the offspring of the interpopulation crosses would be expected to most closely resemble their respective maternal population.

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Bruchidius villosus reared from seeds of *Chamaecytisus prolifer* were significantly heavier, more fecund and had higher overwintering survival than those from *Cytisus scoparius*. Longevity was similar between beetles reared from the two species. The implications of the findings of the whole thesis are presented in Chapter 10.

Chapter 10: General Conclusions

10.1 Summary

The objective of this thesis was to determine why host specificity tests failed to predict that *Chamaecytisus prolifer* would be a suitable host for *Bruchidius villosus*, a biological control agent introduced into New Zealand to control the spread of *Cytisus scoparius*.

Based on results from field tests, the hypothesis that host specificity of *B. villosus*, measured as adult oviposition preference and development, had changed significantly between the pre-release study and the occurrence of post release non-target effects was rejected (Chapter 2). The possibility that the entity known as *B. villosus* was in fact a group of sibling species was eliminated by genetic analysis (Chapter 3). The importance of phenological synchrony in predicting host range was highlighted in Chapter 2, where known European hosts of *B. villosus* were not oviposited on in sleeved no-choice tests or the field trial probably because the oviposition period of the insect and the appropriate phenological stage of the plant species did not overlap.

None of the following factors were found to be the cause of the failure of the tests to predict that *Ch. prolifer* was a suitable host for *B. villosus*: seed size and stage of development (Chapter 4), larval rearing host or adult food type (Chapter 5), lack of overwintering, cage size, orientation of food resource, or amount of oviposition resource (Chapter 6). On the other hand, low replication (small sample size) in the original tests (Chapter 7), coupled with high levels of individual variation in oviposition behaviour among *B. villosus* (Chapter 8) were shown to be the most likely factors accounting for the discrepancy between the original test results and the new findings from the field in New Zealand.

Findings in Chapter 9 suggest that the potential exists for adaptation for improved performance on the non-target host. Further research is needed to determine whether a host race is developing. There is phenotypic plasticity involving differences in size and colour between beetles from the two hosts and several measures of fitness including weight, fecundity and overwintering survival are higher in beetles reared from *Ch. prolifer*. In all experiments conducted in this study no New Zealand native pod-bearing species were attacked by *B. villosus*, indicating that these plants will remain safe. The implications from this work highlight the importance of the sample size (level

of replication) used in host specificity tests to account for potential individual variation within populations, the potential for adaptation and increased utilisation of 'new association' non-target hosts, and the type of host specificity test selected (choice versus no-choice).

10.2 Host range and identity of *Bruchidius villosus*

Because of confusion in early literature over the nomenclature of *Bruchidius villosus* (Hoffmann 1945, Bridwell 1946, Zacher 1952a, Southgate 1963, Aldridge and Pope 1986) at the time of its release in New Zealand some apparent host records were not recognised and others were regarded as suspect (P. Syrett, personal communication). Apart from the identity problems, it was unclear whether some host records were rearing or perching records (Zacher 1952a, 1952b).

Chapter 2 aimed to clarify the field host range of *B. villosus* in New Zealand and investigate the possibility that it might be different from the field host range in Europe (its area of origin). If the host ranges differed between the two areas, it would have suggested that *B. villosus* had changed since pre-release testing. Results showed that the potential field host range of *B. villosus* is likely to be the same in New Zealand as in Europe. Fundamentally *B. villosus* can utilise a wide range of pod-bearing plant species, mainly from the tribe Genisteae, but the realised range it exhibits is dependent on the abundance and phenology of the host plant species available.

Chapter 3 aimed to determine whether the species known as *B. villosus* around the world is genetically one species with a broad host range or a group of distinct genetic sub groups with narrower host ranges. If it was found that *B. villosus* comprised distinct genetic sub groups this could explain the inconsistencies in the field and rearing records as compared with the specificity testing. A mitochrondrial DNA (COI) analysis of beetles from selected sites in the Northern and Southern Hemispheres found there was no significant variation in this gene, strongly suggesting that *B. villosus* is a single species and not a group of distinct genetic entities. It is concluded from a re-interpretation of early host records (Chapter 1) and from recently published work on its host range (Hosking 1992, Syrett and Emberson 1997, Wittenberg and Thomann 2001, Delobel and Delobel 2003, Jermy and Szentesi 2003, Haines et al. 2004, Kergoat et al. 2004) that *B. villosus* has a broader natural host range than was thought at the time of its introduction into New Zealand.

10.3 Examination of Testing Protocols

10.3.1 Effect of seed size and developmental stage

Chapter 4 investigated the effect seed size and pod developmental stage had on *B. villosus* oviposition and on larval survival and development. The developmental stage of certain plant parts is known to affect oviposition preferences in insects (Straw 1989, Hoddle 1991, Lalonde and Roitberg 1992, Bingaman and Hart 1993, Kouki 1993, Smyth et al. 2003a) and larval performance (Smyth et al. 2003b).

The effects of seed size and developmental stage on oviposition were examined to determine if the *Chamaecytius prolifer* pods provided in the host specificity tests conducted in 1985 were at the appropriate phenological stage for oviposition and subsequent development. If it was concluded that pods of *Ch. prolifer* at the right stage and size were not presented in the 1985 tests, this may explain why they received no eggs and therefore why the host specificity tests failed. *Bruchidius villosus* laid eggs on green pods of both *C. scoparius* and *Ch. prolifer*, which were of a similar size and at the same stage of development as those used in the original tests. It was therefore concluded that the use of inappropriate plant material was not to blame for the failure of the original tests.

