Host specificity testing in Australasia: towards improved assays for biological control

Papers from the **Introduction of exotic biocontrol agents—recommendations on host specificity testing procedures in Australasia** workshop, Brisbane, October 1998

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The workshop

The Co-operative Research Centre for Tropical Pest Management sponsored a one-day workshop entitled "Introduction of exotic biocontrol agents—recommendations on host specificity testing procedures in Australasia", which was held in Brisbane, Australia, on October 3rd 1998, in conjunction with the 6th Australasian Applied Entomological Research Conference. Most of the participants were actively involved in biological control of weeds and insect pests in Australia and New Zealand. The first nine chapters of this book are based upon oral presentations given to that workshop. The final chapter provides a synthesis of group discussions both before and during the workshop.

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Preface

Biological control using arthropod agents is an integral part of sustainable pest and weed management methods. There is an almost universal requirement that any biological control agent released will not have adverse economic and/or environmental impacts in the new country. If there is a danger of attack to non-target species, then it must be demonstrated that the potential benefits outweigh any adverse impacts. To address this, candidate agents are subjected to host specificity tests to obtain data that will allow the practitioner to predict the field host range in the country of introduction. The problem exists within this process of host specificity testing, in both Australia and New Zealand, that researchers utilise a range of differing methodologies in order to determine the host range of potential insect biological control agents. Discrepancies between organisations in the use of different types of assays contributes to confusion within and among authorities who grant permission for the release of such agents. How are the reviewers in those authorities to correctly interpret data, which have been gathered in different ways, to determine whether or not the exotic insect would prove to be safe in the new country?

Regulation of the introduction of biological control agents into new countries is of high interest because of its potentially irreversible effects. FAO have had drafted technical guidelines in support of an FAO Code of Conduct for the import and release of biological control agents' with the intention it would provide guidelines for 'best practice' biological control agent introductions. These FAO guidelines do not, however, detail the pros and cons of various methodologies and their relative ability to produce results that can be used to predict likely impacts on non-target organisms in the field.

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The first seven chapters focus on the various methodologies commonly used in the host specificity testing of candidate agents for biological control of weeds. Richard Hill explains the political and scientific usefulness for the commonly utilised no-choice trial. He takes the concept further than just the no-choice starvation test, but also considers extended fecundity and developmental trials of herbivorous insects and mites under no-choice conditions. Both Michael Day and Bill Palmer add to the understanding of the breadth and application of no-choice trials. Michael Day considers how results of trials over multiple generation on non-target hosts can be interpreted, while Bill Palmer reviews the biological control literature and finds no evidence that using cut foliage for no-choice trials rather than whole plants can drastically alter the outcomes, at least for foliage feeding insects. Tim Heard summarises a

technique for host range testing insects that utilise discreet resources, and which have mechanisms, such as the use of an oviposition-deterring pheromone, that tend to prevent them from over exploiting resources. Penelope Edwards defines what constitutes a choice test, considers the usefulness of such tests, and makes recommendations about their role, particularly as pertains to ascertaining oviposition and/ or feeding preferences between plant species. David Briese reviews the literature surrounding open field host range tests, their rationale, and interpretation. His recommendation for a two-phase methodology appears to overcome a number of the current concerns about field tests. Andy Sheppard provides a thorough review of the biological control literature and reveals that, to date, no specific sequence of assay type has been predominant. He has produced an insect biology-based decision flow chart to suggest how the selection of the initial host range assay type could be most appropriately made.

The next two papers discuss methodologies used in host specificity testing of parasitoids for biological control of arthropod pests. Barbara Barratt and co-authors provide an over-view of the requirements for host specificity testing of parasitoids. The regulatory requirements, as well as the complexities of assay design for parasitoids are considered, and some modern technological aids to host range assessment are introduced. Michael Keller discusses the importance of having an understanding of the processes involved in host selection and clearly illustrates the relevant concepts with mainly parasitoid examples.

Finally, Toni Withers discusses prospects for developing an integrated approach to host specificity testing to improve the accuracy of predicting field host range. How the order of host specificity testing assay type can be altered so that applications for release of biological control agents fit within a ‘best practice risk assessment’ framework, is discussed.

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Minimising uncertainty – in support of no-choice tests

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The Environmental Risk Management Authority (ERMA) has recently been established in New Zealand, and among its tasks will be the assessment of new biological control agents for release in New Zealand. It intends to employ the 'precautionary principle' in decisions where the effects are irreversible, involuntary, persistent, and widespread, and where the risks and benefits are uncertain. Uncertainty can arise at steps throughout the host-range testing process, and some of these are highlighted. Applicants will need to minimise those uncertainties to ensure that applications are successful, and that includes reliable prediction of potential host-range by testing. In no-choice tests, the control agents cannot exercise all of the discriminatory behaviours that might cause them to reject a host in a more natural arena. A negative test is therefore strong evidence that a plant is not a potential host. For the same reason, however, no-choice tests can generate false positive results, which can be misinterpreted. Some advocate that no-choice tests should be avoided in favour of more natural test conditions, but it is argued here that no-choice tests may be the most appropriate method when the control agent is small and immobile relative to its host-plant. The paper concludes that regulatory authorities would benefit from having a set of 'best practice' standards by which to judge the validity of test designs, and the interpretation of the results of host-range testing.

Introduction

Pre-release studies to determine the susceptibility of non-target plants to control agents have been part of biological control practice for over 60 years. At first, the standard procedure was to carry out no-choice tests with a number of specified, commercially-valued plant species. Such tests assessed whether the particular plants tested were at risk, but provided little insight into the breadth of host-range. Since the 1970's, tests have been designed to determine the boundaries of the host-range, and to predict whether plants that are economically or environmentally valued lie within that range. The so-called centrifugal phylogenetic method (and modifications of that method), in which plants more- or less- related to the target weed are tested (Wapshere 1974, 1989), has become the standard method for determining that host-range boundary. There is little or no evidence in the existing literature (McFadyen 1998) that this approach has ever failed, which suggests that the prediction method is robust.

Until recently, the minimum standard for accepting the safety of a control agent was usually that no damage was likely to any ecologically or economically valued plant. More sophisticated risk management concepts are developing, and the release of several control

agents has been approved recently, even though tests indicated that non-target plants might be damaged (McFadyen & Marohasy 1990, Hill *et al.* 1995, Olckers 1996, McFadyen 1998).

An Environmental Risk Management Authority (ERMA) has recently been established in New Zealand. One of its many tasks will be to regulate the importation of all exotic organisms, including biological control agents. All evidence suggests that this new organisation will change both the nature of experimental evidence required of applicants, and the standards by which that evidence is judged. In making decisions, the Authority is required to balance environmental and economic benefits of any proposal with the risks and possible costs that might follow introduction. ERMA has published the methodology it will use to make decisions (WWW.ermanz.govt.nz). This states that the 'precautionary principle' will be important, implying that in areas where there are significant uncertainties, ERMA is likely to take a conservative view. The methodology states that the Authority will be particularly cautious when exposure to risk is involuntary, persists over time, spreads uncontrollably, is irreversible, or where the risk is imperfectly understood. These are all features characteristic of classical biological control introductions, and it appears that such proposals may be difficult to justify. The Authority is required to base its decisions on available scientific evidence, but must take into account the views of the public, ethics, values, perceptions, and changing attitude to risk. Where the balance between these factors finally falls will only become known once ERMA has built a case history of decisions. In this new environment, the challenge for applicants will be to make arguments that not only satisfy the Authority (a quasi-judicial body including non-biologists) and the scientific community, but can be understood and accepted by a practically-minded, risk averse, and highly influential public.

Better definition of the risks and benefits associated with a new control agent will be the key to a successful application. Foremost amongst these will be defining the risk of damage to non-target organisms, and minimising the uncertainties associated with the determination of potential host-range. No-choice testing has always been a cornerstone of that process. In this paper I describe the nature of no-choice tests and current attitudes towards their use. I discuss the interpretation of data and discuss areas where interpretation is fraught with uncertainty. I look at how agent size and mobility should determine the choice of appropriate testing methods. Finally, I draw some conclusions about the relevance of no-choice testing in modern risk assessment.

The nature of no-choice tests

There is no universally accepted definition of a no-choice test. Adult or larval feeding tests conducted on the foliage of a single test-plant are clearly no-choice tests, as are oviposition tests in which only single plants are exposed to ovipositing females. However, in the literature, there are conflicting descriptions of what constitutes a no-choice test. Heard & van Klinken (1998) describe any test that contains more than one test-plant species as a choice experiment, because insects can choose between species. If the target host is not present, they describe the test as 'choice-minus-control'. Others define such tests as no-choice tests (e.g. Hill *et al.* 1995), because control agents do not have the opportunity to 'choose' a known acceptable host. For the purposes of this paper, I include such tests amongst no-choice tests.

No-choice larval development or starvation tests measure the ability of a plant to support the development of an immature control agent. No-choice oviposition tests measure the ability of adults to survive and mature, and to lay eggs on a test-plant. No-choice tests can be

conducted in the field by transferring relatively immobile stages onto plants (Hill *et al.* 1991), or by following through the fate of oviposited eggs (Day, this volume). More commonly, small containers or cages are used. In containment, the control agents can respond only to the range of cues closely associated with the plant substrate, and not to the many pre-alighting cues that can moderate host-selection (Marohasy 1998).

In no-choice tests, insects can become highly motivated to feed and/or oviposit before dying, while test-plants may provide continuous, positive sensory stimulation in a small arena (especially when the insect is a small one). Such tests therefore provide a 'maximum challenge' to the host-discrimination mechanisms of the control agent under test. No-choice tests largely ignore the behavioural and ecological constraints that might prevent a control agent using those hosts under natural conditions, but reveal the range of hosts that the agent can use physiologically, in the absence of those constraints (Cullen 1990). This is why it is generally accepted that no-choice tests overestimate the true host-range of potential control agents (McFadyen 1998). The probability of an insect rejecting a host in such tests, but then accepting it in the field are considered low. Marohasy (1998) has described this as a false negative result, but has not identified any phenomenon that could induce such a response. On the other hand, the likelihood of accepting a host-plant in containment that would not be accepted in the field (a false positive) is considered high. Use of a plant in no-choice tests is therefore not evidence that the agent would attack the plant in the field, but rather lack of evidence that it would not.

The use of no-choice tests in current biological control practice is limited. No-choice bioassays that yield negative results (no attack) are confidently and commonly used as a primary screening device to eliminate test-plants from further testing. Any utilisation of a test-plant in no-choice tests implies potential risk in the field, and requires further examination, but because the risk of the positive result being false are high, potentially safe and useful agents should not be rejected on the basis of no-choice tests alone. Plants attacked in no-choice tests are normally included in increasingly sophisticated tests that expose the control agent to the wider range of cues and barriers insects encounter in the natural process of host selection (Wapshere 1989, Hill *et al.* 1995). Cullen (1990) pointed out other circumstances where no-choice tests can help to assess risk to non-target plants, particularly when adults or larvae tend to wander from target hosts on which eggs were laid and could damage neighbouring vegetation, where adult feeding is damaging, and possibly where attempted oviposition itself causes damage. However, whether negative or positive, all no-choice tests must be carefully interpreted in relation to the environmental and ecological conditions that will prevail in the target area (McFadyen 1998), and the physiological state of the control agent at the time of testing (Withers 1997, Marohasy 1998). The relevance of no-choice tests are discussed later in this paper.

Acceptance or rejection of a test-plant in no-choice tests is often not absolute. In adult survival and oviposition tests, commonly used measures of acceptability are life span, feeding damage by adults (if there is any), oviposition rate, lifetime fecundity, and egg hatch. Hatching success is affected by egg quality, but plants can also directly influence egg survival (Schröder 1967). Insects often oviposit on test-plants in no-choice tests, particularly if they lack deterrent properties, but significant use of the test-plant in the field can be discounted because the oviposition response is so poor. Unless the act of oviposition is damaging in itself, deposition of eggs on test-plants is not significant unless it results in significant feeding and development of hatching immatures. Marohasy (1998) recommended a technique to

clarify the significance of oviposition by ranking the relative acceptability of plants using multiple choice oviposition tests.

Measures of test-plant acceptability in no-choice feeding or starvation tests include longevity, developmental stage achieved, damage, amount ingested, and the quality of the insects produced. Newly-hatched insects of the damaging life stage are normally tested. Rapid death indicates little or no risk of attack. Death at an early stage of development also suggests minimal risk to the plant, even if the insect survives for a long period. However, both become important if the agent causes significant damage before it dies. Even if the control agent completes development in no-choice tests, the ability of the insect to successfully colonise the plant in the field may still be limited by the quality and fecundity of the resulting adults. Hill *et al.* (1995) found that one *Agonopterix ulicetella* larva pupated when fed on red clover leaves (*Trifolium pratense*), but it was half of the weight of pupae produced by larvae feeding on the target plant (gorse, *Ulex europaeus*), and did not emerge. Pupal weight may be a reliable indicator of potential fecundity and quality in many insects. Similarly, *Tetranychus lintearius* survived for almost two generations on bean leaves, *Phaseolus vulgaris* (Hill & O'Donnell 1991), but few F2 eggs were laid, development times were extended, and developing individuals lacked the dark red colour characteristic of well-fed mites. Populations could not complete a second generation on bean leaves. The inability of this mite to colonise beans was confirmed in no-choice tests on whole plants and in field plots (Hill & O'Donnell 1991).

Behavioural observations taken during no-choice tests can enhance these end-point measures. Withers (1997) suggested recording oviposition over small time periods during a no-choice test, so that changes in the tendency to accept the plant could be measured over time. Increasing oviposition on test-plants over time might indicate that the result was induced by depriving the agent of an acceptable oviposition or feeding site. Similarly, Withers (1998) recorded different stages of host discrimination behaviour, and was able to show that feeding by *Zygogramma bicolorata* (Chrysomelidae) on sunflower was insignificant, and caused by a high level of feeding responsiveness induced by deprivation. As yet, behavioural studies have not been adopted freely as standard practice in host-range testing, but as long as they are robust, studies such as this will help explain the mechanisms behind uncertain results that frequently plague host-range testing programmes (Marohasy 1998).

Control agents and their universe

The opportunity to directly compare sensory stimuli from potential host-plants in laboratory tests, or in the field, within a reasonable time frame is not real for many phytophagous insects. At any particular moment, the choice is rather to accept a test-plant as a suitable substrate for reproduction or development, or to reject it. It is generally believed that rejection of a plant is accompanied by locomotion (Jermy 1971), and further host-plant sampling. Where this behavioural sequence prevails, no-choice tests may be a more natural and reliable method of determining true host range than choice tests. Factors that affect how insects sample test-plants (and hence the most appropriate test), are the size of the control agent, and its mobility in relation to test-plant size (Figure 1).

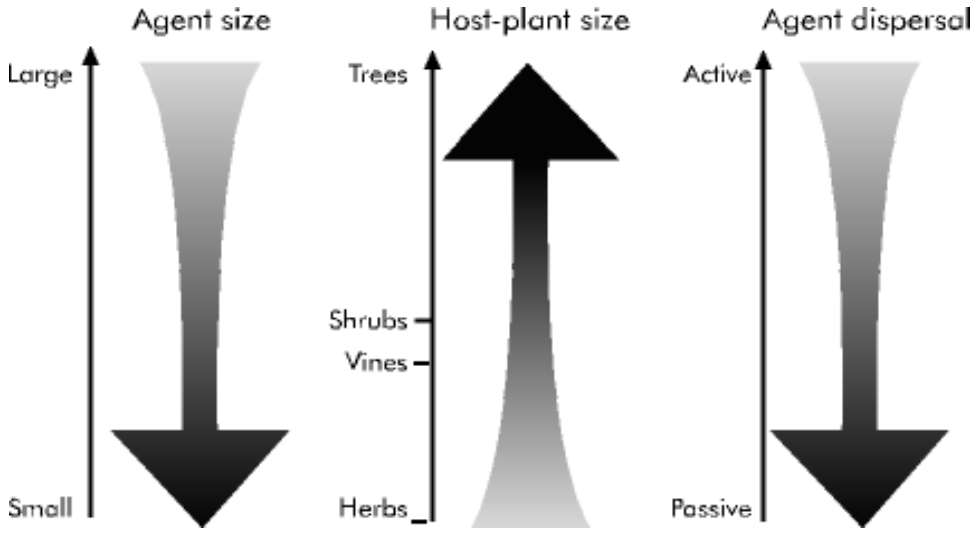
The consequences of an insect's response to sensory cues is influenced by its size. In the case of an eriophyid mite on a leaf, a relatively uniform substrate may stretch 50-100 body lengths in each direction, and a different potential host may be thousands of body lengths distant.

When an insect rejects a substrate and moves, the probability of then sampling a different substrate is lower for a small insect than it is for a larger one. The rate of movement is lower, and hence the frequency of encountering a different substrate is lower. No-choice tests, where the insect constantly re-samples the same substrate, may be a more natural reflection of host-choice behaviour than choice tests for small organisms such as mites. The relevance of no-choice tests is greater for smaller insects than it is for larger ones (Figure 1).

Insects and mites accept a host by arresting movement and initiating behaviour leading to host use. They reject hosts by actively moving, or by simply detaching from the substrate. If the plant is large, the insect is more likely to encounter the same substrate than foliage of a different species. Similarly, the likelihood of encountering a different species in a monoculture is lower than in diverse vegetation. For example, *Bruchophagus acaciae* is a small eurytomid wasp that attacks the seeds of some acacias (Cameron 1910). If this wasp randomly samples foliage within a large tree, rejects the substrate, and moves, then within a given period of time it is more likely to encounter foliage of the same tree rather than a new potential host (R Hill, unpublished data). This is true even when these species are close together, but often the distances between potential host trees are large. Again, no-choice tests may be a better reflection of the process by which insects select or reject large hosts than choice tests (Figure 1). This is especially true if tests are short term. These effects are compounded if the organism is passively-dispersed, and has limited capacity to actively seek alternative hosts. Choice tests may be more relevant for mobile insects than no-choice tests (Figure 1).

In general, no-choice tests may be more ecologically relevant for inactive, small, passively-dispersed organisms attempting to discriminate between large test-plants. Conversely, choice tests may be more appropriate where agents are large relative to the test-plants, and have activity patterns (such as flight) that allow frequent sampling of different hosts (Figure 1).

Figure 1. A representation of changes in the relevance of no-choice tests in determining the host range of biological control agents with changes in agent size, host plant size and agent dispersal ability. The shaded arrow indicates the increasing relevance of no-choice tests.



Some sources of uncertainty in host-range testing

Interpretation of 'outliers'. Often one or more individuals in a no-choice test oviposit more readily or survive much better than the majority of insects tested. This was observed when no-choice feeding tests were conducted on *Pempelia genistella*, a pyralid moth producing larvae that feed on gorse (*U. europaeus*). First instar larvae were transferred to cut shoots of a range of test-plants, three larvae per shoot, five shoots for each plant. On controls, an average of 55% of larvae survived to pupate. On *Laburnum anagyroides*, only 5 of the 15 larvae remained after 10 days, and 2 after 30 days, but a single larva survived for 90 days to pupate (Gourlay & Hill 1994). Did this larva represent natural variation between individuals in the development biology of *P. genistella*, or a genotype potentially capable of a wider host-range than other larvae?

Current methods of interpretation distinguish these options imperfectly. No-choice tests are often conducted on a wide range of test species, but with little replication. If such observations are part of the natural variation in insect performance within the population, then the prediction of host-range would presumably be improved (and uncertainty reduced) by increasing replication. On the other hand, increased survival or oviposition by rare individuals may be the means by which evolving populations test host-range boundaries. If so, observations such as these should be expected when conducting host-range tests (S Fowler, pers. comm.). The ecological and evolutionary significance of 'outliers' merits further examination, but are such results of concern to biological control practice? Any tendency to expand host-range (which is one interpretation that could be placed on such outliers) could become fixed in the field only if the behaviour resulted in successful development and reproduction, and also conferred competitive advantage over the more host-specific part of the population. There are no unequivocal examples of host-specific insects forming sympatric host races on new hosts (Marohasy 1996), although the development of sympatric host-races of *Rhagoletis pomonella* may be such a case (Feder *et al.* 1994). Literature records show that highly host-specific insects can be found in unusual situations in the field, often feeding on novel hosts, but to infer a host-shift from these observations alone is wrong. Marohasy (1996, 1998) argues that all records of apparent host changes recorded in the literature are adequately explained without the need to invoke a genetic 'host shift'. Some can be explained by temporary host substitution, resulting from a threshold change caused by host deprivation. Others are best explained not by a genetic change to enable host-plant use, but by the inherent ability of the insect species to incorporate the new plant into its existing host-range. The observation of the new relationship may be novel, but the mechanisms that allow it are not.

Low levels of attack on less-preferred 'hosts' in no-choice tests may well be ecologically insignificant, but the normal practice is to treat such results conservatively, and to examine the potential risk more closely by further experimentation (Wapshere 1989). Unfortunately, better information on risk does not make the original observation go away, and there is always the possibility that regulatory authorities and the public may overemphasise the uncertainty posed by such 'outliers'.

Cultivars of test plants. Insect performance varies between cultivars of a plant, and the choice of cultivar tested may influence the results of a no-choice test. Gorse spider mite (*Tetranychus lintearius*) was tested against a wide range of cultivars of garden beans (*P. vulgaris)* because *T. urticae*, a species closely related to gorse spider mite, is a pest of this crop. No-choice tests indicated that adult survival, fecundity, and the development of

offspring reared on excised leaves of different cultivars differed greatly (Table 1). Had just one unpalatable cultivar been selected for testing, the mite's ability to survive for almost two generations on certain cultivars would have gone unnoticed. No-choice testing on whole plants, and in field trials later revealed that although gorse spider mites could survive on leaves of several cultivars in the laboratory, they were not colonised under more natural conditions (Hill & O'Donnell 1991).

Adult maturity and no-choice tests. There is generally a low risk of obtaining a false negative result in no-choice tests, but this is not always so. *Phytomyza vitalbae* (Agromyzidae) was recently introduced into New Zealand for the biological control of old man's beard, *Clematis vitalba*. In the course of host-range testing, the ability of the adult fly to puncture leaves and feed on exudate, and to produce mines on leaves of various *Clematis* species was tested. Adult feeding and oviposition in no-choice tests were rare in most no-choice tests, but in others attack approached the level observed on controls (R Wittenberg, unpublished data). Further research revealed that test results varied according to the age of the adults used. On some test species such as *C. montana* and *C. maximowicziana*, newly-emerged flies laid no eggs while flies which had already fed on *C. vitalba* did (Table 2). *Clematis orientalis* was also able to support adult maturation, and although the fecundity, feeding intensity, and survival of newly-emerged flies was significantly lower than that of flies that had fed on *Clematis vitalba*, many

Table 1. Development of *Tetranychus lintearius* on cultivars of *Phaseolus vulgaris*. Eleven other cultivars provided intermediate results (from Hill & O'Donnell 1991).

Species Cultivar	**Total tests**	**Tests Producing:**			
		F1 adults	**F2 fertile eggs**	**F2 teliochrysalids**	**F2 adults**
Ulex europaeus	4	4	4	4	2
Phaseolus vulgaris					
A154	4	4	4	3	0
Purple King	4	4	4	2	0
Sanilac Navy	4	3	3	3	0
Top Crop	4	3	2	2	0
Pinto UI III	4	4	2	1	0
Uzura Cranberry	4	2	1	0	0
Carioca	4	3	0	0	0
Yates Crop	4	2	0	0	0

eggs were laid and larvae completed development (Schwarzlaender *et al.* 1996). In this case, no-choice tests conducted with newly-emerged flies produced a false negative result. To avoid potentially mis-leading results, oviposition tests are best conducted using mature insects drawn from populations reared on the target plant, as recommended by Marohasy (1998).

The validity of current host-testing methods. Current methods strongly indicate whether the plants tested are likely to be attacked in the field. However, extrapolation of these results to plants that have not been tested relies on the assumption that the laboratory host-range revealed by the centrifugal testing method mirrors the field host-range of the control agent under test. Analysis of the long recorded history of biological control of weeds projects suggests that this assumption is reliable (McFadyen 1998) but this hypothesis has rarely, if ever, been experimentally tested. There are rare cases where centrifugal testing would not accurately predict the host-range of oligophagous insects. Cabbage white butterfly *(Pieris rapae*) is oligophagous on brassicas, but can also use the garden nasturtium, *Tropaeolum majus* (Tropaeolaceae). Although the plants are in different families, foliage of nasturtiums and brassicas both contain glucosinolates, which promotes oviposition and larval feeding. Wapshere (1974) proposed methods in addition to the centrifugal method to identify such cases, but these are difficult to apply consistently, and the choice of additional non-target plants to test can be difficult to defend.

Table 2. Feeding, oviposition, and longevity of *Phytomyza vitalbae* flies on selected host-plants with and without access to *Clematis vitalba* leaves for adult maturation (differences within pairs of data; * P<0.05; ** P<0.01)(data from Schwarzlaender *et al.* 1996).

Plant	Adult state	eggs per female	feeding punctures	mean longevity (days)
Clematis vitalba		978	7470	35.2
C. montana	new	0	2*	2.0
	mature	12.8	14*	3.4
C. orientalis	new	177.6**	1495**	8.2
	mature	787.3**	5987**	24.0
C. maximowicziana	new	0*	8	4.0
	mature	4.0*	50	4.0

Conclusions

The strengths and weaknesses of methods for determining the host-range of biological control agents for weeds have been well reviewed (Cullen 1990, McClay 1996, Blossey 1997, Heard & van Klinken 1998, McFadyen 1998, Marohasy 1998). The prevailing view is that test designs that allow a subject to exercise its full complement of host-discrimination techniques provide more accurate prediction of host-range than those that do not, and that oviposition tests reveal more about the ecological host-range of a phytophagous insect than feeding and development tests (Wapshere 1989, Cullen 1990). As a result, choice tests are generally thought to reveal more about the host-range of an insect than no-choice tests. However, it is easy to grasp the concept that if a plant is ever to be susceptible, then a no-choice test will

show it. Negative no-choice tests are therefore convincing to regulators and the public because the risk of that result being false is considered low. On the other hand, the risk of a positive result being false is high, and there is always a risk that no-choice tests will generate data that obscures rather than clarifies the true host-range of the control agents in the eyes of regulators and the public. The prevailing view is that no-choice tests should be used to support choice or field tests, especially if test-plants are closely related to the target weed.
In this paper I have argued that no-choice tests may sometimes be more ecologically relevant than choice tests. This is true when the agent under test has limited opportunity to perceive and react to alternative plant-borne stimuli because it disperses passively, the agent is small in relation to its host-plant, and particularly if tests are conducted for a short period. In this situation, an agent can only accept or reject the host utilisation cues that dominate its universe. This reaches its extreme in tests of pathogens, where all tests are no-choice tests because the agent can only act where and when it is applied. This is one end of the size/mobility continuum. If relative size and mobility are important, then some tests that have been called choice tests in the past, may in fact be better described as multiple no-choice tests. Perhaps it is time to rethink how we design testing procedures, not to reflect how we present the test-plants, but how the agents under test might perceive those plants. New ways of recording how insects respond to test-plants will improve the precision of those tests (e.g. Marohasy 1998, Withers 1998).

ERMA has changed the face of biological control research in New Zealand. The authority will decide whether a biological control introduction should proceed in the context of a "values landscape" of which science is a critical, but not an over-riding consideration. Other determinants will be public attitudes to risk, perceptions of risk, values of the indigenous people, and in particular, levels of uncertainty. There is little that we can do about most of these inputs, but we can both minimise and clarify the uncertainties surrounding host-range determination. Surprisingly, with the exception of the protocols developed by Wapshere (1974), there has been little theory published to underpin better assessment of host-range (but see Marohasy 1998). Areas where agreed frameworks would be useful include:

- Setting standards for the interpretation of choice and no-choice test data.
- Experimental verification that no-choice tests have a low risk of a false negative result but a high risk of a false positive.
- Experimental verification that potential host-range of an agent predicted using centrifugal phylogenetic testing either predicts or overestimates the field host-range.
- Further analysis of the risk of host-range shifts (Marohasy 1998).
- Defining 'best practice' for testing particular insects or pathogens, guilds, or test-plant families.

Without objective standards, applications tend to rely heavily on the excellent historical safety record of biological control of weeds, and ask regulators to trust the applicant to continue to do it right. As the regulatory regime in New Zealand changes, it will become increasingly difficult to bridge any credibility gap without a more consistent framework.

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Continuation trials: their use in assessing the host range of a potential biological control agent

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Continuation trials test whether non-target species can support successive generations of potential biocontrol agents in no-choice trials. A plant species may be considered not to be at risk if adults reared from it did not develop or lay eggs, or if any resulting larvae did not complete development. Alternatively, a plant species that can support successive generations of an agent is potentially an alternative host and therefore at risk if the agent were to be released. A more difficult outcome to interpret is when adults that have developed on a non-target species, are able to oviposit and the larvae can complete development on the non-target species, but the population eventually dies out over subsequent generations. The rate at which the population dies out is the key consideration. The greater the number of generations that an agent can be maintained on the non-target species, the greater the chance of that plant becoming an acceptable host through selection. Whether potential biological control agents that can develop on a non-target plant for successive generations are deemed insufficiently host specific, will depend on the relative importance of the non-target plant species attacked.

Introduction

Determining the host range or preferred host of a potential biological control agent is a critical part of the classical biological control process. Host specificity testing aims to determine whether potential agents have the ability to feed, oviposit and/or develop on plants other than the target weed. Complete specificity is relatively uncommon and some non-target plant species may be subject to some minor feeding and/or oviposition by the agent. In many cases, the damage is insignificant or the larvae fail to complete development and the agents are approved for release (Davis *et al.* 1991; Forno *et al.* 1991; Day *et al.* 1998; Huwer & McFadyen, 1999).

Where the non-target species is also a weed, damage by potential agents may not be considered critical. However, it is the extent of such an impact on desirable species that is fundamental in determining whether an agent is safe to release in the field. If oviposition, larval feeding or adult development occurs on a valued non-target species, further tests should be conducted to determine if the agent could persist on the species. These follow-up tests have been named continuation trials. Continuation trials consist of returning adults that have completed development on one plant species during choice or no-choice trials, onto a fresh plant of the same species in no-choice trials, to test whether the non-target species can support successive generations of the agent.

Plant species that support an agent for more than one generation at greater risk than plant species that can support only one generation of an agent. In the latter case, plants could still be subject to periodic damage by the agent, and the extent of damage should be addressed before approval is sought. However, the more generations a plant species can support an agent, the greater the chance that adaptation may occur and the plant becoming an acceptable host.

There are several examples where insects have adapted to a new host and populations have increased (Prokopy *et al.* 1988; Carroll & Boyd 1992). Prokopy *et al.* (1988) reported that *Rhagoletis pomonella*, naturally occurring on the native *Crataegus mollis*, accepted apples, *Malus sylvestris,* and developed significant populations on the plant after a number of generations. Carroll & Boyd (1992) demonstrated that in Florida, the soapberry bug, *Jadera haematoloma*, which is found commonly on the native *Sapindus saponaria*, has been able to utilise the introduced tree, *Koelreuteria elegans*.

The question of whether selection acting on the agent will produce a strain preferring a new host, is contentious. Marohasy (1996) argues that an insect species that accepts a new host is more likely to have been pre-adapted to that host than to have made a 'host shift' following selection. Franca *et al.* (1994) tried to breed for selection using the Colorado potato beetle (CPB), *Leptinotarsa decemlineata.* After ten generations, there still wasn't any enhanced performance on the cultivated potato, *Solanum tuberosum,* with survival being maintained at 30%. In addition, when offered a choice of potato or its original native host, *Solanum rostratum*, the CPB still preferred its native host. Similarly, Carroll & Boyd (1992) and Prokopy *et al.* (1988) found insects persisting on new hosts, still preferred their original host when given a choice in experimental trials.

The rationale behind continuation trials is that the performance of an agent, especially on a sub-optimal host, may be influenced by the rearing host of its parents (Williams *et al.* 1983). Therefore it is necessary, in assessing the likelihood that a non-target species would support a population of the agent, to determine the number of female offspring produced per female when successive generations are reared on the same species. If the number of female offspring per female is less than one, the population will decrease and eventually die out. It is possible that females and their progeny reared on the non-target species maybe less fit and therefore lay fewer eggs than their counterparts reared on the target weed. In addition, the mean proportion of larvae completing development on the non-target species may be lower than for those reared on the target plant. Consequently, there would be less progeny/female produced each generation and the population on the non-target species may not be able to be sustained.

Alternatively, if the emerging adults can readily oviposit and subsequent larvae can complete development, such that the number of female progeny/female produced in each successive generation is greater than one, then the non-target species is considered to be able to support a population of the agent. Populations of potential agents would subsequently increase on the non-target species.

This paper addresses under what circumstances continuation trials should be conducted, how they should be performed, the possible outcomes of such trials and interprets their usefulness in assessing the likely field host range of biological control agents.

When should continuation trials be run?

Continuation trials should be set up when any adults are produced on a non-target species as a result of oviposition occurring during choice or no-choice trials. They should also be conducted when adults develop as a result of eggs or neonates being placed directly onto plants in no-choice trials. These inoculation trials may have been conducted if the females were indiscriminate in their egg-laying behaviour. Eggs or neonates are placed on the test plants and their subsequent development monitored. One side effect of this method is that there are usually a larger number of plants with larval feeding or possibly adult development, than if oviposition trials could have been used as placing eggs or neonates on plants removes the host finding mechanism of adults (Cullen 1990). There have been several examples where agents tested in this manner, have been reported to have initially fed on a large number of plants yet development was not completed and the agents were approved for release (Forno *et al.* 1991; Day *et al.* 1998).

Methods for conducting continuation trials

The procedure for conducting continuation trials may vary depending on the information required. In the first instance, some simple trials to determine if the non-target can support the agent over a series of generations may suffice. Where the non-target is valued, the decision to reject the agent could be made on the basis that a population was supported over a number of generations though no quantitative data is recorded.

In most situations, quantitative data are desirable to determine whether or not a non-target species is at risk from the agent. Studies should be conducted such that comparisons can be made across generations and between the non-target species and the target weed. The trials should consist of an equal number of pairs of newly emerged adults reared from a plant species during choice or no-choice trials being placed on a fresh plant of the same species. The trials should be replicated where possible to avoid problems such as variation in plant quality etc. The plants should be changed at regular intervals until all or possibly 90% of the adults have died. The number of eggs present on each plant at each change, should be recorded. All plants with eggs are then kept for subsequent larval development. By replacing the plant frequently, the pre-oviposition period, age specific fecundity and adult survival can be calculated.

A logistic problem that may arise during such trials is the large number of plants that would be held, especially if generations overlap. Depending on the number of eggs laid on each plant, not all plants may need to be kept. A sufficient number of plants should be kept to obtain a viable and synchronous population for the next generation. Emerging adults from the new generation can then be returned to a plant of the same species to test for fecundity and larval development. The number of generations that the trials need to be run, should depend on the relative performance of the agent on the non-target species compared with that on the target species. Long term studies such as those outlined above, were used when determining the host range of the chrysomelid *Calligrapha pantherina,* and the stem-boring beetle, *Eutinobothrus* sp., biological control agents for *Sida acuta* (Forno *et al.* 1992; Day *et al.* 1995).

Another problem that may arise is when only a small number of adults complete development on a non-target plant during choice or no-choice trials, such that only one or two pairs emerge

or that their emergence is not synchronised. While one or two pairs of adults is not ideal, continuation trials can still be conducted. Where synchrony of adult emergence is such that mating pairs cannot be obtained, then obviously trials cannot be validly performed. However, it is unreasonable to conclude that the plant is not at risk because only a few adults emerged. In the field, populations will be hundreds of times larger than in the laboratory. Therefore, a small percent survival in the laboratory could translate to a large number of adults in the field. When emergence is low on a non-target plant, extra effort should be made to increase the population size to enable synchronised emergence. This will assist with determining the agent's fecundity and ability to produce a second generation on the non-target plant.

Interpreting results from continuation trials.

There are three possible outcomes of continuation trials, forming part of a continuum. These are:

1) The first generation of adults reared on the non-target species fail to produce offspring.
2) The first generation of adults produce viable adult progeny on the same species on which they were reared. These adults then produce viable eggs from which larvae develop and the population in subsequent generations becomes larger.
3) The first generation of adults produce viable adult progeny on the same species on which they were reared. However, larval survival and/or fecundity of the emerging adults are reduced such that less than one female progeny per female is produced so the population size decreases over successive generations with the result that the population eventually dies out.

In the first scenario, when first generation adults reared from a non-target plant failed to produce viable adult progeny, it may be that the plant was deficient in some nutrients or contained some harmful substances. These insects may have reached adulthood only because they received the necessary nutrients from the eggs of females that had developed on the preferred plant (Williams *et al.* 1983; Rossiter *et al.* 1993). However, the nutrients from eggs alone were probably not sufficient to support the development of second generation larvae.

An example of the first scenario is the cactus mealybug, *Hypogeococcus festerianus,* which developed to adult on *Portulaca oleracea* when cut pieces of the target weed, *Eriocereus martinii,* containing active stages of *H. festerians,* were pinned to test plants. Emerging adults which were returned to *P. oleracea*, failed to lay eggs (McFadyen 1979). In another example, when neonates of *Nephele densoi*, a biocontrol agent for *Cryptostegia grandiflora*, were placed on the non-target species *Carissa grandiflora*, some individuals completed development, but the adults were deformed and not viable (Huwer & McFadyen 1999). Under such circumstances, non-target plants are unlikely to sustain persistent populations of the agents though they may still be at risk from periodic attack from individuals moving off the target weed.

In the second scenario, plant species that can support larval development and subsequent generations of the agent can be considered a potential host. There are several examples of insects encountering and completing development on a plant species, which has been introduced into the insects geographic range (e.g. Prokopy *et al.* 1988; Carroll & Boyd 1992). In addition, Hsiao (1978) reported that the Colorado potato beetle, *Leptinotarsa decemlineata*, which normally occurs on the native *Solanum rostratum*, successfully utilises and is now a pest of the cultivated potato, *Solanum tuberosum*. These insects are considered

to have been pre-adapted to the new hosts and to have incorporated these plants into their host range (Marohasy 1996). It is possible that, if the above plant species had always been found in the insects native range, then feeding and development on these plants would have occurred. It is important to note however, that the above species, when given a choice of their new host or their original host, preferred their original host.

The most difficult outcome to address in terms of assessing the risk to a non-target plant is the third scenario, when an insect population gradually dies out over a number of generations on that species. The decline in total numbers in subsequent generations on a non-target species through reduced performance is possibly a result of trans-generation effects through sub-optimal nutrition. Emerging adults on non-target species may have significantly reduced fecundity and subsequent lower larval survival. For instance, populations of *C. pantherina* could not be sustained on non-target species of *Sida* (Forno *et al.* 1992). First generation adults that had developed from neonates placed onto test plants, took longer to reach sexual maturity and laid significantly fewer eggs than those reared on the target, *S. acuta*. Survival to adult in the second generation was only 15% on *S. atherophera* and 10% on *S. cunninghamii*, while survival on *S. acuta* was 75%. Second generation adults reared on *S. atherophera* and *S. cunninghamii* failed to produce and lay eggs (Table 1). Damage by adults and non-target plants could not support continuing generations of the agent, *C. pantherina* was approved for release although it might occasionally feed on other *Sida* species in the field (Forno *et al.* 1992). There is a certain level of subjectivity in assessing the impact of the agent to the non-target species. Whether genetic adaptation or the effects of experience will result in the enhanced utilisation of the non-target species if it can support a number of generations of the agent is uncertain. In many cases, the final decision to approve the release of the agent may depend on the relative importance of the plant species attacked.

Other examples where agents have been sustained on non-target species for several generations are the stem boring moth, *Neurostrota gunniella*, an agent for *Mimosa pigra* and the stem boring beetle *Eutinobothrus* sp., an agent for *S. acuta* (Davis *et al.* 1991; Day *et al.* 1995). Small populations of *N. gunniella* were able to be supported on four species of *Neptunia* but mortality on these plants was over 70%, compared to less than 25% on *M. pigra*. Damage to the *Neptunia* species by *N. gunniella* was minimal, with only a few pinnae damaged on each plant (Davis *et al.* 1991). Populations of *Eutinobothrus* sp. were able to persist in low numbers on the introduced weed, *S. spinosa* and on the native *S. atherophera,* after three generations (Table 2). The total number of adults produced per female was generally lower on *S. atherophera* and *S. spinosa* than on *S. acuta*. *Eutinobothrus* sp. caused minor tunnelling in the stems of both these non-target *Sida* species but failed to kill any plants (Day *et al.* 1995). The decision to release *Eutinobothrus* sp. was granted as *S. atherophera* occurs only in central Queensland, thousands of kilometres away from the nearest *S. acuta* infestation, and *S. spinosa* is itself a weed. Although occasional damage to non-target species by both *N. gunniella* and *Eutinobothrus* sp. in the field is inevitable, it is likely to occur only when insect populations are high, and/or when the target weed is scarce. More importantly, if any of the non-target species attacked were considered of high value, then it is highly unlikely that release of the agents would have been permitted.

It is also important to consider that in the field, the potential total damage to a plant is the sum of the damage by all the generations including any insects that migrate from the target weed. The total damage to a plant could therefore be considered significant even though damage by insects in the last generation during tests is small.

Although there is uncertainty over the likelihood that selection pressure will cause adaptation of an agent to a non-target plant species, a rapid decline in the insect population reduces the possibility of any such occurrence. It is not necessary to state that non-target species should be considered to be unacceptably at risk if female agents produce progeny after rearing on non-target plants for a certain number of generations. However, it is clear that the slower the decline in population over generations on a non-target species, the more an application for the release of that agent would need to be argued on the basis of other criteria, e.g. the low value of the non-target plant in question, or geographic or habitat separation between the target weed and non-target species.

Table 1. The development of *Calligrapha pantherina* when neonate larvae were placed on *Sida acuta* and non-target species during no-choice continuation trials (Forno *et al.* 1992).

Plant species	Total neonates placed on each species	Total 1st generation adults emerged	Pre-oviposition period (days)	Total egg batches laid per female	Mean eggs/batch	% egg-adult development (2nd generation)	Oviposition by 2nd generation adults?
Sida acuta	30	24	18	36[a]	50	75	Yes
S. rhombifolia	30	20	18	12[b]	42	65	Yes
S. spinosa	30	20	28	2[c]	30	65	Yes
S. atherophera	30	9	21	1[d]	10	15	No
S. cleisocalyx	30	2	-	0	0	-	-
S. cunninghamii	30	6	135	1[e]	30	10	No
S. fibulifera	30	6	-	0	0	-	-
Abutilon otocarpum	30	9	-	0	0	-	-
A. oxycarpum	30	22	-	0	0	-	-
Malva parviflora	30	2	-	0	0	-	-
Modiola caroliniana	30	6	-	0	0	-	-

[a] 2 batches/week for 18 weeks
[b] 2 batches/week for 6 weeks
[c] 1 batch/2 weeks for 4 weeks
[d] only two egg batches were laid
[e] three females laid one egg batch each

Table 2. The survival and fecundity of *Eutinobothrus* sp. when placed on *Sida acuta* and non-target test plants during no-choice continuation trials (Day *et al.* 1995).

	Initial no. females	Pre-oviposition period (days)	Egg-laying period (days)	Total adults produced
1st Generation				
S. acuta	5	18	180	151
S. atherophera	4	84	56	16
S. spinosa	4	18	138	73
2nd Generation				
S. acuta	50	22	261	619
S. atherophera	5	41	202	93
S. spinosa	18	75	169	64
3rd Generation				
S. acuta	20	27	227	469
S. atherophera	14	35	150	39
S. spinosa	8	22	137	66

Conclusion

Continuation trials can play a significant role in determining whether a plant species is at risk from a potential biocontrol agent. Agents that can survive over successive generations on a non-target plant, or those that show an increase in performance on non-target host plant species through induction or selection, might be considered too risky to release. However, the final decision to release, will depend on the value of the non-target species at risk, and the level of damage caused by the agent.

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The use of cut foliage instead of whole plants for host specificity testing of weed biocontrol insects - is this acceptable practice ?

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There are practical and scientific advantages in using cut foliage in feeding tests to determine the host range of an insect being considered for release for biological control. However, the question arises as to whether cut foliage tests adequately reflect the feeding pattern that the insect might exhibit if exposed to growing plants. Factors which might cause aberrant results include changes in plant defences, inadequate sampling of the plant and sub-optimal microhabitats. Nine datasets (of mainly foliage feeding insects) comparing cut foliage and whole plant responses are examined. Although examples are given of instances where cutting the foliage was both beneficial and detrimental to an insect's survival, in general there was good agreement between cut foliage and whole plant results. Cut foliage is considered an acceptable method for determining host range, at least for foliage feeders, but it is recommended that contentious results should be confirmed using a whole plant technique. The technique may be less appropriate for phloem feeding species.

Introduction

Before an insect can be approved for release in a new country as a biocontrol agent it is necessary to provide data indicating that the insect has a narrow host range and that it will not present any danger to plants of economic importance nor to native species. Appropriate data are collected from a number of sources including literature review, examination of host data on museum specimens, discussions with scientists knowledgeable about the insect or its congeners, and survey and observation of related plants in areas where the insect is abundant. However the most critical data are usually the results of formal experimental testing of the insect's host range.