10.3.2 Effect of larval rearing host, adult feeding and no overwintering period

Chapter 5 examined the effect that previous adult feeding experience, larval rearing host, and no overwintering period had on *B. villosus* oviposition. Firstly larval rearing host and adult feeding were investigated. Adult nutrition in insects has been shown to affect reproductive ability (Good 1933, Geering and Coaker 1961, Gilbert 1972, Romeis and Wackers 2002). Hopkin's Selection Principle describes the conditioning that can occur between larvae and host which affects subsequent adult behaviour (Thorpe 1939, Jermy et al. 1968).

Bruchidius villosus was thought to become reproductively mature only after feeding on *C. scoparius* pollen (Parnell 1966, Waloff 1968, Harman 1999). If adult food type or larval rearing host affects subsequent naive oviposition decisions in *B. villosus* the fact beetles were reared from *C. scoparius* and exposed to *C. scoparius* flowers before the choice tests in 1985 may have preconditioned them toward laying only on this host.

Beetles in this investigation were reared from *C. scoparius* and *Ch. prolifer*, overwintered and then presented flowers of either *C. scoparius*, *Ch. prolifer*, *G. monspessulana* or bee pollen. If *C. scoparius* was the only pollen source which could induce oviposition in *B. villosus*, eggs would only be laid in treatments where beetles had been presented *C. scoparius* flowers. Eggs were laid in all treatments, so it was concluded that all pollen types could induce oviposition. If the beetles imported in 1985 were conditioned to *C. scoparius*, beetles in this investigation reared from *C. scoparius* and fed *C. scoparius* would be expected to only oviposit on this host. Beetles which were reared from *C. scoparius*, overwintered and fed pollen from *C. scoparius* flowers, laid eggs on *Ch. prolifer*. Larval rearing host and adult feeding were ruled out as the cause for the failure of the original tests.

Secondly the effect of no overwintering period was investigated. Insects are often transported to countries with reverse seasonality for host specificity testing, and as a consequence are used in tests without overwintering (Syrett and O'Donnell 1987). The beetles used in the 1985 host specificity tests were imported to New Zealand shortly after emerging from field collected pods of *C. scoparius* in the UK. On arrival in New Zealand quarantine they were fed bee pollen and were presented with broom flowers for five weeks before being exposed to small green pods of *C. scoparius*. After two weeks exposure to green pods testing began. The beetles were not overwintered as they were collected at the end of summer in the UK, which coincides with the beginning of spring in New Zealand, the time of year when test plant species are flowering and beginning to bear pods. If no overwintering period affects oviposition decisions this may have affected the outcome of the original tests.

To test this hypothesis, beetles which emerged from *C. scoparius* pods collected from the field in New Zealand were treated, as far as possible, identically to the beetles imported in 1985, with no overwintering period. These beetles laid eggs on *Ch. prolifer*. Lack of overwintering was therefore ruled out as a possible cause for the failure of the 1985 host specificity tests.

10.3.3 Effect of quantity of oviposition resource, cage size and orientation of plant material

Chapter 6 investigated the effect that quantity of oviposition resource, cage size and orientation of test plant material had on *B. villosus* oviposition. Cage size is sometimes

known to disrupt insect behaviour (Olckers et al. 1995, Marohasy 1998, Sands and Van Driesche 2000). Orientation of plant material in host specificity tests has not been widely studied. The rationale for studying it in this investigation was that shoots were presented to *B. villosus* in the original tests lying horizontally on the base of the cage. Presenting the shoots vertically, as was done in this investigation was thought to be more representative of the pods orientation in a natural field situation. Host specificity tests which combine the advantages of choice and no-choice tests are under development. One suggested method is to reduce the amount of control oviposition resource available in choice tests, so that when oviposition sites become overloaded on the control the pressure to lay on the more abundant test plant is increased (Heard 1997). The original tests were conducted in small cages, and cut shoots of the test plant and control bearing equal amounts of pod material were placed horizontally on the base of the cage.

If *B. villosus* needed a large space (bigger cage), vertical orientation of plant material (possibly more natural) or increased resource pressure (control plant resource halved) to oviposit on alternative or less preferred hosts, this may explain why *Ch. prolifer* received no eggs. All combinations of the factors listed above were tested. *Chamaecytisus prolifer* was laid on by *B. villosus* in at least some, and in some cases all, replicates of all the combinations of treatments. There was no difference in numbers of eggs laid in large and small cages and on horizontally or vertically orientated shoots. Halving the ratio of the control oviposition resource compared to the test plant in some replicates resulted in more eggs being laid on the test plant in two cases (one significantly), compared to replicates where equal amounts of control and test plant resource were provided. However beetles laid on *Ch. prolifer* in treatments where amounts of test plant and control resource were equal. Because eggs were laid on *Ch. prolifer* in at least some replicates of all treatments (including one treatment which replicated the same experimental design as in 1985) the effect of cage size, orientation of plant material and quantity of resource provided were ruled out as factors contributing to the false negative result produced in tests conducted in 1985.

10.3.4 Sample size and individual variation

Chapter 7 investigated the effect of sample size on oviposition preference of groups of *B. villosus* beetles. In the cut shoot choice oviposition tests conducted in 1985 the treatment containing *Ch. prolifer* was replicated only twice (a total of ten female beetles).

Beetles were imported into New Zealand from the UK in 2002 and the original tests were repeated as closely as possible, apart from the level of replication, which was increased to ten replicates (50 female beetles). The hypothesis was if UK beetles still refused to accept *Ch. prolifer* for oviposition, while their New Zealand progeny now accepted it, then a host range expansion would be the most likely explanation. The sample size was increased and *B. villosus* laid eggs on *Ch. prolifer* in some replicates, indicating that the beetles released in 1986 had not undergone a host shift in the intervening 16 generations since their release, and that the sample size in the earlier choice test was insufficient to reveal the acceptability of the alternative host.