The objective of such studies is to assess the likelihood that any valued non-target plant is at risk. Plants can be outside the host range of an insect for a variety of reasons. For example, the insect may not oviposit or feed on the plant or cannot complete its development from neonate to reproductive adult. Thus tests commonly examine oviposition or feeding responses to test plants or their ability to support development of relevant life stages. Experimental data may be gathered in the country

of origin but, because of the high costs associated with maintaining overseas field stations and the increased sophistication of quarantine facilities, there is a trend for greater experimentation in quarantine facilities within the importing country.

In these host range tests, it is a common practice to offer the insect portions excised from a growing test plant rather than the whole plant itself. While this practice can be utilised for all types of tests and insects, it is most commonly practised for testing the growth, development and survival of leaf feeding insects.

The "cut foliage" in tests can take a variety of forms. Commonly it consists of a leaf disc or portion of a single leaf, a single whole leaf, a sprig in a petri dish, or a small bouquet of foliage with leaves still attached to cut stems standing in water.

There are many good reasons, both practical and scientific, for utilizing cut foliage tests in host specificity testing. The advantages are listed as follows:

(1) Insects can be kept under closer observation and more detailed measurements made when they are confined to a small area.

(2) The test plant itself (which is often difficult or expensive to replace) need not be brought into the quarantine facility and therefore it is not needlessly sacrificed at the end of experimentation if removal of plants from quarantine is not permitted.

(3) Space and other resources are often limiting within a quarantine facility so testing can be completed more quickly and efficiently if small enclosures such as petri dishes can be used instead of large cages.

(4) Abiotic conditions can be more easily standardized and manipulated.

(5) Basic assays increase the ability to do more replications and therefore achieve statistical meaningfulness.

However the question arises as to whether results from tests using cut foliage accurately reflect the host range of the insect. The is some evidence to suggest that perhaps excising leaf material will alter the outcomes of feeding assays. In an interesting study, Risch (1985) compared the responses of four species of beetles (two polyphagous, two more specialist) offered leaf discs, whole excised leaves and whole, potted plants of corn, bean and squash. The method of testing had a very significant effect on the results, in some cases changing the the level of significance and in others changing the direction of preference altogether. However the feeding preferences of the specialists were less affected by test method than were the more generalist species. There was also a much greater differential in response between leaf disc and whole leaf than between whole leaf and whole plant.

Three areas of particular concern should be considered before utilising a cut foliage technique.

(1) *Effect on plant chemistry or defences.* Perhaps the most important concern is whether the process of removing the foliage from the whole plant somehow makes the foliage more, or less, attractive to the insect.

Many complex physical and chemical changes such as water loss, chemical degradation, loss of soluble carbohydrate, conversion of protein to soluble nitrogen and change in soluble tannins and other secondary chemicals take place when foliage is cut from the plant. Indeed, plant defences are commonly induced by injury to the plants (Barker *et al.* 1995). However the induction of plant defences is usually not instantaneous (Karban & Meyers 1989) but builds up, sometimes over a period of days. The effect on plant chemistry and defences also depends on the method of treating the cut foliage. One would intuitively expect greater differentials using leaf discs than using, for instance, bouquets of foliage attached to excised stems placed in water.

The study of McCaffery (1982) provides an example of the changes in cut foliage of a kind which would be of concern in biocontrol host specificity studies. In this study the grasshopper, *Zonocerus variegatus,* was able to grow, develop and reproduce significantly better when fed excised cassava, *Manihot esculenta*, foliage than when fed on whole plants. McCaffery attributed this to there being less hydrogen cyanide in excised leaves than in attached leaves.

(2) *Sampling.* Cut foliage methods invariably utilize a very small sample of the total foliage of a plant; usually a much smaller sample than a test involving whole plants. A smaller or a more uniform sample may omit material of a particular quality that could be utilized by the test insect thereby possibly leading to a false negative. This can be partially overcome by using sprigs and bouquets, rather than single leaves or leaf discs.

However it should be noted that experimental whole plants such as those in pots may also be samples of a larger system (e.g. a 10m tree) and insects may react differently on a potted plant than on a naturally growing plant. For example, nymphs of the coreid, *Mozena obtusa,* failed to develop on potted *Prosopis glandulosa* which were artificially fertilised with nitrogen rather than natural nodulation (Cuda *et al.* 1995).

(3) *Laboratory artifacts and interactions.* It is important that optimum abiotic conditions are provided for the insects during the host testing process. This becomes more important as the system becomes more confined because the insect is less able to seek favourable microhabitats which might be present in larger arenas. Factors to be considered include the temperature, humidity, light regime, disease control, social behaviour of the insect, frequency and method of laboratory handling, and frequency of foliage changes. All these factors must be carefully considered for cut foliage tests, as well as whether any factor has an interactive effect with plant species.

In many cases reported differences in the results obtained between a cut foliage and whole plant assay may simply reflect the relative imperfections of the two methods, rather than reflecting an inherently different response by the insect to cut versus attached foliage.

Case studies comparing cut foliage and whole plant tests

Nine data sets gathered from experiments undertaken to define the host ranges of prospective agents for the biological control of weeds are examined on following pages.

(1) The geometrid *Isturgia deerraria* (Walker) is an African species found on *Acacia nilotica* (Mimosaceae), various other African congeners and also the introduced Australian species *A. mearnsii* and *A. decurrens* (Kruger 1995). Its ability to develop on twelve leguminous species was tested by placing neonates on both potted plants (approximately 0.5 m in height) and cut foliage (sprigs of foliage placed in petri dishes) replaced every three days. The two methods produced similar results, with the insect being able to complete larval development on nine plants but not on the other three species (Table 1). Both methods indicated that *Acacia farnesiana*, taxonomically close to *A. nilotica*, was a poor host while *Delonix regia* (Caesalpinaceae) was highly suitable. Both methods also indicated that development times were lengthened when the larvae were reared on plants such as *A. flavescens*, *A. farnesiana* and *A. deanei* (Table 2). The insect would have been rejected as a biocontrol agent with either method of host testing (W. Palmer, unpublished).

(2) An Indian population of *Isturgia disputaria* (Guenée) is presently being tested by similar methods. This species has been collected from *A. nilotica,* two other native congeners and also the Australian taxa, *A. decurrens* and *A. mearnsii* (Kruger 1995). So far there is good agreement between the insects raised on potted plants and cut foliage. In both methods neonates developed through to pupae on *A. nilotica, A. bidwillii, A. mearnsii,* and *A. pulchella* while 100% mortality occurred on seven other leguminous species (Table 1). Induced differences in development time to pupation (Table 2) and pupal weight were also detected by both methods (W. Palmer, unpublished).

Table 1. The percentage survival between neonate and pupation, of four geometrids when reared in either cut foliage on a petri dish or on a potted plant (W. Palmer, unpublished data).

Plant	*Isturgia deerraria*		*Isturgia disputaria*		*Chiasmia assimilis*		*Chiasmia inconspicua*	
	petri dish	potted plant	petri dish	potted plant	petri dish	potted plant	petri dish	potted plant
Acacia nilotica	52	58	85	75	69	73	67	87
A. deanei	23	45	0	10	3	0	0	0
A. bidwillii	50	70	7	10	0	0	0	0
A. conferta	10	45	0	0	0	0	0	0
A. farnesiana	3	3	0	0	0	0	0	0
A. angustissima	0	0	0	0	0	0	0	0
A. flavescens	3	8	0	0	0	0	0	0
A. decurrens	60	70	0	20	7	5	0	0
A. plectocarpa	0	0	0	0	0	0	0	0
A. mearnsii	36	60	3	40	0	3	0	0
A. pulchella	-	-	13	100	10	0	40	3
Delonix regia	30	67	0	0	0	0	0	0
Arachis hypogea	0	0	0	0	0	0	0	0

Table 2. The mean development times (days) to pupation for four geometrids when reared on either cut foliage in a petri dish or on a potted plant.

Plant	*Isturgia deerraria*		*Isturgia disputaria*		*Chiasmia assimilis*		*Chiasmia inconspicua*	
	petri dish	**potted plant**	**petri dish**	**potted plant**	**petri dish**	**potted plant**	**petri dish**	**potted plant**
Acacia nilotica	18	17	17	17	15	15	16	19
A. deanei	24	23	-	20	19	-	-	-
A. bidwillii	18	17	33	25	-	-	-	-
A. conferta	27	18	-	-	-	-	-	-
A. farnesiana	22	27	-	-	-	-	-	-
A. angustissima	-	-	-	-	-	-	-	-
A. flavescens	25	31	-	-	-	-	-	-
A. decurrens	15	21	-	28	22	23	-	-
A. plectocarpa	-	-	-	-	-	-	-	-
A. mearnsii	13	15	22	26	-	22	-	-
A. pulchella	-	-	30	25	25	-	22	26
Delonix regia	18	18	-	-	-	-	-	-
Arachis hypogea	-	-	-	-	-	-	0	0

(3) The geometrid *Chiasmia assimilis* (Warren) from Kenya is more narrowly stenophagous than either of the two *Isturgia* spp. Again there was good agreement between the results obtained by potted plant and cut foliage. In this case both methods produced good survival on *Acacia nilotica* , reduced survival and longer development time to pupation on *A. decurrens*, and no survival on eight other leguminous species (Table 1). Both methods also indicated longer development times associated with these marginal hosts (Table 2) (W. Palmer, unpublished data).

(4) *Chiasmia inconspicua* (Warren) is the most specific of four geometrids (case studies 1--4) tested recently as biological control agents for *Acacia nilotica*. In the comparative test, larvae successfully completed development on *A. pulchella* as well as *A. nilotica* when reared on both potted plants and cut foliage while complete mortality was recorded on eleven other leguminous species in both methods (Table 1). Both whole plant and petri dish methods indicated that, in comparison to *A. nilotica*, insects reared on *A. pulchella* had higher mortality, longer development time (Table 2) and lower pupal weights (W. Palmer, unpublished data).

(5) A geometrid, *Prochoerodes truxaliata* (Guenee), was tested as a biocontrol agent for *Baccharis halimifolia*. Survival of neonates on potted plants and cut foliage (excised leaves placed in petri dishes) was evaluated. Survival to pupation on *B. halimifolia* was 60% and 70% respectively for the two methods, while larvae did not survive on any of the other 17 plant species using either method (Ehler *et al.* 1990).

(6) A second geometrid, *Itame varadaria* (Walker), was also tested for biocontrol of *B. halimifolia*. The responses to whole plant and cut foliage (excised leaves in glass vials changed daily) of 19 plant species were compared. Larval survival six days after the unfed neonates were introduced were 70% and 100% for excised leaf and whole plant respectively. Complete mortality occurred with both methods on the other 18 plants (Palmer 1989).

(7) The cassidine beetle, *Conchyloctenia tigrina* Oliver was tested against four species of *Solanum* using both potted plants and cut foliage (excised leaf in petri dish). Significantly greater survival to adult occurred on two of the four species when the insects were reared on whole plants (91% and 62%) than when reared in the petri dish (34% and 25% respectively) (Olkers & Hulley 1994). However these differences would not have been sufficient to alter a biocontrol release decision.

(8) The cassidine beetle, *Gratiana lutescens* (Boheman), was also tested as a prospective agent for *Solanum elaeagnifolium* in South Africa (Olkers & Hulley 1994). It was tested against six species of *Solanum* using both potted plant and cut foliage (excised leaf in petri dish). Neonate larvae were able to develop to adults on all six plants in both methods. However while the cut foliage test indicated that the survival rate on *S. elaegnifolium* was similar to that on four of the other five species, the whole plant test indicated that *S. elaeagnifolium* was a significantly better host than any of the other plants. The authors inferred that the whole plant test was a better test than the cut foliage. Because the whole plant test indicated that other plants were less suitable hosts than the target weed, they suggested that the insect should be further considered (Olkers & Hulley 1994). Further testing (Hill 1999) using a whole plant technique produced high, similar survivals for five of the six plant species indicating conclusively, that the insect was not suitable for release. The original cut foliage test had, indeed, indicated the relative acceptability of the various plants as hosts.

(9) Cut foliage tests have also been used to test oviposition preference (Diatloff & Palmer 1987). When adults of the cecidomyiid, *Neolasioptera lathami* Gagne, were offered a choice of bouquets of seven plant species they oviposited 400 and 550 eggs on *B. halimifolia* and *B. neglecta,* respectively, but nothing on the other five plants, all of which occur commonly within the insect's field range. This reflected the known host associations of the insect in the field (Gagné 1989).

Other Information

Small cut foliage tests are also often used in informal pilot studies which are not reported. On a collecting trip it is not uncommon to test a promising new insect against the foliage of a few key plants in transit between collection sites. Alternatively, one may try the insect on some cut foliage to get a feel for its feeding behaviour before embarking on a formal experiment. My experience has been that tests with cut foliage do indeed give a good indication of what will happen when testing is conducted on whole plants.

No mention has yet been made of the use of cut foliage for testing sucking insects such as hemipterans. In a small study the seed feeding lygaeid, *Ochrimnus mimulus,* was tested using cut inflorescences of six asteraceous species (Palmer 1986), producing results which reflected its field host range.

However, excision of foliage may give significantly different results when testing phloem feeders. Three aphid species have been reared on excised foliage of plants which were unsuitable when offered as a whole plant (O. Edwards, pers. comm.). A psyllid, *Acizzia melanocephala* Burckhardt & Mifsud, has been reared though from egg to adult on cut foliage of *Acacia nilotica indica* while repeated attempts to rear them on whole plants of this taxon have failed (W. Palmer, unpublished). The aphid, *Brachycaudus rumexicolens,* survived better on some plant species when confined by a clip cage (thought to produce a tourniquet effect restricting the flow of translocatable, antiherbivore chemicals thus rendering the plants more suitable for aphid development) than when placed in a dialysis cage (Scott & Yeoh 1998). These results support the analysis of Koricheva *et al.* (1998) which indicated that sucking insects performed best on stressed plants. Cut foliage methods may not be suitable or may be extremely conservative for such sucking insects.

Discussion

Host range tests are used by biocontrol workers to support decisions as to whether an exotic insect can be released safely within a new ecosystem. As such our requirements for sensitivity may differ from those of others working in fields such as insect behaviour (e.g. Risch 1985), host-plant resistance, or modelling. We are interested in knowing whether a proportion of an insect population might be able to utilize a particular host. In some cases it would not matter whether 10% or 60% of individuals survived on a test plant; both scenarios would lead to rejection.

Most of the presented data sets showed good agreement between the results of whole plant and cut foliage methods and there was no clear indication of one method consistently producing higher survival. It must be pointed out, however, that all but one of these cases relate to tests of larval development (or neonate to adult). For development to occur, a plant must be palatable, must contain nutrients in suitable absolute and relative amounts and must lack lethal concentrations of allelochemicals. Thus, it appears that excision does not alter

palatability, nutrient content and/or the occurrence and qualities of secondary plant substances sufficiently to influence the outcome of larval development tests in ways which would affect decisions relating to the safety of candidate agents. Only one of the examples surveyed related to oviposition tests and this was not a formal comparison of whole plant versus excised foliage. Thus, on the basis of the evidence presented, it is not possible to draw firm conclusions about the effect of excision on acceptability for oviposition.

Where appreciable differences appeared between methods it was not possible to ascribe a reason. However, in all probability the differences in most cases may be due to laboratory methodologies and sampling rather than due to intrinsic changes in plant chemistry or defence mechanisms in the foliage.

In all the data sets examined above, data from the cut foliage tests would have produced the same final conclusion on host range of the proposed insect, as that generated by whole plant testing. There thus appears to be no general reason why the cut foliage test should not be considered an appropriate method for determining host range.

However, although cut foliage is an acceptable method, in most circumstances, we should remain alert to the possibility that particular plant groups (e.g. *Manihot* spp.) may be appreciably altered by cutting the foliage. It would be prudent to include relevant comparative data in order to confirm the appropriateness of the method.

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Methods for host testing insects that use an oviposition deterring pheromone or other host discrimination system

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Insects that breed in discreet resources such as flowers or seeds have commonly evolved mechanisms which minimise over-exploitation of these resources. One mechanism is the use of an oviposition deterring pheromone (ODP), but other methods of host discrimination also exist such as an ability to detect and be deterred from further oviposition by the presence of con-specific eggs, larvae or damaged host tissue. The existence of such mechanisms can be exploited in the design of host specificity tests. Potential biocontrol agents of the weed *Mimosa pigra* that utilise flowers and seeds were found to cease ovipositing in their resource when a certain egg load was reached. Host specificity choice trials were subsequently designed to ensure that this level of damage occurred in the target plant within the duration of the trial, thus increasing the rigour of the trial. A simple method is described to assess the level of host damage at which host discrimination and deterrence occurs; and to help design choice trials to ensure this level is attained. This technique may be generally applicable where the insect utilises a discreet resource.

Introduction

Oviposition behaviour is the behaviour most commonly examined in host specificity testing of insects (Heard & van Klinken 1998). The acceptance or rejection of a plant species for oviposition by a female is the critical result required from these tests. However, factors that may manifest themselves independently of plant species affect oviposition behaviour, e.g. plant morphology, levels of secondary compounds, levels of nutrients particularly nitrogen, developmental stage of plant or plant parts. Finally, host plant selection may be influenced by the presence on the plant of conspecific eggs (Rothschild & Schoonhoven 1977) or larvae (Mappes & Mäkelä 1993), damage caused by larval or adult feeding (Fitt 1984), or an oviposition deterring pheromone deposited by the females during or immediately following oviposition (Roitberg & Prokopy 1987). This final aspect, often called host discrimination, is examined in this paper.

Ovipositing females of many phytophagous insects avoid hosts already infested by conspecifics. This behaviour results in a more even dispersal of eggs, a reduction in larval competition, and an increase in larval survival. Host discrimination of this kind is used most commonly by insects that have restricted host ranges, that feed at relatively ephemeral rather than permanent plant parts, that have restricted feeding sites within plants, that are immobile as larvae, and that feed on perennial rather than annual hosts. This is because most of these characters are correlated with situations where the chances of competition between conspecifics are high (Roitberg & Prokopy 1987).

Biocontrol agents that fit these characteristics include phytophagous insects that utilise flowers, seeds and fruits. Insects at the next trophic level also exhibit host discrimination, including both parasitoids (Godfray 1994) and predators, such as Coccinellidae (Doumbia *et al.* 1998), Cecidomyiidae (Ruzicka & Havelka 1998) and Neuroptera (Ruzicka 1997). Many of the conclusions of this paper therefore can be applied to host specificity testing of natural enemies of arthropod pests.

Case study 1. *Coelocephalapion aculeatum* (Coleoptera: Apionidae)

Coelocephalapion aculeatum was released in Australia in 1992 as a biological control agent of *Mimosa pigra* (Mimosaceae), a serious weed in Australia (Forno *et al.* 1994). This insect is host specific. Its larval feeding sites, inflorescences of *M. pigra*, are ephemeral and well separated. Larvae are unable to move from one inflorescence to another. The host is a perennial shrub. All these factors suggest that *C. aculeatum* may utilise an ODP or other mechanism to recognise previous infestations and to avoid oviposition in such inflorescences. Furthermore, adults oviposited into immature inflorescences of varying stages in numbers that correlated with their predicted carrying capacity (Heard 1995a). This further suggested that this insect used an ODP or some other high signal to noise ratio source of information concerning conspecifics.

The basis for this oviposition deterrence was examined by offering inflorescences damaged by adult feeding alone, larval feeding alone and a combination of adult feeding and oviposition. Adults preferred to oviposit on inflorescences that were not damaged by either adult feeding, larval feeding, or oviposition. No evidence for the existence of an ODP was found. In this case, the ability of a single host inflorescence to support the development of many larvae probably selected for the use of these oviposition deterring cues which can convey more quantitative information about the level of previous infestation than can ODPs. Adults fed a similar amount on damaged compared to undamaged inflorescences (Heard 1995b).

Studies of the kind referred to above would be too time consuming to be done within the resources of most biocontrol programs. However, a simple assay to detect the presence and the strength of the oviposition deterrence can be conducted by exposing cohorts of adults to two levels of access to oviposition sites: (1) “restricted”, in which the number of oviposition sites are greater than the potential fecundity of the insect and (2) “abundant”, in which sites are less than the potential fecundity. Food must not be limiting in either group. The plant material is changed daily and the number of eggs counted. The pattern of oviposition for *C. aculeatum* is shown in Figure 1. Mean values for oviposition show that the cohort of adults is capable of laying 46 eggs per day but cease ovipositing in inflorescences when a mean of 18 eggs have been deposited (Table 1).

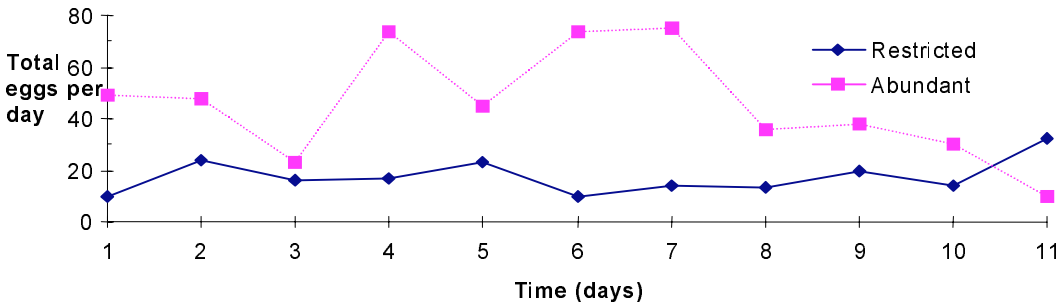

Figure 1. Total number of eggs laid by two cohorts of adult *Coelocephalapion aculeatum* one with access to abundant inflorescences, the other with restricted access (from Heard 1995b). Used with kind permission from Kluwer Academic Publishers.

Table 1. Oviposition response of *Coelocephalapion aculeatum* to abundant or limited oviposition sites (mean followed by the SEM in brackets). Data are the mean daily response over an 11-day period of a group of 25 adults.

	Oviposition sites	
	Abundant	Restricted
Number of inflorescences	8.1 (0.2)	1 (0)
Number of eggs / day	45.6 (6.5)	17.5 (2)
Eggs / inflorescence / day	5.5 (0.7)	17.5 (2)
Number of feeds / day	82.5 (11.9)	30.1 (4.5)
Feeds / inflorescence / day	10.1 (1.4)	30.1 (4.5)

These results assisted the design and conduct of host specificity trials. The observation that females tend to cease laying when the number of eggs per inflorescence reaches 18 provided guidelines on the quantity of plant and insect material that should be included and the duration of host specificity trials. These trials are choice trials, in which several plant species are exposed to insects simultaneously. To maximise the chances of detecting acceptance of alternative hosts, we aimed to achieve egg carrying capacity of the normal host during the trial so that ovipositing females were deterred. After this stage, the motivation of females to oviposit is expected to increase, with the result that they could be expected to accept any less preferred hosts (Singer *et al.* 1992). The number of insects, amount of plant material, and duration of the trial can be managed to achieve carrying capacity before the end of the trial by applying the information on maximum fecundity gained from the abundant treatment.

It is interesting to note that another agent against *M. pigra*, the related *C. pigrae* was shown to behave in a similar way. Adults were deterred from laying more eggs when the number of eggs per inflorescence reached 24 (Heard & Forno 1996).

Case study 2. *Chalcodermus serripes* (Coleoptera: Curculionidae)

Chalcodermus serripes is a seed feeder of *Mimosa pigra.* Adults oviposit only on seeds but feed on growing tips, flowers and pods of its host-plant. One larva develops per seed (Heard *et al.* 1999). This insect satisfies the characteristics of insects which normally use a host discrimination system, namely, restricted host range, feeding at relatively ephemeral plant parts, restricted feeding sites within plants, immobile as larvae, and feeding on perennial rather than annual hosts. All of these characteristics will increase the chances of intra-specific competition and select for host discrimination.

An experiment determined the effect of confining adults to a limited number of oviposition sites and thereby forcing adults to select or reject those sites that had been already damaged by previous oviposition. The experimental design was similar to that above for *C. aculeatum*. Eighteen healthy adults of *C. serripes* were placed simultaneously in each of two treatments in a no-choice design. The first treatment (abundant) consisted of an abundant number of sprigs with inflorescences, leaves, young pods (too young for oviposition but suitable for feeding) and nine older pods of *M. pigra* suitable for oviposition. The second treatment (limited) consisted of a similar abundant amount of feeding material but only one pod suitable for oviposition. The trial ran for 12 days. Every second day

the adults were provided with fresh plant material of the type described for each treatment. The older plant material was removed and examined for eggs and feeding scars.

Adults with access to abundant oviposition sites laid more eggs than those with limited sites (see Table 2: Oviposition response of *Chalcodermus serripes* to abundant or limited oviposition sites in Heard *et al*, 1999). The 3.5 eggs per female per day laid on the abundant-seeds treatment did not result in full exploitation of the resource, with only half of the seeds having eggs. The 1.4 eggs per female per day laid on the limited pods resulted in many seeds with more than one egg. An abundance of inflorescences and leaves ensured that food was not limiting to either group of adults (Heard *et al.* 1999).

The information on fecundity guided decisions on the numbers of adults, quantity of plant material and duration of the host specificity trials. Females cease oviposition when the number of eggs per seed reaches approximately 1.7. The application of this information for host specificity testing is that this level of damage should be achieved in choice tests to force females to search for and accept or reject the test plants. Such a level was achieved in trials on *C. serripes*.

Conclusion and recommendation for host testing

The ability of conspecific signals to deter further oviposition can be used to increase the rigour of choice tests in host specificity testing. A simple preliminary study, similar to the one described in the case studies, can detect the presence and strength of the deterrence. This trial will also determine the maximum fecundity of the insects. Both these pieces of information can then be used in the design of choice tests. The tests should provide the appropriate amount of plant material, number of insects, and duration to ensure that the level of infestation of the target plant that caused deterrence is reached before the end of the test. Ovipositing females are then required to search elsewhere for oviposition sites and are more likely to find and accept alternative plant species present in the trial. This increases the rigour of choice trials making them an acceptable alternative to no-choice trials when resources dictate against the latter.

Many of the conclusions apply to parasitoids and predators and so can be applied to host specificity testing of natural enemies of arthropod pests.

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The use of choice tests in host-specificity testing of herbivorous insects

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Choice tests are often used when determining the host range of potential agents for the biological control of weeds. They may be the only type of test involved, although they are more usually done in conjunction with no-choice tests. They are undertaken by offering the insect a choice of two or more plant species, which may or may not include the target species. Different types of choice tests are described. Examples are taken from the literature to investigate the rationale given for undertaking different types of choice tests, as well as reasons given as to why researchers have undertaken both choice tests and no-choice tests on the same insect life stage. Some of the problems in interpretation of results of choice tests are presented, and the future role of these tests in specificity testing is discussed.

Introduction

The early years of biological control of weeds were characterised by the use of no-choice (or "starvation") tests. However in 1969, Harley (1969) supported the use of "simulated field exposure" (i.e. choice tests which included the target weed) as being more desirable than the "unnatural conditions" of no-choice tests. Choice tests are now sometimes the only type of laboratory test undertaken (e.g. Winder *et al.* 1984), while in other studies, no-choice tests are the only tests done (e.g. Adair & Scott 1991, Woodburn 1993). More often, both types of tests are undertaken, either on different stages of the insect's life cycle, or on the same stage, usually on ovipositing females, or feeding larvae or adults (Heard & van Klinken 1998). No-choice tests frequently indicate a larger potential host range than do choice tests (e.g. Thompson & Habeck 1989), and the challenge for biological control practitioners is to determine which result, if either, allows a reliable prediction of the subsequent field host range to be made. This review will consider (i) the different types of choice tests that are used in host specificity testing, (ii) when and why they are used in the testing process, (iii) problems that can arise in interpreting results, and (iv) some thoughts on their role in the future of biological control of weeds.

Types of choice tests

Choice tests are used in biological control to assess the degree of specificity of an insect to the target plant species, or weed. They are here defined as tests involving the simultaneous presentation to the insect of two or more plant species in the same arena.

This definition excludes tests in which the insects are exposed to a single plant species and then to another single species and so on. These latter tests more properly represent a variant of no-choice tests, where the insects are presented with only one species of plant at any given time.

There are two main types of choice tests. The first type involves the presentation of two or more plant species, of which one is always the target weed. This category can be subdivided into 'multiple-choice' tests, involving more than two species, and 'paired-choice' tests, involving only two species, of which one is the target species (Heard & van Klinken 1998).

The second type of choice test again involves the presentation of two or more plant species, but this time the target species is absent. Heard & van Klinken (1998) termed these tests as 'choice minus control' tests. It is perhaps preferable to use the term 'target species' or 'target', rather than 'control', since the target is not a control, in the usual scientific sense of the word. Furthermore use of the word 'control' may suggest that the researcher believes that this plant is the preferred or only host of the insect. The target species is usually tested separately in these tests, either simultaneously with the choice test, using another cohort of insects, or before or after the choice test, using the same cohort of insects. This is to provide evidence that the insects are healthy or reproductively mature, and to provide a comparison for performance.

The implementation of choice tests is usually quite straight forward, the main variables being the number of test plant species included in the test, the size of the testing arena, the stage of the insect used, the number of insects added, the duration of the tests, and the parameters that are scored. Examples are listed in Heard & van Klinken (1998, Appendix 1). The interpretation of the results of choice tests is far less straight forward, as will be discussed later. The main divergence arises from differing opinions as to the validity of choice versus no-choice tests. Similarly, there are divergent opinions on the validity of tests involving the target species, versus tests excluding the target species.

When and why are choice tests done?

Choice versus no-choice tests. Sometimes choice tests are the only tests that are undertaken during the testing of a potential biological control agent, but more often they are done in conjunction with no-choice tests. When both choice and no-choice tests are undertaken they may be done on different life stages of the insect, and this is usually to clarify whether the insect is able to complete its life cycle on a particular plant species.

Of more relevance to the current analysis is the situation when choice and no-choice tests are done using the same life stage of an insect, such as ovipositing females. One can argue that if a particular test was valid, then why was it necessary to undertake another type of test on the same insect life stage? Or are the tests considered to be addressing different questions? It is therefore pertinent to look at reasons given by authors as to why the two types of tests were done. A selection of approximately 30 papers in which the two types of tests were done on the same life stage was investigated to determine (i) the order in which the two types of test were done, (ii) what rationale was given (if any) for doing either or both the tests, and (iii) what

interpretation (if any) was given if the results of the two tests were not the same. All choice tests used in this analysis included the target species. The selection of papers was unbiased but far from comprehensive. Appendix 1 from Heard & van Klinken (1998) provided about half the examples, the rest coming from a computer-based literature search requiring that both 'multiple-choice test' and 'no-choice test' appeared in the one reference.

In many cases the choice tests (including target) followed the no-choice tests, and were done only on those species on which feeding, oviposition or complete development had occurred in the no-choice tests (e.g. Maw 1976, Nayek & Banerjee 1987, Thompson & Habeck 1989, Peschken & Sawchyn 1993, Gassmann 1996, Fornasari 1997). The stated, or implied, reason for this was to observe which of the acceptable plant species was preferred when the insects were given a choice. Often in the choice situation only the target host was attacked, or was attacked to a far greater extent than any other species, with the authors usually concluding that this demonstrated the specificity (and hence safety) of the insect.

A different reason for doing no-choice tests first was given for the Argentine weevil *Heilipodus ventralis*. It was tested on its natural hosts in a no-choice test to determine if biotypes of the insect occurred, and thereafter the full range of tests plants was tested using choice oviposition tests (Cordo 1985).

In some cases, choice tests (including the target species) were done before no-choice tests. Heard & Forno (1996) gave their reasons for doing this as "to determine the host range", and for "rapid screening", before the "more rigorous" no-choice tests, on the few species on which oviposition had occurred in the first tests. Similarly Palmer & Goeden (1991) undertook choice tests first "to screen" twelve plant species, before doing a no-choice test on one of these. Day *et al.* (1995) did choice trials first, and then did no-choice tests on only those species which supported egg-to-adult development or on which larvae fed in the previous tests.

In other cases it was not clear in which order the tests were done, although some explanation was given (or can be inferred) as to why the different types of test were undertaken. Buckingham *et al.* (1991) did no-choice larval development tests on plant species considered to be at greatest risk, and choice tests on other species considered to be at a lower risk. The reason given for the choice tests was so that they could test "a greater range of species … than was possible in no-choice tests". The assumption appears to be that the no-choice tests are more conservative (i.e. less likely to provide a 'false negative' result, see Marohasy 1998) and hence were used on species considered to be at greater risk. Similarly, Jordan (1995) did oviposition choice tests with *Larinus minutus* on a large number of test species, but did a no-choice test only on globe artichoke, *Cynara scolymus*. Although this species was not attacked in choice tests, it was presumably tested further because of its economic value.

In many cases (not included in the above examples) there were no reasons given for why both tests were undertaken, and no interpretation given when the tests produce different results. This suggests that in many cases biological control researchers may be doing the two types of test routinely, without questioning the rationale behind them, and without considering the implications of what the results will indicate. Furthermore, the fact that some workers do choice tests before no-choice tests, and

others do them the other way round, suggests there is no consensus in how the two types of test should be used and interpreted, and perhaps that there is not full understanding of what the results of the two types of tests are telling us. Another possible reason for the type of tests done and the order in which they are undertaken could relate to logistical constraints such as availability of quarantine space and/or cages of required size, although this is rarely stated.

The use of different types of choice tests. The literature survey by Heard & van Klinken (1998) indicates that choice tests including the target species are more often used than choice tests that exclude the target species. For instance, of the 26 studies on oviposition that involved choice tests, 20 tests involved only choice tests that included the target species, five involved both types of tests, and one involved only choice tests without the target species. In this last study, by Heard *et al.* (1997), *Sibinia fastigiata* were initially placed on the target species in a no-choice situation, before entering the 'choice minus target' configuration. The rationale used by Heard *et al.* (1997) was that this approach combined the efficiency of choice tests (i.e. more rapid processing of species), with the "fewer assumptions implicit in no-choice tests". The approach was extremely effective in this instance, because almost no oviposition occurred on the non-target species. If a non-target species had been more acceptable to the insect, the interpretation would have been more difficult, and the authors may have had to undertake choice tests including the target species.

A somewhat similar procedure was undertaken for *Liriomyza sonchi* by Peschken and Derby (1988) (not included in Heard & van Klinken's 1998 survey). Choice tests were done without the target plant, while the target plant was tested concurrently with a different sample of insects. If any plant in the choice test was attacked, the other plants were re-tested after removing the species that had been attacked. In this way a less-preferred but acceptable species could not be 'protected' by the presence of a more preferred plant. Interestingly, the authors refer to these tests as no-choice tests, presumably because the insects were not presented with a choice between the target species and any other species.

Based on the research of Wiklund (1981) on identifying the hierarchy of oviposition preferences of butterflies, Marohasy (1998) has suggested a two-step choice procedure for testing biological control agents. In Stage 1, test species are tested in a choice minus target configuration, while the target is tested concurrently with another cohort of comparable insects. If no attack occurs on non-targets, the insect is then considered safe to be released. If attack occurs on any test species Marohasy then proposes Stage 2 tests be applied. A provisional ranking of the acceptability of plant species is derived, and the insects are then presented with a choice of the highest ranking species. After an appropriate period for oviposition or feeding, the most acceptable plant species is removed, and replaced with the next most acceptable from the preliminary ranking. The procedure is continued until no feeding or oviposition occurs. Thus one can ascertain the ranking of acceptable hosts, as well as determine those which will not support any attack. Stage 1 is the same as the procedure used by Peschken & Derby (1988), and aspects of Stage 2 are also evident in their procedure.

An example that has used Marohasy's (1998) approach involves *Carmenta ithacae*, which was tested for specificity to *Parthenium hysterophorus* (parthenium weed) (Withers *et al.*1999). The tests involved placing the moths with a group of plant

species comprising parthenium weed and other potentially susceptible species for two days, then removing parthenium weed for two days, and then returning it to the group. In essence this procedure involves a 'choice with host' test, followed by an 'choice without host' test, using the same group of insects for both. The procedure was not as complex as that described by Marohasy (1998), since only one plant species other than the target was found to be acceptable for oviposition.

As mentioned above, five studies on oviposition from Heard & van Klinken's (1998) survey included both choice tests with the target and choice tests without the target. In three of these (Peschken & Harris 1975, DeLoach *et al.* 1976, Cordo 1985), little explanation for the experimental design was provided, although all indicated that the reason for including the 'choice minus target' tests was to put the non-target plants under greater pressure of attack. Wapshere & Kirk (1977) used the two tests in a sequential procedure remarkably similar to the method used by Withers *et al.* (1999). They did not provide comment on the reasoning behind their tests, perhaps because they considered it is fairly self evident. Adult *Dialectica scalariella* moths were given a choice of the target and two test species until damage appeared on the target species. The target was then removed for 6 days, and subsequently returned until damage was again evident. Fresh insects were added to those already in the cage at the time the target plant was removed. The addition of naïve insects to experienced insects could have complicated interpretation, although this did not seem to arise in this study. A similar sequential procedure was employed by Hill *et al.* (1995), with the results of the 'choice with target' tests being used to confirm that the *Agonopterix ulicetella* moths were capable of oviposition in the 'choice minus target' tests. In an interesting variation, they also undertook 'choice minus target' tests with insects that had never experienced the target.

Problems in interpreting results of choice tests

A problem common to all types of testing done in cages is the possibility of indiscriminate behaviour, particularly relating to oviposition. The topic has been addressed by Withers & Barton Browne (1998), who offer possible explanations for this behaviour, as well as recommendations to reduce its effects. Occasionally the problems of cage-induced aberrant behaviour cannot be resolved in the laboratory, and the only solution is to undertake field testing in the country of origin (see Briese, this volume). The discussion that follows assumes that there is no evidence for cage-induced aberrant behaviour, and that the insects have expressed their normal preferences.

Marohasy (1998) lists behavioural phenomena that are likely to incorrectly indicate that a plant is outside the natural range of the insect (a 'false negative') in choice tests as (i) unresponsiveness to lower-ranked species in the presence of higher ranked species, and (ii) central inhibition owing to recent contact with strongly deterrent non-host plants. Presumably this latter factor could also affect the acceptability of the known host. Marohasy (1998) also lists the factors that might lead to the incorrect conclusion from choice tests that a plant is at risk from attack by the insect (a 'false positive') as (i) attack on non-hosts positioned close to target host due to central excitation or sensitisation, (ii) associative learning when the target host and a non-host have some characteristics in common, (iii) habituation following repeated contact

with non-hosts, and (iv) volatiles from the target plant permeating the cage and condensing on non-hosts.

Perhaps the major problem associated with choice tests, particularly those that include the target, is that an acceptable, but less preferred host might be protected during tests by the presence of a more highly ranked host. For this reason it is unwise to base a decision on the safety of an insect for release where the only testing done involved choice tests that included the target.

Some researchers prefer choice tests that include the target, to no-choice tests or 'choice minus target' tests, since the former tests more closely represent the field situation. On the other hand, it can be argued that no-choice tests (and choice tests minus target) are preferable, since it is of interest to know what an insect will do if the target plant is overloaded by the insect or even eliminated from an area, or if the insect finds itself in a region without the target. The real world probably lies somewhere between these two extremes, and the best a researcher can do is to design a choice experiment to take this into account. For instance the ratio of target plants to non-targets could be based on likely field situations, and the plants can be arranged in realistic densities. This approach is also likely to reduce some of the effects mentioned above, such as central excitation due to close proximity of the target plant, and volatiles from the target plant condensing onto non-targets. However limitations of cage numbers and sizes could curtail this degree of refinement, and the approach has more applicability to field studies in the country of origin.

A final comment on the problems associated with choice tests is that, from a behavioural point of view, an insect in fact rarely makes a true choice between two plant species, either in a cage or in the field (L. Barton Browne, pers. comm.). More accurately, an insect encounters a plant, and then makes a decision to alight/feed/oviposit on the basis of stimuli perceived at that instant, and either stays or departs. While this decision can be affected by previous experience and physiological state (e.g. last plant encountered, degree of hunger), it is rarely based on an assessment of two or more sets of simultaneously perceived sensory cues. This latter would be true choice.

Conclusions on the use of choice tests in specificity testing

An obvious advantage of choice testing has already been mentioned, and that is the ability to process many plant species more rapidly than by following a no-choice testing regime. Apart from the advantage of saved time that this confers, it is also practical when numbers of insects are low or when they are only available for a short time of the year. Another advantage is that by using choice tests on the stage that is involved in host selection (often the ovipositing female), the number of plant species that then needs to be tested for the whole life cycle (usually larval and/or adult feeding) can be greatly reduced. A further advantage of choice tests which include the target is that the insect is not 'forced' within the confines of a cage to either attack a non-target or do nothing, i.e. there is less risk of aberrant behaviour. However, as stated above, there is a major risk associated with choice tests. If there is an acceptable but less preferred species, this may not be detected if it is always tested in the presence of a more preferred species. Are there ways this risk can be minimised, without losing the several advantages of choice tests?

It is likely that choice tests will continue to be an integral part of specificity testing for potential biological control agents. While it is not possible to suggest the 'perfect' testing schedule, it is possible to recommended a sound protocol, based on a balance between practicality and scientific rigour. A design that combines several elements from the tests employed by Wapshere & Kirk (1977), Peschken & Derby (1988), Heard *et al.* (1997), Marohasy (1998) and Withers *et al.* (1999) is to undertake a choice test which includes the target species, combined with a concurrent no-choice test on the target. The target species is then removed from the choice test. If no attack (oviposition or feeding) occurs on the remaining species, these can all safely be excluded from further testing. The decision on how long to leave the insects in the 'choice minus target' configuration is a difficult but crucial one. It should not be so short that the insect's physiological threshold has not dropped to a level where it is ready to feed or oviposit again, but nor should it be so long that the threshold has dropped to a level where the insect might feed or oviposit on almost any plant (see Barton Browne 1993, Withers 1999). The process of plant removal can be continued if attack occurs on any non-target, to obtain a ranking of these species. However, since biological control agents are required to have a high degree of host specificity, it may be that if attack occurs on several, or even one non-target then further testing is abandoned. The above procedure enables a large number of species to be processed rapidly, and provides a concurrent check on the quality of the test insects. It also provides the opportunity for the existence of less preferred hosts to be identified, since at some stage they will be exposed without the 'protection' of a more suitable host. Processing of species could be further expedited by combining species thought most unlikely to be attacked, so that as many groups as possible could be quickly eliminated. This approach can be further simplified by starting the testing process with the 'choice minus target' configuration, with a concurrent no-choice test on the target (as in Peschken & Derby 1988, and Marohasy's (1998) Stage 1).

There will always be species for which no prescribed testing schedule will be appropriate. An example of this is provided by "*Tortrix*" sp., a species that has been tested for specificity to *Chrysanthemoides monilifera* (Anon *et al.* 1999). Cage-testing, and field choice tests with and without the target all produced results which were inconsistent with the observed host range of this insect in the country of origin. It was not until field testing was done by releasing the insects as pupae placed within a target plant, that subsequent host plant selection was comparable to that seen naturally in the field. The first egg batches were laid on the release plant (which in nature is the target), and then subsequent egg batches were laid on other target plants within the experimental arena, but not on other test species. Any experimental design which did not incorporate this essential first step in the insect's adult life did not provide conclusive and unambiguous results.

Ultimately it will depend on the availability of quarantine space, the supply of insects and plants, information on the host range in the country of origin and the experience of the researcher as to which tests are undertaken. The researcher must be alert to the possibility of aberrant behaviour of the caged insects. The tests must be appropriate to the particular insect species being tested, and the prospects for achieving this are improved when the behaviour and biology of the insect are well understood. The final test of the validity and reliability of the testing procedure is not obtained until an insect is released in the field, but as this discussion has suggested, much can be done

before this point to provide a sound scientific basis to assist in making an informed decision on the safety of the insect.

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Open field host-specificity tests: is "natural" good enough for risk assessment?

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Open-field host specificity tests are considered by many to offer the most realistic method for assessing host-range, as agents are not restricted in their movement by some form of caging. Despite this, relatively few candidate agents have been subjected to open-field testing, and then usually to clarify ambiguous results produced by caged testing. Their use has mainly been limited by logistic considerations, such as the need to conduct them in the country of origin of the test agent, and quarantine problems associated with using non-native plants in that country. Recently, open-field tests have been criticised, along with cage tests, as possibly producing false results due to a failure to consider insect behaviour in experimental design. This paper will examine the different rationales used for choosing open-field tests, the different ways in which these have been carried out and how these have helped interpret host-range problems in the past. It will also consider the question of insect behaviour, including the potential to gain further insights on the host-selection process through manipulative field experiments or monitoring agent movement using mark-release-recapture methods. It is argued that open-field testing should not be viewed as an alternative, but as an adjunct to more traditional laboratory-based testing.