In order to find out how likely it was that the low level of replication failed to identify *Ch. prolifer* as a host, Chapter 8 determined oviposition preferences of individual *B. villosus* beetles. All previous testing in this study, as well as all other host specificity testing with this insect, had been done in groups, potentially masking individual behaviour. In the group testing conducted in Chapter 7 some replicates of beetles laid no eggs on *Ch. prolifer*. If there was individual variation in oviposition behaviour within the *B. villosus* population, by using a small number of female beetles, as was done in the original tests, the likelihood of not getting a representative sample is reasonably high.

Significant individual variation in oviposition behaviour was found, sufficient to account for the discrepancy between results from the two tests carried out in 1985 and 2002. Some beetles laid on *C. scoparius* only, others laid on both *C. scoparius* and *Ch. prolifer* and a few laid only on *Ch. prolifer* or neither plant species. According to Ruxton and Colegrave (2003) "Replication is a way of dealing with the between individual variation due to the random variation that will be present in any life science experiment. The more replicates we have, the greater the confidence we have that any factors we see between our experimental groups is due to factors that we are interested in and not due to chance". If a larger sample size had been used in the original tests, the acceptability of *Ch. prolifer* for oviposition by *B. villosus* may have been revealed.

10.3.5 Host race formation

Host range expansion was ruled out as the possible cause of the unpredicted colonisation of *C. prolifer* in New Zealand in Chapter 7, but this idea was examined further in Chapter 9. The likelihood that host race formation had occurred or was likely to occur in New Zealand, following the release of the beetles was investigated, by comparing the fitness of

B. villosus reared from *C. scoparius* and *Ch. prolifer*. For a host range expansion to occur a host race must first develop (Marohasy 1996). Phenotypic plasticity involving size and colour was observed between beetles reared from the two hosts, but it was not clear whether this was indicative of differences in fitness, which may indicate host race formation.

Bruchidius villosus reared from *Ch. prolifer* were larger, more fecund and survived overwintering better than their counterparts reared from *C. scoparius*. However, they took longer to develop from egg to adult and their longevity was not significantly different from those reared from *C. scoparius*. An alternative hypothesis for the size differences is that they are related to seed size and not related to fitness. In another seed feeding beetle, *Stator limbatus*, larger seeds produce larger beetles than smaller ones (Fox 1997). It is possible however, that this may result in increased fecundity, as many studies on insects have shown strong correlations between body size and fecundity (Varley et al. 1973, Credland et al. 1986, Clutton-Brock 1988, Garcia-Barros 2000, Smith et al. 2002, Tammaru et al. 2002). There was some evidence that maternal effects could affect some life history traits in *B. villosus*, ie. body size and also oviposition preference (section 9.5.1 and 9.5.5). If some individuals that accept *Ch. prolifer* perform well on this 'new association' over time, a 'strain' of *B. villosus* may evolve that uses *Ch. prolifer* preferentially (Hokkanen and Pimentel 1989). Further work would be necessary to assess this and the heritability of fitness variables.

10.4 Implications for weed biological control

10.4.1 The role of choice and no-choice tests

The original choice tests conducted in 1985 failed to predict that *B. villosus* would find *Ch. prolifer* an acceptable host in the field in New Zealand. This is described in the biological control literature as a false negative (Type II error) result. Choice tests are prone to producing false negative results as they often indicate only a strong preference for the target plant, but have been preferred because they are considered to simulate more natural conditions than no-choice tests (Harley 1969). If no-choice tests had been conducted in 1985 it is possible that the acceptability of *Ch. prolifer* would have been recognised. No-choice tests are considered less likely to produce false negative results (Cullen 1990, Heard 1997). Paynter et al. (2004) recommend that they be used especially

when there is asynchrony in phenology between target and non-target plant. However, because no-choice tests are known to overestimate field host range, choice tests are often preferred to reveal the relative levels of preference (Marohasy 1998, Edwards 1999).

From the results presented here, and from other work, it is clear that for improved prediction of the host range of introduced biological control agents for weeds more sophisticated testing methods will be required than have commonly been employed by practitioners. Choice tests will play an important role in future host specificity testing protocols, although choice tests in which the target weed is eliminated have been shown to have particular merit (Marohasy 1998).

Marohasy (1998) suggests three steps for assessing the host specificity of agents: 1) determine if any test plant species are susceptible to attack, 2) determine the rank order of susceptible species (because the extent of oviposition in a field situation will depend on the acceptability of the plant species relative to other plants and the target weed) and 3) quantify the length of the discrimination phase of potential biological control agents (to help predict under which field conditions attack on a lower ranked host may occur). Test plants (without the target plant) are tested individually or in groups for durations suitable to the biology of the insect. Cages containing the target weed are set up as controls, and run concurrently with those containing test plants to ensure agents are in a physiological state such that oviposition will readily occur on the host plant. By continually removing the most highly ranked plant from a group of test plants and replacing it with the next most highly ranked species (not already present in the assay) until no oviposition occurs, rank order can be established among test plants. The discrimination phase of the insect can be calculated by measuring the length of time taken between hosts for the insect to oviposit.

In an extensive review by Edwards (1999) the use of choice tests in host specificity testing of insects is explored and a testing design from a culmination of tests employed by host specificity practitioners is proposed. A choice test with the target weed present, combined with a concurrent no-choice test on the target weed is recommended. After a certain time period (dependent on the biology of the agent), the target plant is removed from the choice test, and if no oviposition occurs on the remaining test plants they are excluded from further testing. If oviposition does occur on any test plants, those species can be removed and host ranking can be calculated. The timing of the test when

the control is removed is crucial, so the results are not biased by the insects' physiological condition.

10.4.2 Improving existing protocols

Learning from past mistakes will be crucial in improving host specificity testing protocols and in this way retrospective analyses are an important way to increase future safety of weed biocontrol (Louda et al. 2003a). It is important to continue to improve safety testing methods through appropriate application of theory and experience (Withers et al. 1999). Agents such as *B. villosus* that use discrete, seasonal resources present particular challenges both in design of host range tests, and in interpreting data from the field in the native range.

An important consequence of the work described in this thesis is the significance of individual variation. It is recommended that this is measured, so in crucial tests at least, individuals should be tested rather than groups. A similar high level of variation in oviposition preference as that shown here between *C. scoparius* and *Ch. prolifer* by *B. villosus* was found between *C. scoparius* and *Sophora microphylla* by the weevil *Pirapion immune* Kirby in which only a few females laid on *So. microphylla*, but some laid a large number of eggs (Syrett et al. 1995).

Louda et al (2003a) suggest that host specificity testing needs to be expanded so that the impact of potential agents can be predicted. In their review on case studies of non-target impacts, they found that host specificity testing was highly informative on fundamental host range, if the tests included potential hosts. However, choice preference tests did not predict actual host impact in many cases, so they recommend the incorporation of information on realised (ecological) range (Arnett and Louda 2002). Van Klinken and Edwards (2002) agree that the fundamental host range of a test population is generally easily determined experimentally by host specificity testing: by excluding environmental factors that might limit its expression, such as prior experience and time dependent effects, through use of naive insects and long duration no-choice tests.

In a review on host specificity testing Sheppard (1999) presents a flow chart generalising the decisions involved in selection of an initial host specificity testing procedure for a biological control agent. Sheppard (1999) recommends the collection of field evidence and basic screening in the native range, followed by assessment of the biology of the insect to determine appropriate testing. If the testing fails to elucidate

adequate specificity, the agent can either be rejected or subject to a second round of testing which is more stringent or natural. It is likely that incorporating these suggestions into current practical host testing procedures will substantially increase the cost, and reduce the viability of some projects. Increasingly however, regulatory authorities are demanding demonstration of positive benefit/risk ratios (ERMA 1998) so reliable measures of potential agent impact are likely to become more important to balance against the costs of the weed problem.

Because of non-target effects in which only a small proportion of insects within a population utilise the non-target host, it is not adequate to test a large number of potential host plants with only low levels of replication. In order to detect a 1% rate of utilisation (with 95% confidence), at least 300 individuals should be tested (300 females in oviposition tests) (Sedcole 1977) (section 2.5.2). This means that, especially in no-choice tests, follow up testing is likely to be required for a large number of plant species, and at this stage it is recommended that testing be conducted with individual insects to gain a real understanding of individual variation. It is concluded from the current study that no-choice tests are necessary to gain a measure of the fundamental host range of a potential biocontrol agent, even though they may over estimate the realised host range.

10.4.3 Host shifts

The greatest perceived risk to non-target plants through weed biological control is from changes in the pattern of use of hosts identified through host specificity testing as being within the fundamental host range of the agent. It is necessary to be able to predict whether increased use of a non-target species is likely to evolve, and whether those changes are likely to result in non-target impact reaching unacceptable levels. Physiological and behavioural factors that result in differential performance on target and non-target hosts can usually be identified and quantified through host specificity testing. However, to broaden the scope of prediction, a full assessment of the genetic variation present, likely gene flow, and selection pressure within an ecological context (eg. relative distribution and abundance) is likely to be necessary (van Klinken and Edwards 2002).

There has been considerable concern that weed biological control may be unsafe if rapid evolution in host use results in improved performance on non-target host species (van Klinken and Edwards 2002). *Chrysolina quadrigemina* Suffrian, a chrysomelid beetle, was released in California to control *Hypericum perforatum* L. Almost 30 years

after its release it was found attacking a closely related non-target native plant, *H. calycinum* L. which was already within its fundamental host range. Larval survival in populations collected from *H. calycinum* was higher than in populations collected from the target weed, suggesting that ecological specialisation had occurred in the population on *H. calycinum* (Andres 1986). However at the time the field attack was observed, the abundance of the non-target host was increasing (Andres 1986), so this observation may reflect a change in relative host availability (Andres 1986, Marohasy 1996). Nevertheless, the level of impact in situations such as this may be substantially greater than initially predicted. Results obtained here suggest that *B. villosus* is making greater use of *Ch. prolifer* than is likely to have been predicted by host specificity testing, even if they had been conducted according to current protocols.

10.5 Overall conclusions

Host specificity testing of potential agents for weed biocontrol can never provide complete certainty and guarantee no adverse outcomes. It does, however, provide the information required for a process of risk assessment to be undertaken (Blossey 1995, McClay 1996, McFadyen 1998). Presumably we have to live with a certain level of uncertainty and that even with the best methodology there is a slight risk of an unpredicted outcome. Biological control is one of the few cost effective and environmentally accepted means of dealing with large scale weed problems – it may be 'risky but necessary' (Thomas and Willis 1998). Nevertheless, classical biological control has recently come under greater scrutiny than ever before: Louda and Stiling (2004) ask 'whether the outcomes can be predicted precisely enough *a priori* to know with some certainty that the benefits will outweigh the environmental costs'. In order to address the criticism it is certain that more stringent testing, and more careful interpretation of both laboratory and field test results will be necessary. This study has emphasised the importance of individual variation in host acceptance. Even if only a very small number of individuals from a population are able to use a non-target host, releasing them in the field may lead to a highly significant impact. It is both challenging and expensive to design experiments sufficiently powerful to detect such small effects, but it will be crucial to do so in order to dispel the concerns regarding environmental risks posed by introduced biological control agents.