Introduction

In weed biological control, host-specificity testing is driven by two potentially conflicting needs; not to introduce an agent that may cause unacceptable damage to a non-target plant and not to reject a potentially effective agent unnecessarily. Traditionally, testing has been based almost exclusively on the results of laboratory studies in which variables can be more easily controlled. However, because of the conservative results they generate, laboratory-based tests are biased towards addressing the first need. There is no evidence to date of an agent having attacked a species in the field that was found to be not attacked during a laboratory testing regime. Nonetheless, such tests always require some form of containment for the agent, and a major criticism has been that restricting agent movement can lead to changes in host-choice behaviour (see Marohasy 1998), and therefore to the misidentification of some non-target plants as potential hosts (e.g. Hasan & Wapshere 1977, Cullen 1990, Dunn & Campobasso 1993). Emphasising the first need of safety may, in such cases, lead to the rejection of valuable agents that in reality do not pose a significant risk to non-target plants.

Open field host-specificity tests have been suggested as a way of avoiding problems due to containment of the agent (Cullen 1990, Blossey 1995, Clements & Cristofaro 1995), as the agent is free to express locomotor behaviour associated with host rejection. Thus, open-field tests are pitched more at addressing the second need, that of avoiding the rejection of valuable agents that in the field will not pose a risk to non-target plants. Despite the view that data obtained from open-field testing is more reliable (Blossey 1995) and propositions that it form part of standardised testing protocols (Wapshere 1989, Harris & McEvoy 1995), its use has been fairly limited. This may be due in part to the fact that field tests are usually last in the hierarchy of testing protocols and may not be required in many cases, and in part to a number of political and logistic constraints (Clements & Cristofaro 1995) (see below).

Clements & Cristofaro (1995) reviewed 24 studies describing open-field host-specificity tests, the majority of which have been published in the last decade. They concluded that open-field tests have demonstrated their value in that the host-range data derived from studies on agents "under more natural conditions than those obtained via cage tests" were critical to decisions to release 20 agents. Several of these agents would have been rejected on the basis of laboratory testing alone. As well, three agents were definitively rejected on the basis of open-field tests, confirming their accuracy in identifying non-target plants that can serve as hosts. Nonetheless, open-field tests have been criticised on several grounds. McFadyen (1998) argued that agent densities in such situations may be much lower than potential populations in the country of introduction and any changes in host-choice induced by conditions of extreme intraspecific competition and/or starvation would not be replicated. Marohasy (1998) has also argued that two types of error found in cage tests can also occur in open-field test; a "false negative" should agents be unresponsive to lower-ranked potential hosts and a "false positive" should the presence of the target weed elicit central excitation of the agent and consequent attack on neighbouring non-host plants.

This paper examines a data set of 40 open-field experiments (Table 1), comprising those reviewed by Clement & Cristofaro (1995) and more recent examples. Within this set there is considerable variation in the way individual experiments have been set up. Tests have been conducted for different purposes and structured in different ways, with different emphases on the data generated by them. The focus of this review is not so much outcomes, but more on the rationale for conducting the tests, the experimental design and the type of data collected, with a view to answering criticisms of open-field tests, examining ways in which they can be made more rigorous and determining more precisely what their role should be in overall host-testing procedures.

How and why have open-field tests been done?

Rationale for open-field testing. From the list of tests carried out to date, three main reasons were distinguished for setting up open-field tests of weed biological control agents;

- screening - in which no prior assumptions are made about the host-choice of the agent,

- clarification - where laboratory-based tests have given results that appear to be false, usually on the basis of known field host records.
- refinement - where the host-range is generally known, but differences in preference are suspected between either close target relatives or biotypes of the target species

Of the 15 studies that screened agents (Table 1), seven involved testing the agent against a broad range of non-target plants, ranging from congeneric species to plants from different families, with up to 70 plants being tested. Eight of these studies focussed on a smaller number (2 to 8) of key species, either important crop plants or closely related native plants, that were critical for acceptance to release the agent. Such studies are, in effect, open-field analogues of the routine laboratory-based testing against prescribed plant test lists. Apart from being more "natural", they have the advantage that several candidate control agents can be tested simultaneously, with up to four species being studied in individual experiments (Table 1). Clement & Cristofaro (1995) have, however, identified a number of constraints (Table 2) which would limit the wider use of open-field trials for general screening of test plants.

In 20 of the studies, the authors have sought to clarify apparent anomalies in laboratory-based testing by removing some of the behavioural constraints to host-selection processes (Table 1). Typically these involve only one, or rarely two, potential agents. In most cases, they are directed against a small number of key host plants (Table 1), usually those for which laboratory results were ambiguous, but sometimes supplemented with other close relatives. Three experiments involved candidate agents that exhibited little discrimination in laboratory choice tests and hence used a broader range of test plants (Table 1).

In a number of cases, there is some evidence that an agent known to be safe for introduction, may in fact discriminate between the introduced form of a weedy species and other known forms of the weed from the native range, or between congeneric weedy species (Clement 1994). Hence, five of the open-field studies specifically addressed the issue of refining the known host range in order to improve biological control efficiency rather than to address safety concerns (Table 1). These cases are less relevant to the present review, which is concerned primarily with the risk of introductions.

Table 1. List of open-field tests conducted to evaluate the host-range of weed biological control agents (see text for a discussion of the terms used). While covering the majority of studies, the list is representative rather than exhaustive.

Weed	Non-target species tested			Experimental design			Agent data collected		Result	Reference
	Rationale	Type	No.	Structure	No. agents tested	Agent [a] population	Stage [b]	Adult [c] movement	Host range acceptable	
Lantana camara	screening	broad range	60	interspersion	2	natural	A O L		yes	Harley (1969)
Lantana camara	screening	broad range	70	interspersion	1	natural	A O L		yes	Harley & Kunimoto (1969)
Hypericum perforatum	screening	broad range	23	interspersion	1	natural	A L		yes	Wapshere (1984)
Tamarix spp.	screening	broad range	10	interspersion	4	natural	A L		yes	Sobhian *et al.* (1998)
Crupina vulgaris	screening	broad range	12	interspersion	1	natural	L		no	Sobhian *et al.* (1996)
Tripleurospermum perforatum	screening	broad range	18	interspersion	1	natural	A2		no	Hinz (1993)
Tribulus terrestris	screening	broad range	10	reverse interspersion	1	augmented (T)	A	observed	yes	Andres & Angalet (1963)
Carduus nutans	screening	key species	8	interspersion	1	augmented (O)	A L	MRR	no	Dunn & Campobasso (1993)
Carduus nutans	screening	key species	7	interspersion	1	augmented (O)	A L	observed	yes	Dunn & Campobasso (1993)
Carduus nutans	screening	key species	6	interspersion	1	natural	O L		yes	Rizza *et al.* (1988)
Centaurea spp.	screening	key species	5	set design	2	augmented (O)	A O A2	MRR	yes	Maddox & Sobhian (1987)
Centaurea spp.	screening	key species	4	set design	3	augmented (O)	A A2	MRR	yes	Groppe *et al.* (1990)
Centaurea spp.	screening	key species	3	set design	2	augmented (O)	A2		yes	Clement & Sobhian (1991)
Onopordum spp.	screening	key species	2	set design	4	augmented (H/T)	A O A2	MRR	yes	Briese *et al.* (1995)
Onopordum spp.	screening	key species	3	set design	4	augmented (H/T)	A O A2	MRR	yes	Briese *et al.* (1995)

Table 1. continued

Chondrilla juncea	clarification	broad range	12	interspersion	1	augmented (T)	L		yes	Hasan & Wapshere (1977)
Chrysanthemoides monilifera	clarification	broad range	36	no-choice	1	augmented (T)	1		?	Anon. (1999)
Chrysanthemoides monilifera	clarification	broad range	34	set design	1	augmented (O)	O	observed	?	Anon. (1999)
Lythrum salicaria	clarification	key species	15	interspersion	1	natural	L		no	Blossey & Schroeder (1995)
Lythrum salicaria	clarification	key species	2	interspersion	1	natural	A L		yes	Blossey *et al.* (1994a)
Lythrum salicaria	clarification	key species	2	interspersion	2	natural	A L A2		yes	Blossey *et al.* (1994b)
Cynoglossum officinale	clarification	key species	9	interspersion	1	natural	A2		yes	Schwarzlaender (1996)
Cynoglossum officinale	clarification	key species	4	interspersion	1	natural	A2		yes	Schwarzlaender *et al.* (1997)
Tripleurospermum perforatum	clarification	key species	6	interspersion	1	natural	A2		?	Hinz & Geisen (1995)
Tripleurospermum perforatum	clarification	key species	8	interspersion	1	natural	A2		yes	Hinz *et al.* (1996)
Potentilla recta	clarification	key species	4	interspersion	1	natural	A L		yes	Schaffner & Lauro (1997)
Heliotropium europaeum	clarification	key species	2	interspersion	1	augmented (O)	A O		no	Cullen (1989)
Carthamus lanatus	clarification	key species	1	interspersion	1	natural	A2		yes	Aeschlimann (1997)
Heliotropium europaeum	clarification	key species	2	no-choice	1	augmented (T)	A O		no	Cullen (1989)
Carthamus lanatus	clarification	key species	1	set design	1	natural	A2		no	Aeschlimann (1997)
Carduus nutans	clarification	key species	1	set design	2	augmented (H)	A L	MRR	yes	Rizza & Spencer (1981)
Heliotropium europaeum	clarification	key species	3	set design	1	augmented (O)	A O		no	Cullen (1989)
Euphorbia esula	clarification	key species	1	set design	1	augmented (O)	A O L		yes	Pecora *et al.* (1992)
Cynoglossum officinale	clarification	key species	2	set design	1	augmented (T)	A A2	MRR	yes	Schwarzlaender (1996)
Potentilla recta	clarification	key species	4	set design	1	augmented (O)	A L		no	Schaffner & Lauro (1997)

Table 1. continued

Centaurea diffusa	refinement	conspecifics	2	set design	1	augmented (O)	O A2	n/a	Sobhian *et al.* (1992)
Euphorbia esula	refinement	conspecifics	12	set design	1	augmented (O)	O L	n/a	Pecora *et al.* (1991)
Euphorbia esula	refinement	conspecifics	8	set design	1	natural	L	n/a	Pecora *et al.* (1989)
Centaurea solstitialis	refinement	conspecifics	8	set design	3	natural	A2	n/a	White & Clement (1987)
Centaurea solstitialis	refinement	conspecifics	8	set design	3	natural	L A2	n/a	Clement (1994)

a supplementary agent placed (O) = off-plant, (H) = on known host plant, (T) = on test plants, (H/T) = on both known host and test plants
b A = adult feeding, O = oviposition, L = larval development. A2 = emergence of second generation adults
c MRR = mark, release and recapture data taken

Table 2. Possible constraints to the implementation of open-field host-specificity tests listed by Clement & Cristofaro (1995)

Political
• limited to native range of the agent or areas where it has been previously introduced
• possible restrictions on the use of non-indigenous test plants in the country of origin
Logistic
• added cost of foreign-based host-testing
• impracticality of testing large numbers of non-target plants in the field
• difficulty in controlling agent density
• possible difficulty in discriminating between agent and other herbivore damage

Experimental design. Clements & Cristofaro (1995) produced a framework, based on physical structure, of experimental designs used for open-field testing in order to assist researchers in their planning. They distinguished three types of experimental design; interspersion, where test and control plants were randomly placed in a natural infestation of the target weed containing a population of the agent to be tested, segregation, where blocks of transplanted test or control plants were placed away from the local infestation of the target weed, and set design, where control and experimental plants are placed out in the field in experimental set-ups such as randomised blocks and latin squares. This framework has been modified in the present review to better reflect the purpose of the design rather than structure alone, and to distinguish those designs that may have an influence on agent behaviour. Four types of experimental design are recognised here:

- **Interspersion -** where test plants (often paired with potted controls of the target weed) are randomly placed in a natural infestation of the target weed. The relative density of the target weed is therefore much greater than that of the test plant(s). Typically, this type of experiment relies on natural populations of the agent, though in a few cases agent populations have been augmented (Table 3).

- **Reverse interspersion -** where the target weed is planted out within a natural or crop plantation of the test species. Although there is only one published case approaching this design (Andres & Angalet 1963), it is listed separately as the relative density of the test plant is in this case much greater than that of the target weed and this could have implications for agent behaviour and host-choice. As a natural infestation of the target weed is not present, addition of the agent is required.

- **Set design (choice) -** where combinations of target weed and test plants are planted out in a fixed experimental design, including randomised or non-randomised blocks and Latin squares. While these are often planted out near natural agent populations, agent numbers have been augmented by directly placing them on the experimental plots in most cases (Table 3).

- **Set design (no-choice) -** where the target weed is excluded from the planting. In this case, the agent needs to be added, but unlike laboratory-based no-choice tests, is free to leave if it rejects the test plant as a suitable host. This is a more severe and more artificial version of reverse interspersion.

Table 3. Frequency of use of different experimental designs for open-field host testing of weed biological control agents.

Structure of experiment	Agent population		
	Natural	Augmented	Where placed
Interspersion	16	4	1 on test plants, 3 off-plant
Reverse interspersion	-	1	1 on test plants
Set design (choice)	4	13	1 on host plants, 1 on test plants, 2 on host and test plants, 9 off-plant
Set design (no-choice)	-	2	2 on test plants

The experimental design used is determined to some extent by the purpose of the test. The majority of screening tests use interspersion, all tests aimed at refining the agent's host range have used various set designs, while those aimed at clarifying the results of prior laboratory testing have used both types of experiment with similar frequencies (Table 4). Further variation in design can occur through the use of different methods of augmentation. In most of the 20 cases in which natural agent numbers have been supplemented, the additional agents have been placed off the plant (Table 3), either on the soil or other vegetation as adults, or by being left to emerge from pupae placed in the field plot. This avoids by-passing any pre-alighting cues by enabling agents to freely choose between the target weed and test plants (see Marohasy 1998). In a few instances, though, agents have been placed directly either onto the known host (Rizza & Spencer 1981) or test plants (e.g. Andres & Angalet 1963, Cullen 1989), or both (Briese *et al.* 1995) (Table 3). In these cases, the purpose has usually been to bias the experiment by forcing the agent to choose deliberately to leave or stay on particular plants. Which situation is more "natural" and/or preferable depends on the biology of the particular agent and the questions being asked of the experiment. Finally, in 38 of the 40 cases, the experiments have been designed to address choice by the adult agent, either through feeding and/or, more importantly, oviposition. In only two cases (Hasan & Wapshere 1977, Anon. 1999), has the behaviour of mobile larvae been investigated in the field.

Data collected. The data collected from open-field experiments is also fairly diverse. Invariably, it involves some measurement of agent activity, such as adult feeding, the presence of eggs, larval development and/or emergence of the next generation of adults (Table 1), and less frequently measures of adult longevity or movement. In general, the results have been fairly clear and have demonstrated a more restricted

host-range than that shown under confined conditions. Where tests were conducted to clarify previous cage test data, the agent was found not to attack the key non-target plant or to have a greatly reduced host range in 10 cases, while in four cases attack on the non-target plant was confirmed. Anomalous results, however, are not solely the domain of laboratory-based testing and in a few cases, different field experimental designs have produced conflicting results for the same agent. Such cases are highly instructive when assessing the value of open-field testing (Table 5).

Table 4. Frequency of use of different experimental designs for open-field host testing of weed biological control agents.

Reason for test	Experimental design	
	Interspersion	Set design
Screening	10	5
Clarification	11	9
Refinement	0	5

Table 5. Comparison of host-choice behaviour for interspersion vs set design open-field tests involving the same agent.

Agent-plant system	Agent origin	No. test species	Effect on test plants relative to target weed [a]		Reference
			Interspersion	Set design	
Root-weevil on *Cynoglossum*	Augmented [b]	8	1/8 attacked 20%	4/8 attacked 3-17 %	Schwarzlaender (1996)
Bud weevil on *Potentilla*	Augmented [b]	4	1/4 attacked 5%	3/4 attacked 35 - 91%	Schaffner & Lauro (1997)
Seed fly on *Carthamus*	Natural	1	no attack	42%	Aeschlimann (1997)

a = Agent effect on attacked test plants recorded as percentage of attack level on target plants.
b = Extra agents introduced from elsewhere to increase population density.

Schwarzlaender (1996) used both an interspersion design with a natural agent population and a set design using only introduced adults of the agent, the weevil *Mogulones cruciger*, a candidate agent for control of hound's-tongue, *Cynoglossum officianale*. The test plants were key species, all of which were able to support larval development of the weevil under caged no-choice conditions. He found that there was a clear preference for the target weed by the natural field population of weevils, whereas in the set design with introduced newly emerged weevils placed near but not

on the experimental plants, oviposition and development occurred on four of the eight test species. While this was to a much lower degree than occurred on the target weed, it poses the question of why host-choice behaviour has changed. Some possible influences are the difference in relative densities of target weed vs non-target plant, differences in the prior experience of adults, or agent mobility. The fact that some wild *Mogulones* adults also attacked non-target plants suggests that the relative density of plant species may be more important than prior experience.

Two further cases also support this. Schaffner & Lauro (1997), using designs similar to those of Schwarzlaender mentioned above, found that natural field populations of the weevil *Anthonomus rubripes*, a candidate agent for control of sulphur cinquefoil, *Potentilla recta*, also clearly preferred the target weed, whereas in the set design most weevils, which appear to be poor dispersers, accepted non-target plants in close proximity to where they were released, irrespective of species. The weevils that were introduced into the set-design experiment had been field collected from the target weed and so had had prior experience of it.

This is also supported by the results of Aeschlimann (1997) who observed the same phenomenon for the seed-fly, *Urophora mauretanica*, a candidate agent for the control of saffron thistle, *Carthamus lanatus*. The key test species, safflower *Carthamus tinctorius*, was not attacked by a natural population when a small number of test plants were interspersed within an infestation of the target weed. However, safflower was attacked, though to a lesser degree than the target weed, when the experiment consisted of a set design comprising an equal number of target weed and safflower plants. In this case, the agent source in the set design experiment was a mobile natural population, which suggests that relative abundance of target weed vs test plant may have again influenced behaviour rather than any prior experience of the flies. The results indicated that the risk of releasing the fly into Australia for control of saffron thistle were unacceptably high.

Blossey *et al.* (1994b), using interspersion experiments and natural agent densities, compared adult host choice in two species of *Galerucella*, a defoliating chrysomelid beetle, under conditions of high target weed density (purple loosestrife, *Lythrum salicaria*) compared to a shortage of the weed following heavy larval defoliation in the previous season. They found that when the target weed was abundant, there was no attack on two key non-target species that had been found to support development in caged tests. However, when large numbers of F1 adults emerged to a shortage of target weed, the teneral adults did feed to some extent on both non-target plants. Despite this, there was no oviposition or larval feeding on these species, indicating that they do not support completion of the beetles' life-cycle, and that any damage to the non-target plants was transitory.

A major criticism of caged testing is the restriction in movement imposed on the agents, open-field tests provide an opportunity to examine in more detail movement patterns of mobile insects. Not all agents are amenable to marking, but in some of the studies where this was possible (Table 1), mark-release-recapture data for adult agents introduced into the study plots have provided valuable information on adult behaviour. In general, these show movement from non-host to host plants and movement between host-plants within a plot (e.g., Maddox & Sobhian 1987, Clement & Sobhian 1991). In one experiment, Briese *et al.* (1995) extended this to look at

movement between widely isolated plots of mixed target weed and non-target plants. They observed net movements of two candidate agents (the weevils, *Larinus cynarae* and *Lixus cardui*) away from one plot where soil and moisture conditions had resulted in smaller, apparently less attractive target plants toward a plot in which the plants were larger and more vigorous. The poor condition of the target plants did not lead to attack on non-target plants within the first plot, but to migration in search of other hosts. Non-target plants were not attacked in either plot, giving confidence that host-selection behaviour was not modified by host-plant availability or quality.

Briese *et al.* (1995) also found that the relatively mobile agents, *L. cynarae* and *L. cardui*, shifted more quickly away from a non-target plants. Unlike these two weevil species, a plant-hopper, *Tettigometra sulphurea*, did not show any interplot migration in the experiment described above, and remained for a longer time when placed on non-target plants, even laying some eggs, from which hatching larvae did not survive. In another example, Anon. (1999) set up 5 satellite plots separated from and around a central release plot, containing both non-target and target plants. The agent, a mobile moth *Tortrix* sp., oviposited on 6/34 non-target plants in the central plot near where they had emerged, but oviposited only on the target plants upon dispersal to the satellite plots. These data indicate the newly emerged adults might not be as discriminating, and suggests that consideration needs to be given to both the manner of release and placement of supplementary agents.

Specific criticisms of open-field testing

Non-realistic agent densities. As natural enemy constraints can be removed when an agent is released in a new environment, McFadyen (1998) argued that agent densities in the field may be much lower in the native range, where tests would be conducted, than could eventually be attained in the country of introduction. As a consequence, any changes in host-choice induced by conditions of extreme intraspecific competition and/or starvation would not be expressed. There is evidence that deprivation induces a temporary broadening of host range (see Withers 1997). This is supported by the findings of Blossey *et al.* (1994b), described above, that the adult feeding behaviour, though not oviposition, of a chrysomelid beetle did change when agent densities were very high relative to the preferred host, and by the inference that relative host density may play a role in those cases where different results are produced by interspersion vs set design experiments.

There are, none-theless, ways in which behaviour under such circumstances can be assessed in field tests. An example is the study by Blossey *et al.* (1994b), which took advantage of natural high numbers leading to destruction of the host-plant. Dunn & Campobasso (1993) and Rizza *et al.* (1988) partially showed how this could be done in set-design experiments. They used two-phase field tests, in which naturally occurring host-plants were killed once agents were present and feeding on them, forcing the agents to move and select between planted test and control host plants. If high numbers of agents can be established on known hosts in a set-design and then all host-plants be killed, the agents would be forced to either leave the plot or attack the test plants. Similarly, a reverse interspersion experiment could simulate this effect, where a few heavily infested host-plants are placed in a large population of the test plants and then killed to force an exodus of agents. Andres & Angalet (1963) approached this method by placing cut heavily-infested target weeds amongst

commercial plantations of test plants. In this case, agents chose to leave rather than feed on the test plants.

False negatives and false positives. According to Marohasy (1998), a false positive occurs when "feeding or oviposition occurs on a test-plant species which would not be attacked in the field". It is argued that this might occur in field situations if the presence of the target weed elicits central excitation of the agent with consequent attack on neighbouring non-host plants. While an obvious problem in cage-testing, a false positive is, by its own definition, not an issue for open-field testing. If central excitation is a natural phenomenon that leads to attack on certain test plants under natural field conditions, then those plants form part of the field range. Whether or not they are true hosts and the degree of risk this poses to the non-target plant depends on whether the agent can complete its life-cycle on the plants. Phylogenetic evidence (see Briese 1996) suggests that host specialisation is a very conservative trait, and the effect of phenomena such as central excitation and sensitisation would seem more likely to apply to polyphagous arthropods. Any characteristic leading to indiscriminate reproductive behaviour in specialists, such as candidate biological control agents, would seem an evolutionally unsound trait. The feeding by teneral adults on non-target plants observed by Blossey *et al.* (1994b) may have been due to such a feeding stimulus which persisted even after no more host foliage was left, or to a deprivation induced lowering of the threshold for host-selection. Neither case would have posed a long-term cost, either to the plant or the agent, as it was not associated with reproductive behaviour.