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Appendix 1

The following paper (which is Chapter 7 of this thesis), was presented in 2003 at the 11th Symposium on Biological Control of Weeds and published in the proceedings of this conference in 2004:

Haines, M., P. Syrett, R. Emberson, T. Withers, S. Fowler, and S. Worner. 2004. Ruling out a host range expansion as the cause of the unpredicted non-target attack of tagasaste (*Chamaecytisus proliferus*) by *Bruchidius villosus*. Pages 271-276 *in* J. M. Cullen, D. T. Briese, D. J. Kriticos, W. M. Lonsdale, L. Morin, and J. K. Scott, editors. Proceedings of the 11th International Symposium on Biological Control of Weeds. CSIRO Publishing, Melbourne, Canberra, Australia, 27 April-2 May, 2003

Ruling out a host-range expansion as the cause of the unpredicted non-target attack on tagasaste (*Chamaecytisus proliferus*) by *Bruchidius villosus*

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Summary

Scotch broom (*Cytisus scoparius*) is a woody shrub of European origin that is an invasive weed in New Zealand. *Bruchidius villosus* was released in New Zealand in 1986 as a biological control agent of Scotch broom, after tests indicated that it was specific to this species. However, in 1999, *B. villosus* was discovered developing in the seeds of an unpredicted host, tagasaste or tree lucerne (*Chamaecytisus proliferus*). Although the original choice tests carried out in quarantine failed to predict acceptance of *C. proliferus* by ovipositing females, the current population in New Zealand clearly finds this species an acceptable host. An investigation of the original host-testing procedures revealed a number of possible limitations in the tests conducted in the 1980s. Concerns that a host-range expansion might have occurred in a weed biological control agent led to this study in which beetles from the original population (Silwood Park, United Kingdom) were reimported and the original handling and host choice tests were replicated. Despite showing a strong preference for Scotch broom, the beetles tested in this study accepted *C. proliferus* for oviposition. These results allow us to rule out the possibility that a host-range expansion has occurred.

Keywords: *Bruchidius villosus*, *Chamaecytisus proliferus*, *Cytisus scoparius*, host-range expansion, host-specificity testing.

Introduction

Scotch broom, *Cytisus scoparius* (L.), Link is a woody shrub of European origin that is an invasive weed in many countries, including New Zealand, Australia and North America. The broom seed beetle *Bruchidius villosus* (F.) (previously referred to as *B. ater* (Marsham)) was identified as a potential biological control agent for New Zealand's Scotch broom weed problem because it was thought to attack only *Cytisus* species. Host-specificity testing began in the Inted Kingdom (UK) in 1985 and consisted of no-choice oviposition tests with adults being confined to either whole potted plants or to single branches of larger plants inside cotton mesh sleeve cages (Syrett & O'Donnell 1987). All hosts were required to be bearing young green pods (the stage of pod on which the broom seed beetle oviposits) at the time of testing. Thirteen species of non-target plants were tested, also seven species of potted non-target plants were tested together with *C. scoparius* in a choice test within a field cage in the UK (Syrett & O'Donnell 1987). In all these assays, eggs were only laid on *Cytisus* species (*C. scoparius* and *C. praecox* cv. Allgold). The insect was released as a biological control agent in New Zealand in 1986.

In 1985, *B. villosus* was imported into quarantine in New Zealand as newly emerged beetles from the UK *C. scoparius* pods. Normally in the UK, such beetles would overwinter for about six months, feeding on flowers in the following spring, pbefore becoming

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reproductively mature. However, because New Zealand is in the Southern Hemisphere, the beetles were fed on arrival with bee pollen, honey water and/or fresh flowers for approximately eight weeks (without overwintering), bringing them into reproductive maturity for the oviposition tests. The results of the choice tests with nine species of pod-bearing plants (six New Zealand natives and three exotics), indicated that *B. villosus* would be host-specific to *C. scoparius* in New Zealand (Syrett & O'Donnell 1987). One of the exotic species was tagasaste, *Chamaecytisus proliferus* (L. f.) Link (known as *C. palmensis* (Christ) Bisby & K. Nicholls in New Zealand).

In spring 1994, adults of *B. villosus* reared from *C. scoparius* from New Zealand were imported into quarantine in Australia. The results of choice tests on 18 native Australian species and 10 exotic species supported the New Zealand results, also indicating that *B. villosus* was host-specific to *Cytisus* species (A. Sheppard, unpublished data).

In 1999, however, *B. villosus* was found emerging from *C. proliferus* seeds in New Zealand, and further studies showed that this plant was a suitable and commonly utilized alternative host (Syrett 1999). At the time *C. proliferus* had only been tested in choice tests with *C. scoparius* as a control, in quarantine in both New Zealand and Australia. It was not included in the UK no-choice and choice tests because it does not produce pods in the colder climate of the UK. *C. proliferus* is native to the Canary Islands, which have a significantly warmer climate than the UK, and is grown abundantly in New Zealand where it has naturalized extensively. It is regarded as weedy in some places in New Zealand (Williams & Timmins 1990), but also has benefits including use as fodder in high country farms when there is drought (Douglas *et al.* 1996), as a pollen source for beekeepers (Dann & Trimmer 1986), and as a supplementary food source for the threatened native pigeon in New Zealand (McEwan 1978).

Although it has now been shown that choice tests including the target species are not the most robust method for observing acceptance of lower ranked alternative host plants (Marohasy 1998, Edwards 1999, Hill 1999, Heard 2000, Purcell *et al.* 2000, Barton Browne & Withers 2002), we are nevertheless surprised that the original choice tests in New Zealand did not reveal the relative acceptability of *C. proliferus*. It seemed plausible that a host-range expansion (Dennill *et al.* 1993), otherwise referred to as a host shift (Howarth 1991), had occurred in the population of established beetles in New Zealand some time in the 14 years since its introduction (Syrett 1999). Many purported host-range expansions, defined by Marohasy 1996 as "feeding by biological control agents on plant species other than those on which they were known to feed prior to their release", have been reported in weed biological control. Marohasy (1996) argued that these were caused by other phenomena, such as preadaptation (established behavioural concepts), threshold change as a result of host deprivation, or effects of experience (learning). This study investigates the possibility that a host-range expansion may have occurred in *B. villosus*. Oviposition acceptance behaviour of the current New Zealand population of *B. villosus* was compared with beetles collected from Silwood Park, the same field site where the original beetles had been collected for shipment to New Zealand in the 1980s. Our hypothesis was that, if British beetles still refused to accept *C. proliferus* for oviposition, while their New Zealand progeny now accepted it, then a host-range expansion would indeed be the most likely explanation.

Materials and methods

In June 2002, adult *B. villosus* were beaten from *C. scoparius* at Silwood Park, UK. These beetles were placed into 1 m diameter by 2 m long, 1 mm mesh sleeve cages on branches of *C. scoparius* bearing young pods. In July, infested pods were picked from the sleeves and held in a glasshouse in mesh bags until emergence. The emerged adult beetles were reimported into quarantine in New Zealand in August 2002. Repeating the same procedure as carried out in 1985, 150 adults were maintained in Perspex cages with ample bee pollen and honey water, followed by *C. scoparius* flowers, under a 22:16°C (day:night) temperature regime with a day length of 14:10 L:D. Relative humidity was approximately 70%.

Host-specificity tests undertaken in the original study in 1985 were replicated as far as possible in 2002 using UK beetles, and in 2001 using New Zealand beetles (field collected from *C. scoparius*). The procedures recorded in the original quarantine laboratory books were followed as closely as possible, however minor differences were required with regard to timing and experimental design.

Perspex boxes (220 × 130 × 100 mm), with flexible push-on lids and four, 25-mm diameter gauze-covered holes for ventilation, were used as test cages. Moistened blotting paper was placed at the bottom of the cage, and several pieces of tissue paper were included to absorb excreta. *Cytisus scoparius* twigs, approximately 200 mm long, bearing young green pods, were placed in vials of water in each test cage. A disc of plastizote, 6 mm thick, with the twigs pushed through its centre, acted as a stopper for the vial, which was supported at an angle to ensure the shoot remained in the water. Twigs of each test plant were selected such that they had approximately equal amounts of pod material and pods judged to be at an equivalent developmental stage to the *C. scoparius* pods. Test material of the different plant species, prepared in the same way as the *C. scoparius*, was placed in each cage with an equivalent amount of *C. scoparius*, to constitute paired choice tests comprising *C. scoparius* and a test plant (Syrett & O'Donnell 1987).

Test-plant material was collected from at least three different plants for each species. Beetles were held in each test cage for 6 days during the tests. Beetles were fed pollen and provided with cotton dental rolls soaked in a honey–water solution. After the beetles were removed, all plant material and cages were carefully examined for eggs. The numbers of eggs found on the pods of *C. scoparius* and each of the test species were recorded. Each phase of the experiment was conducted when each of the test-plant species had pods available at the appropriate stage of development (Table 1). Not all plant species were tested at the same time, therefore. Every attempt was made to ensure laboratory conditions, cage type used, number and sex ratio of beetles, bee pollen source, twig size, approximate number of pods presented, the presentation of pod material, duration of assays, and approximate timing of presentation of various host plants, were the same as in the 1985 experiments (Table 1).

In the original choice tests conducted in 1985, one or two replicates were used for each test plant species, whereas 4 replicates for each test plant were used in 2001 and 10 in 2002. Each replicate contained five male and five female beetles.

Results

In 1985 female *B. villosus* laid a mean of between 4.2 and 18.4 eggs each on *C. scoparius* and 0 eggs on the test plants (Table 2). In 2002 tests, *B. villosus* laid a mean of between 3.0 and 12.3 eggs each on *C. scoparius*, and 0.7 eggs on the test plant *C. proliferus*. The range of eggs laid on *C. proliferus* in 2002 was between 0 and 2.6 eggs per female and only 4 out of 10 replicates had eggs laid on them at all. In the 2001 tests using beetles field caught from *C. scoparius* in New Zealand, female beetles laid a mean of between 18.1 and 25.5 eggs on *C. scoparius* and a mean of 1.0 egg each on the test plant *C. proliferus*. The range on *C. proliferus* was between 0.2 and 2.6 eggs per female and in each of the 4 replicates at least 1 egg had been laid.

In the four replicates of the 2001 tests with New Zealand field-collected beetles, a total of 20 eggs was laid on *C. proliferus* by a maximum of 12 females. In

Table 1. Timing of two-choice tests with material presented to *Bruchidius villosus* from various origins. The tests included the target weed *Cytisus scoparius* and the following plant species: *Carmichaelia australis* G. Simpson, *Carmichaelia petriei* T. Kirk, *Carmichaelia stevensonii* (Cheeseman) Heenan, *Carmichaelia williamsii* T. Kirk, *Chamaecytisus proliferus*, *Clianthus puniceus* (G. Don.) Sol., *Cytisus multiflorus* (L'Her) Sweet., *Genista monspessulana* (L.) L.A.S. Johnson, *Laburnum anagyroides* Medikus., *Sophora microphylla* Aiton, and *Sophora prostrata* J. Buchanan.