A false negative occurs when "a test indicates a plant species is outside the host range of the species, when in reality it might be attacked in the field" (Marohasy 1998). In field situations such errors can occur if agents are unresponsive to lower-ranked potential hosts. Such a possibility is dependent on the relative density of target host vs test plants. For example, the fact that, at normal agent densities, host-range is sometimes more limited in interspersion tests than in set-design tests, could occur if the higher relative density of the target weed made agents unresponsive to some test plants. Thus, the false negative phenomenon is another way of viewing the problem of non-realistic host densities described above, and represents the opposite extreme in behavioural response to that shown by agents under deprivation. This would suggest that the use of set designs with augmented agent populations provides a more accurate idea of the extremes of agent behaviour than the use of interspersion with natural agent populations. From a safety perspective, these designs appear to be the more conservative type of open-field test.

Conclusions

Open-field experiments have an important role in host-range testing of weed biological control agents, and while not essential in all cases, they are critical in some. This review has shown that differences in the design of field experiments can affect results and identifies a number of issues that need to be considered before setting up a design:

- set design experiments appear less likely to produce false negatives than interspersion experiments in natural infestations of the target weed,

- the relative abundance of target and non-target plants in the design may influence behaviour,
- the initial release method and placement of supplementary insects may influence interpretation of results,
- agent movement should be monitored when this is feasible,
- agent feeding on test plants may occur under some circumstances without agent reproduction, and
- the quality of plants may influence their acceptability to agents.

As indicated earlier, open-field tests frequently address the risk of unnecessarily rejecting a safe and potentially effective agent. Care needs to be taken not to downgrade the issue of risk to non-target species in doing so. Observations made on test plants exposed to natural field populations of the agent usually enable us to a evaluate only the host range of the agent under ideal conditions. Potential risk to non-target plants may depend more on host-acceptance behaviour during more extreme situations. As McFadyen (1998) has pointed out, an effective agent would be expected to reach very high densities relative to that of its host if it is to control the target weed, and hence its host-choice behaviour should be tested at these levels. The application of deprivation in a test should reveal the expression of any host-range expansion. No-choice cage testing of agents is ideal for doing this, and therefore will always remain a key step in the rapid screening of test plant species (Cullen 1990, Hill, this volume, Sheppard, this volume). However, no-choice tests force behaviour to unnatural limits through containment of the agents, which means that negative results are extremely robust but positive results should be viewed with caution. The use of more realistic tests, such as caged host-choice tests or open-field tests, thus becomes essential.

Within the constraints of agent biology, a well planned set-design field test can reveal natural host-selection behaviour without any form of containment. As an example, the idea of two-phase tests is appealing, in which the first choice phase examines the behaviour of high agent densities relative to the target weed, in the presence of test plants (Fig. 1). The second no-choice phase would involve killing or removing the target weed to reveal the agent's behaviour with regard to non-target plants at high agent densities in the absence of the target species. This would simulate the situation where a successful agent builds up in numbers and locally destroys its host weed. Such a design addresses the two possible problems listed by Marohasy (1998) that are of importance in the open field, i.e. lowered responsiveness to other plants when the target plant is unlimited, and changes in behaviour in the absence of the target plant. Coupled with the ability to monitor agent movement and the use of satellite plots to trap emigrating agents, such tests would be very powerful and would provide the most reliable measure of risk to non-target plants. In interpreting the results of these tests, appropriate weighting should be given to results indicating transitory feeding by food-stressed agents on non-target plants compared to those which indicate that reproduction is possible.

Most testing protocols argue that open-field tests should provide the final filter in a hierarchy of procedures (Wapshere 1989). Certainly, open-field tests should not replace laboratory-based tests for broad-scale screening of test plants, unless there is a problem in maintaining agent cultures in laboratory conditions. However, in view of the potential for political and logistic constraints to doing such tests, it would seem wise to be opportunistic in the case of "clarification" and conduct pre-emptive tests on

key species whenever this is feasible during initial studies. In many cases, those plant species likely to form part of the host-range of an oligophagous agent can be identified from phylogenetic relationships. The more realistic result produced by the open-field test can only strengthen confidence in any risk assessment.

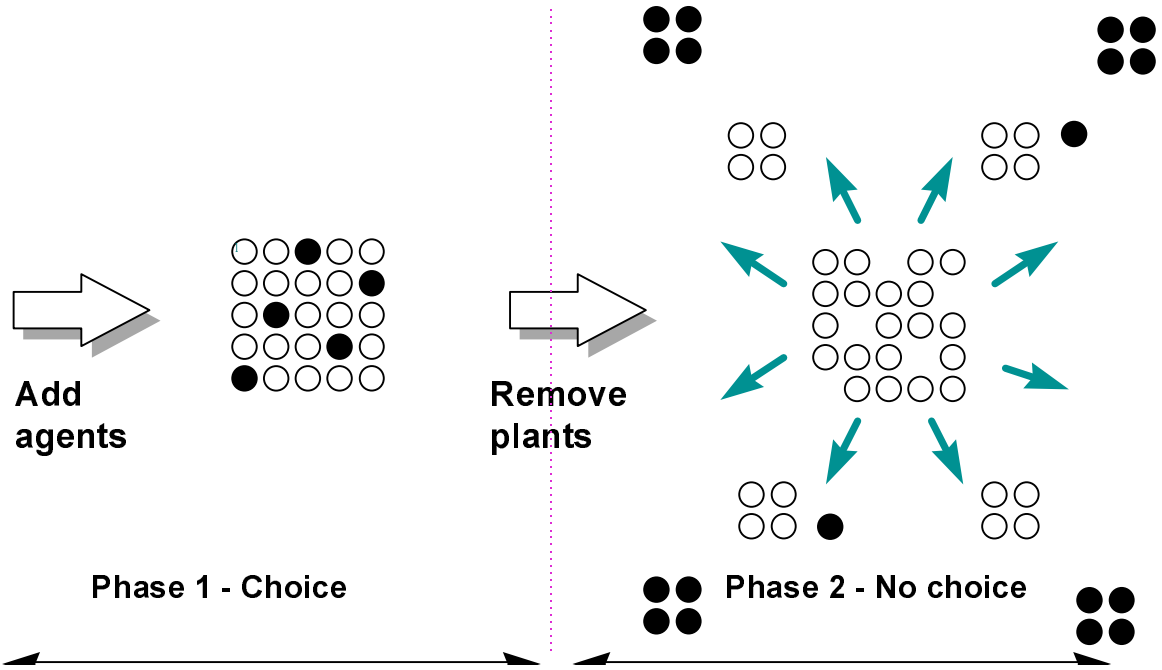

Fig. 1. Two-phase set design experiment which incorporates many of the features considered to improve the rigour of open-field tests (Black circles represent target plants and patterned light circles different test plants. Small groups in Phase 2 are satellite trap plots and arrows indicate possible emigration of agents once target plants are removed. See text for more detail).

In general, there is no universal best design for open-field tests. The pioneers of this form of testing have provided a box of design tools that can be used to customise any future test. This flexibility is needed to accommodate different agent biologies and to address particular questions concerning agent host-choice. As such, open-field tests should continue to form an important adjunct to the more traditional laboratory-based host-testing and, where anomalies exist, continue to reduce the chances of missing effective agents without compromising safety.

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Which test? A mini review of test usage in host specificity testing.

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Procedure in host specificity testing for the biological control of weeds is perceived as being anything from rigid and sacrosanct to being at the whim of the testing practitioner. In North America host specificity testing aimed at ensuring no risk to non-targets has been traditionally based on no-choice tests against all test plants. Elsewhere release permits for biocontrol agents are increasingly being issued on the basis of the results of oviposition tests. Field tests have broadened in role from a tool in host range refinement to an initial screening procedure. This paper surveys 147 host specificity studies and reviews them in relation to the order in which tests were used, the relationship between agent and test type and perceived problems associated with tests. Practitioners rarely adopted a procedure of starvation test followed by choice test followed by field test when defining host range. No particular test type or sequence dominated. The starvation test was the main test in 4.5 out of 7 invertebrate orders and in 50% of cases. Where starvation tests were not used, oviposition tests were the alternative when appropriate to the agent's biology. There was no evidence that problems associated with insect behaviour in choice tests affected any release decisions. A simple biology-based decision flow chart was generated which illustrates how initial test type selection can be made.

Introduction

Host specificity testing provides an objective mechanism for assessing the "maximum likelihood" host range for biological control agents, and such tests are necessary for obtaining a release permit into new countries of introduction. The no-choice feeding test (usually referred to as a starvation test in North America) aims to determine whether the agent can complete full reproductive development by feeding solely on a particular test plant. Used against an approved test plant list, this has provided a traditional bench mark and, at least in North America, is still the basis for assessing acceptance for release (Harris and Zwölfer 1968; Wan *et al.* 1996). It has long been stated that such no-choice tests are usually "inadequate" (Harris & Zwölfer 1968) by all too frequently defining a "physiological" host range (Cullen 1990), that is broader than the observed host range in the field. This is explained through disruption of natural behavioural processes of host selection, which are usually a complex sequence of 'sieves' for invertebrates (Harris & McEvoy 1995; Marohasy 1998; Withers & Barton Browne 1998) or results from likelihood of field exposure under suitable climatic conditions for pathogens (e.g. Hasan & Delfosse 1995). Host specificity testing of invertebrates has incorporated a suite of alternative host choice and substitution tests and incorporated spatial scale to tests ranging from single-leaf Petri dish tests to open field experiments on whole stands of test plants (e.g. Wan *et al.* 1996; Briese, this volume).

Nonetheless in the minds of most biocontrol workers worldwide, the no-choice feeding test with all its inadequacies still remains a standard test, applicable to all types of agent (Cullen 1990; Harris & McEvoy 1995).

There are good reasons for this. Such testing is quick and easy and agents from many genera will show sufficiently high specificity in these "maximum likelihood" tests. The test provides assurance that, however unlikely, non-targets will not be at risk of becoming hosts of introduced biocontrol agents. Does it necessarily follow, however that because no-choice feeding tests are a standard and frequent practise that they should be a requirement of all testing procedures? Being organised and consistent in approach may appear outwardly more scientific, but if, as a standard test, the results vary from total (e.g. Cordo *et al.* 1995; Sagliocco & Coupland 1995) to little semblance to true host range, how can the results provide a reliable baseline for comparing potential host ranges between species? Biologists interested in the mechanisms behind host selection have argued that understanding true host range requires a rigorous and consistent scientific process, i.e. setting a maximum likelihood host range by standard starvation tests and then refining this with increasingly complex test conditions (P. McEvoy, pers. comm.). To abandon no-choice feeding tests or indeed to start testing procedure with any other testing approach therefore compromises scientific rigour. Practitioners, however, have not consistently seen no-choice feeding tests as mandatory (e.g. Cullen 1990; Harley & Forno 1992) nor indeed have all agencies that authorise release permits.

This paper adopts a simple review of past practise to look at frequency and consistency in the use of the no-choice feeding test in host specificity studies for the biological control of weeds. It also reviews general test type usage in relation to agent taxonomy and biology and uses the results to propose a simple conceptual decision model that may be followed for selecting the initial test type.

Review of past practise

All articles in the CABI Abstracts on-line database which included the key words "specificity" and "weed" and described host specificity tests of weed biocontrol agents were searched, together with four unpublished studies. Agent family was recorded along with the test type(s) applied; as larval or adult, no-choice or choice, feeding or oviposition, laboratory or field. For categorical simplicity all feeding tests encountered which individually tested all plants in the test list and which lasted long enough to determine test plant acceptability were included in the "feeding test" category. The definition of "choice" in tests in the papers reviewed referred to either more than one plant species offered or availability of test species together with the target weed. The definition of choice test adopted here includes any combinations of test plant species offered concurrently as well as sequential tests, where agents are re-offered the target weed after the test plant. While it might be argued that such sequential tests have few biological affinities with tests involving several test plants at once, there was a need for large categories given the simple nature of this review. Conversely, no-choice tests are therefore defined here as all tests in which only one test plant species was available to candidate agents for the duration of the test and therefore included tests with a separate control group on the target species. All tests where agents were deliberately not exposed to the target weed prior to the test were ignored in the survey as they were very few in number and were always accompanied by a similar test including exposure to the target weed.

This literature survey was not directly concerned with issues of insect behaviour. If non-target plants received eggs during oviposition tests and development of resulting larvae was followed through only on this subset of test plants, a separate no-choice feeding test was not recorded. Field tests were accepted whether with or without the use of cages as this distinction did not affect the general conclusions of the survey.

This survey included 147 separate studies since 1971 from 20 journals involving 161 potential agents from 33 invertebrate families and 16 fungal pathogens. Unfortunately all studies were probably not complete i.e., did not include all the relevant test results required for the release permit, but clear preliminary studies were omitted. Nonetheless the survey provided information on the pattern of test types used for a given agent type. All pathogens were tested using no-choice procedures. Test type by invertebrate group is summarised in Table 1.

Agent type versus test type.

Generally the type of test adopted was strongly related to the biology of the agent (Table 1). There was no consensus to the order in which different test types were used. No-choice feeding tests were not more frequently the first test described in a study.

For Lepidoptera, no-choice feeding tests were standard. Where oviposition tests were used only in 5 out of the 13 cases did this generate narrower specificity and only in one case was specificity justified only from oviposition tests (Wapshere & Kirk 1977). In two cases field oviposition trials were used to support and refine laboratory-based studies and in both cases revealed a narrower host range (Hill *et al.* 1995; Anon. 1999).

For Coleoptera with internally feeding larvae (weevils, longhorn, seed feeders, flea beetles etc.), early studies and nearly all studies from North America used larval transfer no-choice feeding tests in combination with adult no-choice feeding tests. Most recent studies particularly for Australia and South Africa adopt adult oviposition tests in place of larval transfer no-choice feeding tests, making this the numerically dominant test for defining capacity to support complete agent development. Most of these tests have been of the sequential rather than simultaneous choice variety. In at least 19 cases oviposition and not no-choice feeding tests were applied to the complete test list and used to justify high specificity. Complimentary field tests were common for Coleoptera with internally feeding larvae (one fifth of studies) and was usually part and parcel of the whole test procedure: a clear indication that for this group, at least, such tests are now conducted whenever possible. In at least three cases, involving either flea beetles or weevils, field choice tests were used as part of the initial selection process (Dunn & Campobasso 1993; Briese *et al.* 1995; Sohbian *et al.* 1996).

For Coleoptera with externally feeding larvae (e.g. non-flea beetle chrysomelids), larval and adult no-choice feeding tests were standard. In one case involving a nitidulid, testing for release could be based entirely on adult oviposition choice tests as the larvae were incapable of moving between plants (Swirepik *et al.* 1996).

Among the Diptera (mainly tephritids and cecidomyiids), standard testing was almost entirely based on adult oviposition with sequential testing on the target being the main practice adopted. These have been backed up by field choice tests, in one case the field test being part of the initial selection process (Briese *et al.* 1995).

Table 1. Test types applied under laboratory and field conditions sorted by invertebrate group. Number of cases (=agent species) in parentheses. Most common test type used in bold. Feeding tests include all tests where survival and/or ability to complete reproductive development was assessed. Choice here is defined as between multiple plant species in time or space irrespective of the presence of the target weed. No-choice tests were all testing procedures in which agents were exposed to only one test plant for the duration of the test.

Invertebrate Group	Laboratory test type	Field test type
Lepidoptera (38)	**Larval feeding** **- no-choice** (36) - choice (1) Oviposition - choice (13)	**Oviposition** **- choice** (2) Larval feeding - no-choice (2)
Coleoptera – larvae internal (54)	**Adult feeding** **- no-choice** (34) - choice (16) **Oviposition** **- no-choice** (20) **- choice** (19) Larval feeding - no-choice (27) - choice (2)	**Adult feeding and oviposition** **- choice** (11) Adult feeding - no-choice (1)
Coleoptera – larvae external (17)	**Adult / larval feeding** **- no-choice** (17) - choice (4) Oviposition - no-choice (3) - choice (4)	**Adult feeding and oviposition** **- choice** (2)
Diptera (15)	**Oviposition** **- no-choice** (7) **- choice** (3) Larval feeding - no-choice (2)	**Oviposition** **- choice** (4)
Hemiptera (11)	**Adult/ larval feeding** **- no-choice** (11) Oviposition - no-choice (5) - choice (1)	**Adult feeding and oviposition** **- choice** (1)
Eriophyiidae (5)	**Adult / larval feeding** **No-choice** (5)	None in survey
Hymenoptera (3)	**Oviposition –** **Choice** (3)	None in survey
Thysanoptera (2)	**Adult / larval feeding** **no-choice** (2)	None in survey

For the Hemiptera, testing for population survival on the non-target has been standard practise. This effectively means trying to maintain a viable culture on the test plant (Day, this volume). Only once was a choice oviposition test conducted (Willson & Garcia 1992) and the results indicated a wider host range than did the population no-choice feeding tests. Five studies using mites were also all based on no-choice feeding tests, although one field choice

test has recently been carried out also indicating high specificity (Q. Paynter, pers. comm.). The two cases involving Hymenoptera both only used sequential choice tests for oviposition, while the two cases involving thrips both used no-choice feeding tests.

No-choice feeding tests

For Lepidoptera, Coleoptera with externally feeding larvae, Hemiptera, Eriophyiidae and Thysanoptera, the no-choice larval feeding test was the commonest procedure used to define whether development to maturity could be achieved on the test plant. Despite the obvious problems associated with such tests (Cullen 1990; Harris & McEvoy 1995), is this really likely to change? Where larval choice tests were occasionally used (Table 1), they did not reveal narrower specificity. It is not relevant to test only adults when larval stages have the capacity to actively or passively disperse or when adult behavioural problems of egg dumping or an overriding escape response (found commonly amongst the Lepidoptera) in cages cannot be overcome. In such cases, the only usual fall back option other than no-choice feeding test results that may fail to agree with phylogenetic or observational evidence for specificity, is a field test (see Briese, this volume). The low frequency of its usage, however, either suggests the no-choice feeding test is adequate or that field tests rarely provide an acceptable outcome, at least for the five groups for which the starvation test is used as a benchmark.

No-choice feeding tests of adults amongst the Coleoptera, Hemiptera, Thysanoptera and Eriophyiidae have a long history, but need not be mandatory prior to release where it is the larvae that are most damaging (e.g. Swirepik *et al.* 1996). The pre-release requirement of such tests should be limited to situations where the consequences of cosmetic feeding damage on non-targets may be serious. The no-choice feeding test provides assurance for the situation where large numbers of agents are present without their natural host (McFadyen 1998).

Oviposition-based testing

Testing of invertebrate agents, where adults select the host plant and larvae remain in or on it throughout development, has concentrated on tests associated with adult female oviposition. Testing of Diptera and the many such Coleopteran groups is increasingly based on oviposition tests. Studies of this agent type for North America still use the larval feeding test, due to a more traditional requirement there for this (Wan *et al.* 1996), elsewhere many agent release applications rely entirely on oviposition tests.

Marohasy (1998) and Withers & Barton Browne (1998) have recently argued that the approach to oviposition testing needs to be more scientific, because test conditions may lead to oviposition on non-hosts or avoidance of true hosts due to condition-related behavioural responses. No case was found in this survey in which the difference between the host range as indicated by caged oviposition and feeding tests, and host range as indicated by field evidence led to rejection of an agent. Nonetheless, caged studies of agents where field evidence is lacking, and where there are no controls for such effects, have the potential to generate false results. For example, a bruchid beetle (*Bruchidius villosus* F.) released into New Zealand for the control of Scotch broom (*Cytisus scoparius* (L.) Link) has been found ovipositing on *Chamaecytisus palmensis* (Christ) Bisby & Nicholls despite failing to oviposit on this species in caged choice oviposition tests (P. Syrett, pers. comm.). The two main behavioural effects likely to be generating false results are; a) habituation of agents to non-hosts in no-choice tests leading to their acceptance for oviposition (false positive), and b) acceptable hosts being

avoided through unresponsiveness in the presence of a strongly attractive host or repellent test plant (false negative) (Marohasy 1998).

The number of test plants that can be offered in any test will be dependent on their availability. Simple improvements in test rigour against such effects will come from running choice tests both with and without the target weed in parallel followed by careful interpretation of any differences found (see Marohasy 1998). Other testing procedures to counter these and other behavioural effects have been discussed elsewhere (Solarz & Newman 1996, Withers & Barton Browne 1998). Nine studies in this survey used oviposition or adult feeding trials in which two tests were used, one with and one without, the target weed. Host range indicated by results from these tests were compared to see if this provided evidence that habituation and unresponsiveness may be operating in caged host specificity tests. Differences in indicated host range from the two different test procedures were evident (Table 2). None of the differences observed, however, appeared to have affected the respective authors' conclusions on agent suitability for release (see references in Table 2). Clearly, testing procedures are generating false results, but the degree of error in most cases is not sufficient to influence whether or not agents are released.

Table 2. Results from studies where adult feeding or oviposition tests both with and without the target weed were used, showing the host range (as number of test species) as indicated by each test type. Refer to references for target weed and agent names.

Agent type	Total no. plants tested	Number of test plants per test		Cage size m^3	Host Range		Reference
		– target	+ target		- target	+ target	
Adult feeding							
flea beetle[b]	6	1	6	0.001	3	4	Syrett 1985
leaf beetle[c]	11	1	4	0.25	10	11	Wan *et al.* 1996
leaf beetle[c]	5	3	4	0.5	2	1	Marohasy 1994
weevil[b]	15	1	10	0.001	8	6	Kok *et al.* 1992[a]
weevil[d]	2	1	2	0.01	4	3	Fornasari *et al.* 1991
Oviposition							
tephritid[d]	4	1	2	0.001	3	2	Sohbian & Pittara 1988
longhorn[b]	45	1	2	0.75	12	12	Kirk & Wapshere 1979
leaf beetle[c]	5	3	4	0.5	1	1	Marohasy 1994
moth[c]	14	7	8	0.25	6	6	Hill *et al.* 1995
weevil[b]	15	1	10	0.001	2	3	Kok *et al.* 1992 [a]
weevil[b]	3	1	3	0.25	3	2	Kok *et al.* 1992 [a]

[a] target species used from this study was both *Lythrium virgatum* and *Lythrium salicaria* as both are natural hosts of the agent.
[b]internal feeder, [c]external leaf feeder, [d]flower head feeder.