Weeks	1985 UK import (1 or 2 reps)	2001 NZ origin (4 reps)	2002 UK import (10 reps)
1–5. (2nd week Sept – 3rd week Oct)	*C. scoparius* flowers and bee pollen		*C. scoparius* + *C. proliferus* flowers and bee pollen
6. (4th week Oct)	*C. scoparius* flowers, green pods and bee pollen	Beetles collected continuously off *C. scoparius*	C. scoparius vs *C. proliferus* No eggs laid
7. (1st week Nov)	*C. scoparius* flowers, green pods and bee pollen First eggs laid	*C. scoparius* vs *C. proliferus* First eggs laid	C. scoparius vs *C. proliferus* No eggs laid
8. (2nd week Nov)	*C. scoparius* vs *C. proliferus*	*C. scoparius* vs *S. microphylla*	*C. scoparius* vs *C. proliferus* First eggs laid
9. (3rd week Nov)	*C. scoparius* vs *S. microphylla*	*C. scoparius* vs *S. prostrata*	*C. scoparius* vs *S. microphylla*
10. (4th week Nov)	–	*C. scoparius* vs *C. multiflorus*	*C. scoparius* vs *C. australis*
11. (1st week Dec)	*C. scoparius* vs *C. australis*	*C. scoparius* vs *G. monspessulana*	*C. scoparius* vs *C. petriei*
12. (2nd week Dec)	*C. scoparius* vs *C. petriei*, *C. williamsii*, *G. monspessulana*, *C. puniceus* & *C. multiflorus*	*C. scoparius* vs *L. anagyroides*	*C. scoparius* vs *C. williamsii*
13. (3rd week Dec)	Repeated *C. scoparius* vs *C. multiflorus*		*C. scoparius* vs *C. puniceus*
14. (4th week Dec)	–		*C. scoparius* vs *C. multiflorus*
15. (1st week Jan)	–		*C. scoparius* vs *G. monspessulana*
16. (2nd week Jan)	–		*C. scoparius* vs *C. stevensonii*
17. (3rd week Jan)	*C. scoparius* vs *C. stevensonii*		C. *scoparius* vs *L. anagyroides*

Note: in Syrett and O'Donnell (1987), *C. proliferus* was referred to as *C. palmensis* (Christ) Bisby & Nicholls, *C. australis* as *C. ovata* G.Simpson, and *C. stevensonii* as *Chordospartium stevensonii*.

the 10 replicates in 2002 with UK imported beetles, a total of 33 eggs was laid by a maximum of 19 females in only 4 of the replicates. There was no significant difference in the overall mean number of eggs laid per female per replicate on *C. scoparius* between the sequential choice tests conducted in 1985 and 2002 with beetles imported from the UK (*t*-test, $P = 0.5$, df = 12). The overall mean number of eggs per female per replicate was 9.1 and 7.9, for beetles in 1985 and 2002, respectively (excluding *Laburnum anagyroides* which was an extra plant in the 2002 sequence). The overall mean number of eggs laid per female per replicate in the 2001 tests on New Zealand field-collected beetles was 22.3, which is more than double the mean in the other tests.

Discussion

For an expansion in fundamental host range to occur in phytophagous insects, so that an insect can move from one host plant to another, a "host race" must first develop. To be classified as a host race (defined in Marohasy 1996) populations must first fulfil the following criteria: (1) be non-interbreeding and sympatric; (2) differ in biological characteristics, but not (or only marginally) in morphology; and finally (3) be prevented from interbreeding as a result either of preference for different host-plant species, or as a consequence of physiological adaptation to different host-plant species.

So which of the above criteria have either been fulfilled or have the potential to be fulfilled in New Zealand with *B. villosus*? Firstly, it seems that *B. villosus* adults emerging from both *C. scoparius* and *C. proliferus* are interbreeding. Beetles emerging from each species of pods at similar times have been observed mating (M. Haines, personal observation). Furthermore, both plant species frequently grow in the same area, and within the same habitats in New Zealand (no geographical isolation). *Bruchidius villosus* shows high mobility, and therefore it appears the insects continue to interbreed after emerging from different host pods. Seasonal asynchrony is, however, a possible mechanism that could also lead to sympatric speciation. Certainly *C. proliferus* flowers earlier than *C. scoparius* in spring and is the first available pollen source to *B. villosus* when it emerges from its overwintering period (Fowler *et al.* 2000). However, *C. proliferus* flowers for a longer period and simultaneously with *C. scoparius* over summer, suggesting seasonal asynchrony in New Zealand may be insufficient to lead to sympatric speciation or to prevent interbreeding.

Secondly, the possibility that *B. villosus* has begun to develop different biological characteristics on the two host plants has also started to be investigated. Field observations and initial data gathering in 1999 (M. Haines, unpublished results) and in 2000 (Wittenberg & Thomann 2001) have suggested there is phenotypic plasticity in body size and colour of *B. villosus* depending on the host-plant seed in which they have developed. Adults emerging from seeds of *C. proliferus* are generally larger and sometimes browner in colour than those emerging from the usual host *C. scoparius*, which are smaller and blacker in colour. Whether this phenotypic plasticity is suggestive of different performance or suitability of genotypes according to host plant has yet to be ascertained, but the development of different biological characteristics cannot be ruled out. Laboratory studies will be used to investigate whether or not lines of *B. villosus* reared from different host-plant pods retain oviposition preferences for the species of pod in which they spent their larval development.