Field tests

This mini survey in line with other reviews revealed that field-testing is an increasingly common practise (Clements & Cristofaro 1995, Heard & van Klinken 1998, Briese this volume), but only within certain invertebrate groups. A greater consideration of their use especially with Lepidoptera may provide further beneficial outcomes for problems associated with host specificity testing (see Briese, this volume). Field tests are rarely a cheap option and have largely, but not always, been adopted following laboratory studies which produced unexpected results. Field tests had significant value when there were only a few key test plants and they can allow several potential agents to be tested simultaneously. It makes economic sense to conduct a field test if the alternative requires several years' investment in the development of a rearing protocol before any testing could be initiated. Such problems are exacerbated when agents also need to be resynchronised between hemispheres. They may also be associated with a failure to successfully grow test plants to a condition suitable for rearing and testing agents under quarantine conditions. In such cases a field test in the native range may provide the only test option (e.g. Syrett & Emberson 1997).

Which test? - a simple guide

This survey has found links between the inherent biological characteristics associated with taxonomic groups and the testing procedure adopted. By ignoring taxonomic group and focussing on biological characteristics and other sources of information on specificity, Fig 1 illustrates a flow chart that generalises the decisions involved in the selection of an initial testing procedure (see Steps 1 & 2 below). Plant pathogens are not included simply because the passive nature of spore dispersal always requires no-choice inoculation tests.

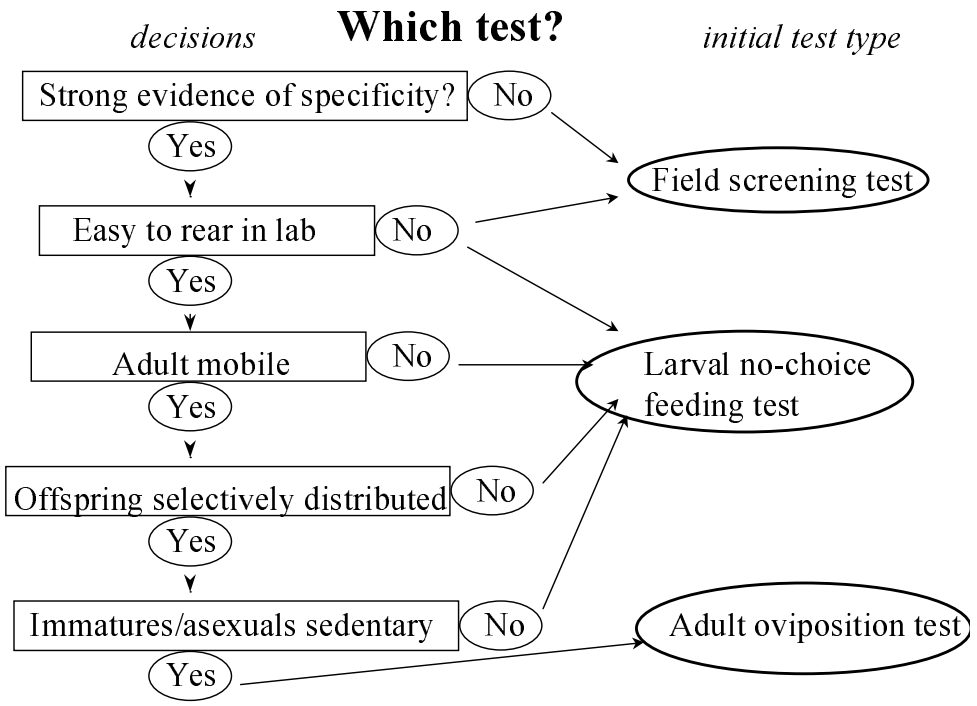

Fig 1. Flow chart generalising decisions involved in the selection of an initial host specificity testing procedure for the biological control of weeds.

Step 1: Gathering of field evidence (e.g. Syrett & Emberson 1997), together with phylogenetic considerations (e.g. Briese 1996) of feeding preferences, are the necessary first steps. Feeding preferences and specificity therein have very strong associations to taxonomy at all levels making this a vital tool to providing initial indications of specificity. Were this not the case, biological control agents would be coming from potentially all invertebrate orders. It would be risky and wasteful to import a candidate agent in the absence of such information, so the first step is to conduct some basic screening in the native range. To take advantage of the available natural conditions, initial screening studies should, if possible, be carried out in the field. For agents which are hard to mate or rear this may include screening the whole test list. If field studies are not possible then the testing environment becomes the quarantine glasshouse or laboratory.

Step 2: If adults are not mobile or the distribution of eggs/progeny by adults under laboratory conditions is unselective or if immature or asexual stages can move off their host plant then the larval no-choice feeding test must be the initial and dominant testing procedure to efficiently reject the majority or all test plants as potential hosts. If host choice (even under laboratory conditions) is made by the adult female and larval/asexual stages are sedentary then the initial and dominant testing procedure would be based on oviposition site selection by adult females. Suitable protocols for such testing are discussed in other contributions to this workshop.

Step 3: Results from specificity tests carried out in step 2 that fail to indicate adequate specificity would lead either to agent rejection or a second round of testing under more stringent or natural conditions. For agents where the first type of tests used were no-choice feeding tests (see Step 2), returning to the field testing environment to refine host specificity still presents the only option preventing agent rejection (see Briese this volume). For agents where the first type of tests used were oviposition tests there are two options for further testing. First, no-choice feeding tests used on the short list of test plants shown to be acceptable for oviposition. Second, field testing again offers the final option either directly after the oviposition tests or as a third round following the second round of no-choice feeding tests.

Conclusions

The results of current host specificity procedures have been sufficient to largely prevent unexplained or unpredictable and damaging host shifts in the history of the discipline (Marohasy 1996). Indeed there is no published scientific evidence of any off-target effects on the population dynamics of non-targets following agent releases for the biological control of weeds (Hopper 1998). Nonetheless this does not preclude the need to improve scientific rigour in such testing procedures. This survey of past practise shows that there is no standard testing procedure in host specificity testing, despite rigid adherence to no-choice feeding tests as a traditional standard in North America. It is argued here that for the purpose of ensuring minimum risk to non-targets (i.e. obtaining a release permit) having no standard test that can be applied to all potential agents irrespective of their biology does not add any inherent risk to weed biological control. The type of test applied is highly related to the biology of the agent and therefore choice of test is the result of some simple decisions based on this information and outlined here. Recent criticisms of scientific rigour in testing procedure need to be addressed and this can be done via simple modifications to current protocols. The use of field testing is also still in its infancy and under-utilised for some types of agents, such as Lepidoptera, which often appear quite oligophagous under laboratory conditions. The greater

use of properly designed field and oviposition testing should be encouraged in replacement of no-choice tests where possible.

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Parasitoid host specificity testing to predict field host range

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Pre-release host specificity testing of parasitoids proposed for biological control is discussed in the context of environmental safety and the trend towards more stringent regulatory requirements. The paper covers aspects of containment testing including standardisation of methodology, selection of non-target hosts for host specificity testing, options for experimental design and interpretation of results. Research into new methodologies for host range determination which involve separation of behavioural and physiological host-parasitoid compatibility are described. The importance of field verification of predictions made in pre-release tests in enhancing the value of laboratory testing is discussed.

Introduction

In contrast to the use of pesticides, organisms released in classical biological control programmes are self-perpetuating, self-dispersing and the release is normally considered irreversible. These attributes are seen as beneficial for cost effective pest management, but they are also factors that have alerted researchers to potential environmental risks. While it has often been stated that no adverse impacts from biological control releases have been demonstrated e.g. DeBach (1974); Caltagirone & Huffaker (1980); Onstad & McManus (1996), workers such as Howarth (1991); Howarth & Ramsay (1991); Cullen (1993); Samways (1994); Simberloff & Stiling (1996b) have pointed out that this lack of evidence can be attributed to a lack of study of effects rather than an absence of such impacts. Waage (1997) has also noted that, while there is little evidence for negative effects from biological control agent introductions, pre-release testing, when carried out, has rarely included non-target indigenous species.

In the USA, biological control practitioners still urge caution in establishing mandatory host range testing for entomophagous biological control agents, although consultation with insect conservationists is recommended (e.g. van Driesche & Hoddle 1997). However, changes in regulation and funding strategies may eventually bring about an integration of effort combining the interests of effective biological control with minimising unwanted impacts.

Parasitoid host specificity testing

Regulation of biological control agent introduction is required in the public interest because of its irreversibility (Harris 1990; Waage 1997), and the potential for biological control agents to disperse to habitats other than those where they were released (e.g. van Lenteren 1997). New Zealand has recently reformed legislation in this area with the introduction of the Hazardous Substances and New Organisms Act 1996 (HSNO) under which the Environmental Risk Management Authority (ERMA New Zealand 1997b) has been established. ERMA New Zealand have made it clear that the provision of information to support applications to introduce new organisms into New Zealand is the responsibility of the applicant (ERMA New Zealand 1997b), and that the outcome of the application will be to a large extent contingent on the quality of the information provided. In the case of biological control agents, one of the most difficult tasks faced by an applicant for a new biological control introduction will be supplying adequate information on potential impacts of the new organism in the environment.

Pre-release host specificity testing is carried out to assist in the prediction of the likely post-release field host range and impact of introduced biological control agents. Zwolfer (1971) has pointed out that strictly monophagous parasitoids are rare and in some taxonomic groups, non-existent. While it is often stated that a high degree of host specificity is desirable in the interests of minimising non-target effects, there is also a view that the availability of alternative hosts can be important for the success of biological control in the long-term (e.g. Nechols *et al.* 1992). These authors contended that release of biological control agents known to attack non-target species does not conflict with conservation objectives as long as the host preferences of the biological control agent are well known. Realistically, however, the decision by regulators to allow any biological control release is a compromise based on incomplete knowledge, and must be the result of a risk/benefit analysis. This in turn raises its own dilemma. The benefit is usually easy to determine in economic terms, for example, the value of the pest in terms of crop losses. In contrast, it is far more difficult to place an economic value on indigenous species, environmental integrity and biodiversity.

Simberloff & Stiling (1996a) have expressed the view that protocols for biological control agent host specificity testing as a prerequisite for release in the environment could be very much improved. A shift in emphasis could usefully occur amongst policy makers whereby biological control agents are considered 'guilty until proven innocent', a comment that has sparked further debate (Frank 1998; Simberloff & Stiling 1998).

Considerations in general methodology

The extremely varied nature of host-parasitoid relationships and the large number of taxa, precludes the establishment of a single prescriptive set of protocols for parasitoid-host specificity testing. Assuming that exploratory investigations have resulted in the selection of one or more candidate parasitoids that appear suitable, there are a number of factors that should be considered in the design of a series of tests:

Regulatory requirements. The requirements of the regulatory authority that will approve the application to import and release a proposed biological control agent clearly have to be taken into account, and the minimum standards required by them must be met. This is likely to include the standards required for the containment facility in which host specificity testing will be carried out, requisite biosecurity clearance, information on environmental safety, and economic risk/benefit data. While these requirements seem obvious, in fact detailed directives for meeting all aspects of the pre-release information standards are not usually specified because of the need for a case-by-case approach. Consultation with regulators about the investigation before initiating experimentation is advised by ERMA New Zealand (ERMA New Zealand 1997a), and required in Australia (Paton 1992).

Available information. Information on host range from the country of origin of the parasitoid, while seldom sought, can be indicative of the likely extent of the range of species which might be 'at risk' in the area proposed for release. Literature on the parasitoid and/or related species that have been used as biological control agents elsewhere may also provide some guidance on host specificity. However, apparent monophagy/oligophagy in the natural range of the parasitoid, or other areas where it has been released as a biological control agent cannot necessarily be relied upon. To give some examples, when the braconid *Microctonus aethiopoides* was introduced into New Zealand in 1982 to control the adult stage of the lucerne pest *Sitona discoideus* (Coleoptera: Curculionidae), it was thought to attack only two genera of weevils, *Sitona* and *Hypera* (Loan & Holdaway 1961; Loan 1975). Subsequent to its release in New Zealand, *M. aethiopoides* has been found parasitising weevils from four indigenous and four exotic genera in the field (Barratt *et al.* 1997b). Shaw (1988) suggested that braconid parasitoids from the subfamily Euphorinae are more likely to acquire new hosts when they occur in the same micro-habitat and have similar feeding habits. Similarly, the pteromalid *Pteromalus cereallae* previously considered a monophagous parasitoid of the grain moth *Sitotroga cerealella,* attacked and developed successfully in all 12 potential host species to which it was exposed in tests, which included species from four coleopteran families (Brower 1991). In contrast, however, *Cotesia rubecula* reported from Europe to be 'almost specific' to white butterfly (*Pieris rapae*) was tested in containment in New Zealand and failed to develop in all nine non-target species to which it was exposed, and post-release field studies have confirmed host specificity (Cameron & Walker 1997). Hawkins & Marino (1997) analysed biological and ecological variables of parasitoids which have acquired native hosts in North America to determine whether it was possible to predict whether or not an exotic parasitoid will attack native species, and concluded that there are no 'rules of thumb' to assist in such predictions.

Quality of test insects. Reliable and repeatable results in host-specificity evaluations can only be achieved only if the parasitoids and potential host insects used in the tests are sourced from vigorous and disease-free colonies. Often containment protocols for rearing parasitoids and the target host are well developed, but not for the non-target species. Depending upon the taxa and stages of development required, it may be necessary to collect non-target species from field populations for testing, or it may be possible to develop a laboratory colony. The latter has the advantages of convenience and availability of parasitoid-free insects as and when required, but the development

of 'laboratory strains' which behave abnormally may occur if they are kept in culture for a large number of generations. Non-targets collected and used straight from field populations need to be disease-free, cleared of natural parasitism to avoid confusion in tests, and be maintained on a suitable diet (e.g. Grainger 1995). Survival, feeding activity, larval development and fecundity of adult stages can be used as indicators of vigour of the insects in the laboratory (Evans & Barratt 1995).

Selection of species for host specificity testing

The choice of hosts for parasitoid host specificity testing depends upon the objectives of the programme. If the intention is to meet minimum requirements of the regulating authority, then it may be that only non-target species that are on an endangered species list, and/or beneficial species need to be considered. However, if the purpose of testing is to anticipate adverse environmental, as well as economic and social impacts of parasitoid release, then the selection of non-target species requires more careful consideration. The difficulties of risk assessment of entomophagous biological control agents compared with those considered for weed control have been outlined (Barratt *et al.* 1999b). However, given certain limitations, a similar process using phylogenetic and ecological affiliations between target hosts and non-target species can be applied to some extent with parasitoids, although other factors may also be significant. For example, Duan & Messing (1997) were able to select non-target species for testing against braconid biocontrol agents for fruit fly, based on studies which indicated that only tephritid flies that live in fruit are attacked by ophiine braconids, and that fruit shape, size and colour are essential stimuli to host recognition.

Some general factors to be considered in selecting test species for host specificity testing have been discussed elsewhere in detail (e.g. Goldson & Phillips 1990; Goldson *et al.* 1992; Barratt 1997; Goldson *et al.* 1998; Barratt *et al.* 1999b). However, in brief, a possible process may be to:

1. Examine phylogenetic affinities between the target host and non-target species and rank, as far as knowledge permits, species/genera from those closely related to those distantly related.

2. Examine ecological affinities between native fauna and target hosts by listing species that occupy a similar niche or feed on related plant species e.g., leaf miners, seed feeders, grassland dwellers, canopy feeders etc. irrespective of phylogenetic affinities (e.g. Neale *et al.* 1995).
3. Investigate the extent to which potential non-target species and target hosts occur in mixed populations and use this as a guide to taxa most immediately at risk. This may involve field survey (e.g. Barratt *et al.* 1998).

4. Use points 1-3 to prioritise the list of test species and expose those considered most 'at risk' to parasitoids in laboratory tests. Include beneficial species in a priority list.

5. Evaluate the results of initial tests to determine the need for further testing.

The number of species which should be tested needs to be decided on a case-by-case basis, depending on the length of the 'at risk' list, the availability of test insects, the number of positive results in the initial set of tests, and regulatory requirements. Clearly, knowledge of the non-target fauna, and resources and objectives of the programme will dictate the extent to which such a process can be followed.

Standardisation of experimental methods

Normally a complete series of host specificity tests for a particular parasitoid cannot be undertaken simultaneously, and so to ensure that results are comparable, test protocols need to be standardised. However, biologically appropriate standardisation of methods is clearly essential and experimental design requires good knowledge of the host/parasitoid relationship being invesigated. Criteria to be considered include factors such as:

Cage environment. Similar structured containers ensure that the volume and surface conditions in which host activity and host location by the parasitoid take place are similar for all tests. The size of the container in which adult weevil hosts were exposed to parasitoids (*Microctonus* spp.) was found to influence resulting levels of parasitism (M. McNeill, unpublished). Other considerations include the effect that 'patch dynamics' may have on host location behaviour of the particular parasitoid and the selection of an appropriate substrate on which to present non-target species. Many factors influence the length of time a parasitoid will search in a patch i.e., physical structure, chemical stimuli, encounters with unparasitised hosts, encounters with parasitised hosts, encounters with each other (van Alphen & Vet 1986).

Host-parasitoid ratio and exposure period. The number of hosts available to a given number of parasitoids and exposure time will clearly influence the levels of parasitism achieved, not necessarily in a linear fashion. Barratt *et al.* (1996) exposed cages of 20 field collected weevils to 0, 1, 3 or 6 parasitoids for 0, 1, 12 or 48 hours, and found that increasing parasitoid number, and to a greater extent, exposure period of parasitoids to their hosts increased parasitism levels. Varying host-parasitoid ratios in tests with target hosts can establish a standard which can be compared with parasitism rates with non-target species. It is important to be aware that interactions between parasitoids can influence their behaviour in cage experiments. For example, some female parasitoids avoid odour trails left by others (Rogers & Hassell 1974), so that too large a number of parasitoids could interfere with parasitism behaviour. High parasitoid-host ratios leading to superparasitism, and resultant premature mortality was observed with the braconid parasitoid *M. aethiopoides* exposed to alfalfa weevil, *Hypera postica* in the laboratory (Neal 1970).

Food plants. It is important to provide consistent quantities, quality and species of food plants in the test containers, with a standard food replenishment regime to avoid varied food plant odours in different cages confounding results. For example, in host range tests with the braconid *M. aethiopoides*, the target host feeds on lucerne (*Medicago sativa*) whereas most of the non-target species tested required a white clover/ryegrass mixture. Consequently, when host specificity tests were carried out, all three plant species were provided for each test species (target and non-target) in no-choice tests for the parasitoid exposure period, so that as far as possible, similar

odours were present in all cages. Some parasitoids rely extensively on host and/or host food plant odour or host frass for host location and oviposition success (Vet *et al.* 1990; Duan & Messing 1997), and so this would need to be taken into consideration when designing tests. Providing a food source for the parasitoids themselves can also be important (Lewis *et al.* 1998) and may influence longevity and fecundity (Phillips 1998). The artificial conditions of caging place limitations on reproducing a 'natural' environment, but if conditions are standardised such that the target host is effectively parasitised in the test environment, then the likelihood of achieving comparable results with test species is enhanced.

Environmental conditions. Temperature, humidity, and photoperiod clearly should be suited to the parasitoid under consideration, and uniform for all tests which are to be compared.

Physiological state of test species and parasitoids. It is important to understand the host/parasitoid relationship before deciding how to standardise most appropriately the age, stage of reproductive maturity, feeding experience, previous oviposition experience etc. of both parasitoids and test species used in tests, so that host location behaviour and oviposition effort will be comparable, and preferably maximised. Since host behaviour and availability for parasitism is also a possible variable in relation to age, physiological state, previous history and treatment, uniformity can help to eliminate such sources of inconsistency. For example, in the braconid *M. aethiopoides* ovipositor insertion in adult weevils is facilitated if the host is active. In weevils that are in diapause or aestivation, the level of activity is reduced, and consequently wasps may have fewer opportunities to attempt oviposition (Phillips 1996). Similarly, prolonged storage of hosts can modify behaviour reducing attractiveness of hosts to parasitoids (Fusco & Hower 1973). Furthermore, some parasitoids undergo sympathetic diapause with their hosts, so if parasitism does occur, development times may be considerably protracted.

Measurements, records and analysis. The result required from host specificity tests is usually the level of parasitism achieved by the parasitoid in a group of non-target species in comparison with that achieved in the target host. These data are frequently obtained by maintaining test species until parasitoid emergence occurs, so that an estimate of successful parasitism can be made. However, dissection of surviving test species, and those which died during the maintenance period provides additional information such as incidence of unsuccessful parasitism, including encapsulation as a result of a host immune response. Recording information such as condition of the reproductive system, mature egg load, presence of food in the gut, can indicate effects of pseudoparasitism. This is a term coined by Jones *et al.* (1986) for incomplete parasitoid attack, or stinging of hosts without oviposition, which may be responsible for reduced host survival (Goldson *et al.* 1993) or physiological changes such as reductions in fecundity and feeding activity (Barratt *et al.* 1996). Incidence of host feeding is another important measure of impacts of parasitoids on hosts in addition to parasitism, which can also be influenced by host fitness (McGregor 1997).

Such information has potential value in predicting sub-lethal impacts of field parasitism. A record of sex ratio of parasitoid offspring can also provide a useful indication of the suitability of test conditions and quality of alternative hosts. For

example, factors such as temperature, humidity and photoperiod, female parasitoid age, density and diet, and host size, sex and density can affect parasitoid sex ratios (King 1987). Recording developmental periods for the parasitoid in target versus non-target hosts can be useful in interpreting host suitability (Goldson *et al.* 1992).

Experimental design

Replication and control. As noted above, the design of experiments for parasitoid host specificity testing is dictated by the host/parasitoid system under investigation. In general, replication which gives an acceptable level of variability needs to be determined in advance of the testing programme. Including replicate cages containing the parasitoid exposed to the target host species, each time a host specificity test with non-target species is conducted, provides confidence that the parasitoid is behaving as expected (Barratt *et al.* 1997b; Duan & Messing 1997). Addition of control cages containing hosts unexposed to parasitoids provides a standard by which host survival, feeding, fecundity, growth etc. can be compared (Barratt *et al.* 1997b).

Choice versus no-choice tests. The decision to use choice or no-choice experiments depends upon the intent of the test, and both methods used in combination can be useful. In no-choice tests, the main objective is usually to determine the range of species in which a parasitoid can or cannot successfully develop by maximising the likelihood of attack. A choice test where one or more non-target species is available in conjunction with the target host can give additional information about parasitoid host preferences (Duan & Messing 1997) and help to determine whether the behavioural threshold for acceptance of an alternative host can be changed in the presence of a known preferred host. For example, *M. aethiopoides* achieved a higher level of parasitism in the non-target weevil *Sitona lepidus* in the presence of the target host, *S. discoideus,* than when only *S. lepidus* was present and parasitism was very low (Barratt *et al.* 1997a). Although the two host species would rarely be present in mixed populations in the field, the choice test helped determine whether *M. aethiopoides* was indeed capable of parasitising *S. lepidus*. In contrast, in the absence of its natural host, a fruit fly parasitoid, *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae), was found to search more microhabitats of non-target species, than when in their presence (Duan & Messing 1997).