Thirdly, we need to establish that *B. villosus* is in the process of being prevented from interbreeding as a result of a preference developing for the new host-plant species. *Cytisus scoparius* remains the preferred host over *C. proliferus* in all choice tests to date (M. Haines, unpublished results), suggesting that no preference has yet developed for *C. proliferus*. The 2001 test results confirm this (Table 2), as beetles randomly collected from the field laid on average 25 times as many eggs on *C. scoparius* as on *C. proliferus*.

So it appears that the criteria that would indicate that a host race has developed, or is in the early stages of developing in *B. villosus*, are not met. The fact that reimported UK beetles accepted *C. proliferus* suggests that a host-range expansion has not occurred in New Zealand, but that for some reason the 1985 tests failed to elicit oviposition on *C. proliferus*.

There are at least two possible explanations for the discrepancy in laboratory testing results between 1985 (no eggs were laid on *C. proliferus*) and 2002 (some eggs were laid on *C. proliferus*). There were differences in the number of *B. villosus* tested (smaller sample sizes in 1985), and beetles may have been treated subtly differently between tests despite best attempts to replicate conditions (Table 1). For example, the 1985 beetles were held for two weeks before testing with very small pods and flowers of *C. scoparius*, which may have caused an unusual degree of excitation towards *C. scoparius* in 1985. In 2002, beetles were held before testing with pods of both *C. scoparius* and *C. proliferus* at the same time, to check for onset of oviposition. In both cases, all beetles imported from the UK had never experienced *C. proliferus* pods before being imported into New Zealand quarantine. All testing was conducted sequentially, but in both 1985 and 2002, the same groups of beetles were reused for each test plant, whereas in 2001, independent groups of beetles were used for each test plant in the sequence.

Having ruled out a host-range expansion, why did the original choice tests not indicate some acceptability of *C. proliferus* pods? The hierarchy-threshold model of host selection (Courtney *et al.* 1989) hypothesizes that insects rank hosts in a hierarchical fashion and that selection of diet by individual insects is determined by

Table 2. Mean number of eggs laid per *Bruchidius villosus* female per replicate in paired cut-shoot choice tests run sequentially over time. The tests included the host plant *Cytisus scoparius* and the following test-plant species: *Carmichaelia australis* G.Simpson, *Carmichaelia petriei* T. Kirk, *Carmichaelia stevensonii* (Cheeseman) Heenan, *Carmichaelia williamsii* T. Kirk, *Chamaecytisus proliferus*, *Clianthus puniceus* (G. Don.) Sol., *Cytisus multiflorus* (L'Her) Sweet., *Genista monspessulana* (L.) L.A.S. Johnson, *Laburnum anagyroides* Medikus., *Sophora microphylla* Aiton, and *Sophora prostrata* J. Buchanan.

Plant material	1985 UK import		2001 New Zealand field		2002 UK import	
	test plant	*C. scoparius*	test plant	*C. scoparius*	test plant	*C. scoparius*
Chamaecytisus proliferus	0	18.4	1	25.5	0.7	12.3
Sophora microphylla	0	6.2	0	19.3	0	4.8
Carmichaelia australis	0	12.2	–	–	0	7.4
Carmichaelia petriei	0	7	–	–	0	9.2
Carmichaelia williamsii	0	4.2	–	–	0	8.5
Genista monspessulana	0	10	0	18.1	0	9.5
Clianthus puniceus	0	7.6	–	–	0	4.3
Cytisus multiflorus	0	12.2	0	21.7	0	9.0
Carmichaelia stevensonii	0	4.2	–	–	0	5.3
Sophora prostrata	–	–	0	25.2	–	–
Laburnum anagyroides	–	–	0	24.8	0	3.0

the host's "acceptability". One prediction of the model is that female oviposition behaviour is influenced by female egg load, such that when egg load is high, so is the tendency for a wider range of hosts to become acceptable (Courtney *et al.* 1989). The overall mean number of eggs laid per female was significantly higher in the New Zealand field-collected beetles tested in 2001 (more than twice that of both 1985 and 2002 UK imported beetles), suggesting these beetles had a higher egg-load than their imported counterparts. So could egg-load explain why the original host tests were not indicative of field host range? The number of eggs laid per female in the 2002 imported beetles was almost three-fold less than that of the 2001 New Zealand field-collected population. Yet, the lower-ranking host *C. proliferus* was still accepted for oviposition at an equivalent rate despite the reduced egg-laying. In both experiments, the minimum number of eggs laid per female on *C. proliferus* was 1.7, and more eggs were laid on *C. proliferus* by beetles with a comparatively low egg-load in an equal number of replicates. So, it appears unlikely that egg-load is responsible for the discrepancy in test results.

We conclude that a host-range expansion has not occurred, but that the 1985 host testing failed to detect the non-target impact of *B. villosus* on *C. proliferus*. From the 2001 and 2002 test results indicating that *B. villosus* laid 18–26 times as many eggs on *C. scoparius* as on *C. proliferus* (Table 2), we might have predicted that its non-target impact in the field would be minor, but the level of seed attack by the beetle in New Zealand is in fact substantial (M. Haines, unpublished data). The implication for biological control releases is that we cannot assume non-target impacts will be insignificant on the grounds that results of choice tests indicate a strong preference for the target plant. On a more positive note, despite the non-target attack on the exotic plant *C. proliferus*, *B. villosus* remains a useful agent against *C. scoparius* in New Zealand as all the test results consistently predict that no native Fabaceae are under any risk of attack (M. Haines, unpublished data).

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