Sequential testing. In a programme investigating biological control of fire ants in Texas, a sequential system of exposure of phorids (Diptera: Phoridae) to target and non-target ants was used (Gilbert & Morrison 1997). A single phorid was exposed to a group of the target species, then a group of a non-target species, and finally re-exposed to the target species. The sequential exposure regime was used as a method of avoiding olfactory confusion that might arise in choice tests where the presence of the target might stimulate parasitism of a non-target species which would not normally be attacked. Re-exposure to the target host in this test was intended to determine whether phorids were still motivated to attack. When single females of *M. aethiopoides* reared from the target host, *S. discoideus* were given access to a non-target weevil species, *Nicaeana cervina,* or to *S. discoideus* for 12 h and then to a choice of *S. discoideus* plus *N. cervina* for 36 h, wasps previously exposed to *N. cervina* were significantly more likely to attack *N. cervina* than the target host *S. discoideus*. Similarly, those exposed to *S. discoideus* showed a preference to attack *S. discoideus* during the second exposure (A. Cresswell, 1999). This occurred whether or

not successful oviposition was achieved in the first exposure, although attack rates were higher when a successful attack had occurred previously. Results from sequential tests, and indeed any host specificity tests can, therefore, be confounded by previous experience of the parasitoid, whether this is a result of previous oviposition experience or exposure to potential host species.

Interpretation of results

If in no-choice or choice tests, a parasitoid fails to attack a non-target species (as determined by dissection as well as adult emergence) yet attacks the target host successfully, then some confidence that the test species is not a suitable host can be derived from the result. Dissection of surviving non-target species can establish whether parasitoid oviposition occurred but larval development failed, however, finding evidence of this if the insects have been reared for a period after parasitoid exposure may not always be possible. Dissection of a sub-set of tested species immediately after the parasitoid exposure period may improve the chances of identifying a host immune response. A 'negative' parasitism result also requires consideration of sub-lethal effects, or physiological effects of pseudoparasitism as discussed above.

A suggestion that encapsulation may be a useful indicator of parasitoid host range was tested in two closely related endoparasitoids of larval Lepidoptera, one generalist and one specialist parasitoid, but the reliability of encapsulation as an indicator of host range was not supported by the results (Brodeur & Vet 1995). However, encapsulation of *M. aethiopoides* (polyphagous) in comparison with *Microctonus hyperodae* (oligophagous) when exposed to a similar range of non-target weevil species averaged 12% and 32% respectively for individuals of all species tested, indicative of successful levels of parasitism in the laboratory and in the field (Barratt *et al.* 1997b; Barratt *et al.* 1999b). It should be recognised, however, that even if a parasitoid fails to develop successfully in a non-target species, adverse impacts on this insect may be manifest in terms of reduced reproductive output or sterility in adult insects, and in reduced feeding activity (Barratt *et al.* 1996) which could reduce survival in the field. Consequently, measurement of percent parasitism as an index of parasitoid impact can be misleading in the absence of life table analysis (van Driesche 1983; van Driesche *et al.* 1991).

Pseudoparasitism cannot be dismissed as being of no consequence since it may result in host sterilisation. Munster Swendsen (1994) demonstrated that pseudoparasitism of a larval tortricid by braconid parasitoids reduced the size and fertility of the hosts in the adult stage. Brown and Kainoh (1992) found that pseudoparasitism of lepidopteran eggs by a braconid parasitoid prevented the initiation of gonad development in the developing larvae. Goldson *et al.* (1993) concluded that possible pseudoparasitism reduced survival of the weevil *Listronotus bonariensis* following contact with *M. hyperodae* which had not resulted in parasitism. This might also have explained an 8% mortality of unparasitised *S. discoideus* and 11% of the native *N. cervina* which occurred after exposure to *M. aethiopoides* in the absence of detectable oviposition (Evans 1997).

Extended developmental periods for parasitoids in non-target hosts has been used as an indication of host suitability, for example Goldson *et al.* (1992) found that total development times for the immature stages of *M. hyperodae* in non-target weevil species was increased by up to 27% in comparison with that in the target host *L. bonariensis*.

New initiatives

Some new options for host specificity testing are being investigated. One of the problems acknowledged by biological control practitioners is that host-specificity testing in containment often over-estimates host range because of the artificial testing environment (Goldson *et al.* 1992; Sands 1993; Secord & Kareiva 1996). Parasitoids attack species in the laboratory, which they would not attack in the field, and conversely, in caged conditions, a parasitoid might not be able to use its normal host location mechanisms, and parasitism may not occur when it would in the field. Furthermore, behavioural conditioning or adaptation may occur post-release, so that parasitoids could expand or change their range of hosts to some which would not have been attacked in pre-release tests, despite being physiologically compatible. For example, *M. aethiopoides* was tested in quarantine against the weed biological control agent, *Rhinocyllus conicus* in 1982 and there was no recorded parasitism from rearing or dissection of weevils (Stufkens, pers. comm.). However, in 1994, a specimen of *R. conicus* parasitised by *M. aethiopoides* in the field was recorded (Ferguson *et al.* 1994), and since then parasitism levels of up to 16% have been recorded in field populations in restricted localities in New Zealand (Ferguson *et al.* 1998).

An attempt is being made to develop methods to separate behavioural from physiological host-parasitoid compatibility to help interpret results of parasitoid-host exposure tests. It was considered useful to establish whether a parasitoid is capable of host immunosuppression, even if behaviourally it is not stimulated to attack, and conversely, whether an oviposition attempt has been made in a non-target species, but has failed, or the egg has been encapsulated or destroyed by the insect.

Experiments have been carried out using the bacterial entomopathogen, *Serratia marsescens,* as a marker in conjunction with parasitoid adults to determine whether ovipositor insertion into a test insect has occurred. Rapid mortality results from injection of the bacterium into the haemocoel of the host during ovipositor insertion. This technique has proved successful in determining that while successful parasitism of *S. lepidus* by *M. aethiopoides* rarely occurs, parasitism attempts are frequent, indicating that there is no behavioural inhibition of the parasitoid by this species, but that an effective host immune response occurs (M. McNeill, B. Barratt & A. Evans, unpublished data).

Studies on the mechanism of host immunosuppression, which could be used to assist in host range prediction are also in progress. Some braconids and ichneumonids disrupt the host immune system of their hosts by injecting with the egg a polydnavirus which prevents encapsulation (Stoltz *et al.* 1984). An ultrastructural investigation of *M. aethiopoides*, which has a broad host range in New Zealand, has shown that it has associated with the ovarial epithelium, a virus-like particle (VLP) superficially similar to polydnavirus (Barratt *et al.* 1999a). Similar VLPs have not been found in *M.*

hyperodae, and the New Zealand native *Microctonus zealandicus*, which have narrow host ranges. Further studies will investigate methods by which physiological compatibility between host and parasitoid can be determined and whether this could be a useful additional tool in host specificity testing which can give an objective indication of physiological host range independent of the inherent problems of cage testing or behavioural inhibitions.

Field verification and monitoring

It is well accepted that post-release verification of predictions made from host specificity testing in containment is a valuable means by which future predictions can be improved (Blossey 1995; Strand & Obrycki 1996; McEvoy 1996; Cullen 1997; van Driesche & Hoddle 1997; Barratt *et al.* 1997b; Thomas & Willis 1998; Goldson *et al.* 1998). If establishment and impact of a released parasitoid on the target host is to be investigated, then the opportunity to monitor impacts on non-target species can also be taken. Only by accumulating such information on a number of host/parasitoid models can the value of laboratory testing be enhanced. When comparing the laboratory and field host ranges of *M. aethiopoides* and *M. hyperodae*, it was concluded that the former were indeed indicative of field host range of these parasitoids (Barratt *et al.* 1997b).

Conclusions

Host specificity testing, appropriately designed and standardised for the particular host/parasitoid interaction, and with carefully reasoned selection of non-target test species (including consideration of phylogenetic, ecological and behavioural affinities) is likely to give a reasonable indication of the extent of the post-release host range in most cases. The likelihood of host range extension of a parasitoid post-release, establishment in unexpected environments, and competition leading to displacement of native parasitoids are not easily predicted from quarantine tests. A combination of no-choice and choice tests can in some cases provide additional information which regulators might use to facilitate decision support. Novel techniques for determining behavioural acceptance and physiological compatability of parasitoid – host relationships may in the future assist in prediction of post-release impacts. However, the development of a database of case histories where pre-release testing has been validated by post-release studies is probably one of the best means by which laboratory host specificity methodology can be assessed, and improved in the future.

The question of increased financial cost of biological control programmes that would result from a requirement to undergo more extensive pre-release host specificity testing has often been raised (e.g. Hopper 1995). While this would be inevitable, increased costs of weed biological control introductions because of mandatory host specificity testing has been associated with a higher success rate post-release (Ehler 1990). Inevitably, environmental, social and regulatory requirements will increasingly dictate that biological control programmes will be more expensive and slower to implement, but the success rate and environmental safety of programmes should consequently increase. Ideally, research teams should be assembled to address target and non-target impacts of biological control agents so that predictions can be made

with more confidence, and realistic risk/benefit analyses can be made for the consideration of regulatory agencies.

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Understanding host selection behaviour: the key to more effective host specificity testing

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Host selection in herbivores and parasitoids can be divided into a hierarchical series of steps commencing with locating host habitats, locating hosts, accepting hosts and, in some cases, regulating hosts. The suitability of acceptable hosts is the last step in the hierarchy that determines host range. The term suitability refers to both the provision of nutrients and the ability of the natural enemy to overcome host defences during development. Prey selection fundamentally follows the same hierarchical process. Most host specificity testing of biological control agents has focused on host acceptance and suitability but, as this considers only part of the host selection process, it could incorrectly indicate a wider host range in the field following release and establishment. A more comprehensive evaluation of host selection that includes host-locating and habitat-locating behaviour should lead to more effective specificity testing. It would avoid exclusion of valuable agents that fail less comprehensive tests of specificity. To achieve this, careful attention must be given to the abiotic and biotic conditions of the bioassay arena and to the state of the test organisms.

Introduction

The specificity of natural enemies has emerged as a key concern in biological control. Agents that specifically attack only the target pest should provide the greatest degree of assurance against undesirable effects on non-target organisms. These effects can include direct damage to crops and indigenous flora and fauna, among other potential problems (Howarth 1991). Moreover, the prospects for effective control appear to be greater when there is a tight linkage between the populations of the natural enemy and the target pest which arises from the specificity of the enemy to the pest. Thus it is important to be able to evaluate experimentally the specificity of an exotic natural enemy before releasing it into a novel environment.

This chapter focuses on the evaluation of the specificity of arthropods used in biological control. Their specificity arises from behavioural, and in most instances physiological, adaptations which limit the range of organisms that they will attack and utilise as hosts or prey. Behavioural adaptation precedes physiological adaptation in the evolution of specificity (Futuyma & Moreno 1988), so understanding the behavioural basis of host specificity is important. The primary focus here is on broadening the evaluation of behaviour in specificity testing. In a recent review,

Marohasy (1998) broadly discussed the evaluation of the specificity of arthropods with a focus on herbivores used in the biological control of weeds, and readers should consult this review to obtain a broader perspective of the topic. Special attention is given here to the conditions of specificity assays, and a case study of host selection by a parasitic wasp is presented to illustrate the potential for expanded consideration of behaviour in host specificity testing.

Host selection

Host selection in arthropods is thought to involve a hierarchy of behavioural activities and decisions (Vinson 1984; Miller & Strickler 1984). Salt (1935) first postulated a division between ecological and psychological components of the host selection of parasitoids, which broadly describe searching and accepting hosts, respectively. This conceptualisation of host selection by parasitoids has evolved through the contributions of other authors (e.g. Laing 1937, Doutt 1959; 1964, Vinson 1976; 1984). Today a description of this hierarchy commonly includes four levels: locating habitats, locating hosts, accepting hosts, and regulating hosts. The first three steps in this hierarchy involve behavioural decisions. The last is predominantly physiological and reflects the dynamic interaction between parasitoid and host. The nutritional status of the host and its ability to defend against attack combine to influence the development of the parasitoid. As such the final step in the host selection hierarchy may more correctly be described as "utilising and regulating hosts."

The host selection hierarchy is essentially a filtering process. At each level in the hierarchy, the range of hosts that is potentially attacked is reduced. The filtering process is qualitative at each level, rather than resembling a series of increasingly fine sieves. This point is important because evaluation of the final step in the process, successful development on a range of hosts, is not necessarily reflective of the true host range. Earlier steps may be more restrictive than the last. In some instances the true host range might be estimated only through development of a comprehensive understanding of the hierarchy of host selection, however difficult that may be.

The host selection behaviour of parasitoids is not generally fixed. The experience of the individual during its lifetime can influence which cues it utilises while searching and the levels of response it will display to the variety of cues it perceives (Vet *et al.* 1990). It is not surprising that this is so, since parasitoids exert strong selection pressure on their hosts to avoid detection and, if detected, to avoid attack (Vet & Dicke 1992). Foraging parasitoids are faced with a spectrum of cues that are easily detectable in the habitats of hosts, but which do not reliably indicate host location or even presence. The odour from a damaged host plant is one such cue. Conversely, any cues that reliably indicate the location and identity of the host are selected against such that they will over time become difficult to detect. Some parasitoids cope with this reliability-detectability trade-off by learning to associate detectable cues with hosts and thereby increasing the efficiency of their foraging (Vet & Dicke 1992). For example, positive oviposition experience on certain host plants can increase the subsequent attractiveness of those plants, as well as the intensity of search and search times on them. As such parasitoid experience can affect the outcome of host specificity testing and testing of naïve individuals is preferred. If host specificity testing is to consider a broader range of host selection behaviour, then such testing

must be undertaken with an appreciation of the flexibility of behaviour and the factors that influence its expression, including the role of learning (Vet & Dicke 1992).

It is important to note that the hierarchy of host selection in parasitoids applies in principle to both predators and herbivores (Miller & Strickler 1984). Whether actively or passively, the behaviour of arthropods determines which habitats they will inhabit and which organisms are susceptible to attack within these habitats. Once an organism is attacked, its nutritional status and chemical defenses influence the ultimate outcome.

Host specificity testing

It is the later steps of host and prey selection that have been the primary focus of host specificity testing, with limited attention having been given to evaluation of how arthropods locate habitats and hosts or prey. However, greater attention could be given to evaluation of the entire hierarchy of searching behaviour of arthropods when evaluating their specificity. If this aspect of their biology is ignored, then a distorted picture of their specificity may emerge. The only possible consequence of narrowly considering host selection at the final stages of the hierarchy is to attribute a host or prey range that is broader than would occur in the field. Some may argue that this would give greater safety to biological control, but the danger is that it will unnecessarily limit the range of species available for release. What is needed is a testing methodology that more fully evaluates host and prey specificity without relaxing the stringencies necessary for environmental safety.

An understanding of the host selection hierarchy could be used in host specificity testing in two ways. Firstly, evaluation of the pre-alighting behaviour of flying species or the arrestment behaviour of non-flying species in the presence and absence of hosts or prey should be the first step in the evaluation of host specificity. This will indicate locations and conditions where hosts or prey are likely to be attacked. For herbivores, no-choice developmental trials should only be conducted with those species on which it can be shown that a healthy natural enemy will oviposit. Where this advice has not been followed, the apparent host range shown by rearing insects placed on potential hosts may appear unacceptably large to allow release. In such instances moving back up the hierarchy to earlier steps may show that other behavioural responses to hosts would prevent consumption of some species.

The second way involves biological control directed against arthropods. It is impractical to test a broad range of potential hosts (Sands 1997), so Sands & Papacek (1993) have suggested that only a short list of species closely related taxonomically to the target pest should be "carefully selected" and tested. Selection of species for testing could be made through elucidation of the enemy's host selection behaviour. The aim of this testing should be the determination of which species would most likely be encountered by a searching natural enemy. To achieve this, behavioural assays could be used to assess the hierarchy of host selection cues that determine which habitat niches are likely to be searched, and these data could be used to limit further testing to those species that occur in such niches.

Habitat selection is poorly understood. It probably involves responses to both physical and biotic cues and, in many instances, may be due to reduced a tendency to leave favourable habitats rather than actively searching for them. Much more research on this aspect of host selection is needed (Rosenzweig 1991), but practical problems associated with measuring the movement of small organisms over potentially long distances make this an especially difficult topic of study. Therefore it is unlikely that habitat selection will become a routine part of specificity testing in the near future.

Even in the absence of direct behavioural evaluation, habitat use can be assessed indirectly by examining the frequency with which potential hosts are attacked when they are placed in different habitats. Walter *et al.* (1998) used this approach to demonstrate that an exotic predatory mite, *Phytoseiulus persimilis*, does not invade subtropical rainforest in eastern Australia, so potential prey species in this habitat are not threatened by this predatory mite. Such studies could be conducted in the country of origin to determine which habitats are most likely to be invaded by candidate biological control agents before they are introduced into new locations (Nechols *et al.* 1992).

Behavioural mechanisms and environmental cues

If assays of specificity are to incorporate a greater range of behaviour, then it is essential to understand the factors that influence behavioural decisions and provide an appropriate environment. Otherwise behaviour observed in experiments may not mimic what is observed in the field. The danger of observing behavioural artifacts is illustrated by two examples. Many moths will readily oviposit inside plastic bags in the absence of any cues from host plants (Peterson 1960), yet they locate and oviposit on selected host plants in the field. Likewise, the parasitic wasps are often thought to be relatively specific, but the *Cotesia rubecula* will fly to unusual plants if not given a choice when flying in a wind tunnel (Agelopoulos & Keller 1994). Such seemingly aberrant behaviour can be understood by considering the rolling fulcrum model of Miller & Strickler (1984). The entire spectrum of internal and external factors that either stimulate or inhibit behaviour combine to determine whether it will be expressed at any given time. Thus in the case of the moths in plastic bags approaching the end of their life, even in the absence of a host plant oviposition may give their eggs a slim chance of survival compared to the alternative of certain death without reproducing. For *C. rubecula*, the odours generally associated with green plants appear to provide sufficient positive stimulation to attract females in the absence of other more attractive alternatives or strong negative stimuli.

Careful attention should be given to the physical and biotic conditions of the bioassay. Three factors are given special attention here, the movement of air, lighting and plant nutrition. Most arthropods use chemical cues as primary sources of information when searching for resources like feeding and oviposition sites. Navigation in response to volatile chemicals involves anemotaxis, i.e. the insect turns and moves upwind in response to perception of an attractive odour (Cardé 1984). Moving air is crucial to this behaviour. Many laboratory arenas in which specificity assays are conducted (e.g. screened cages) have no provision for moving air, so normal searching behaviour is not possible. This problem can be overcome by conducting choice assays in the field, in a wind tunnel (Keller 1990), or at least in cages adjacent to fans where some

movement of air is possible. A wind tunnel need not be sophisticated. A crude wind tunnel without any filters was constructed from a domestic box fan and has been used effectively in several experiments (e.g. Salehi 1998). A similar arrangement has been used to study the behaviour of parasitoids in glasshouses (Steinberg *et al.* 1992).

Air movement should not be assumed within enclosures, because screens can substantially reduce or virtually eliminate it. Low velocity air movement should preferably be measured with a hot wire anemometer. Air movement can be easily visualised with smoke, but the heat from burning can give a false impression of the path of odour movement. Chemical smoke which has a neutral density overcomes this problem (Lindgren *et al.* 1984). Equal parts of ethylene diamine and acetic acid applied as single drops to a cotton swab can produce a smoke which is easily visualised to assess air movement. Drops of titanium tetrachloride can also be used for this purpose. It reacts with water in the air to produce smoke. In both cases, the resulting smoke can cause respiratory irritation, so appropriate protective masks should be worn when this smoke is produced indoors.

Many insects behave in an apparently normal manner under a wide range of lighting conditions (M. Keller, unpublished data). However, some insects (e.g. butterflies and flies) appear to rely on visual stimuli to a greater extent than other insects. For them, lighting can have a significant influence on behaviour (Shields 1989). For example, a culture of the pierid butterfly *Pieris rapae* did not produce many eggs on overcast days until lighting conditions were appropriate (M. Keller, unpublished data). In this case lighting was provided by two fluorescent globes (True Light Power Twist, Duro Light Corp., Fairfield, NJ, USA) which provide virtually the same spectrum as sunlight and the lamps were powered by solid state ballasts which flicker at 30 kHz (Helvar, Finland). This high frequency flickering is invisible even to the insect eye (Shields 1989). Similar solid state ballasts should be used whenever flourescent lighting is provided.

Apart from the physical environment, the condition of plants also has the potential to influence the outcome of specificity testing of herbivores, and perhaps parasitoids. It is well known that the nutritional quality of plants can affect their suitability for herbivores (Scriber & Slansky 1981, White 1993). However, the nutritional status of plants can also affect oviposition by herbivores. For example, the moth *Cactoblastis cactorum* avoids oviposition on prickly pear cacti that are stressed by poor nutrition (Myers *et al.* 1981), and the moth *Samea multiplicalis* prefers to oviposit on lush foliage of salvinia that has a high nitrogen content (Taylor & Forno 1987). In combination, nutrition and differential oviposition combine to cause higher levels of damage to vigorous plants (Room *et al.* 1989).

Given the known effects of nitrogen on insects (White 1993), greater attention to plant nutrition is warranted at present. Many commercial potting mixes provide high levels of mineral nutrients which could produce unusually vigorous plants. Perhaps soil fertility in which plants for testing are grown should have moderate levels that mimic what occurs in native ecosystems. This is a vexing problem since plant quality is multifactorial, but plants used in specificity testing should at least have a physical appearance that is comparable to their wild counterparts. Wherever possible factors

like percentage dry mass and nitrogen should be evaluated and shown to be equivalent to the levels seen in wild plants.

The extent to which plant nutrition can affect the outcome host specificity testing of parasitoids is unknown, and research in this area is needed. However, there is no doubt that different plant species can affect the behaviour of searching parasitoids (Vet & Dicke 1992, Kitt & Keller 1998), so host insect and plant combinations should be chosen carefully. Many insects can be reared on factitious hosts, e.g., citrophilous mealybug, *Pseudococcus calceolariae*, can be reared on butternut pumpkins which are not attacked in the field (Baker & Keller 1998). Unnatural combinations of host insects and food plants like this should be avoided whenever possible because they do not provide the appropriate cues to searching parasitoids. Parasitoids reared from pumpkin-reared hosts may either learn to use cues from pumpkins to guide their searching or fail to learn appropriate cues from citrus (the targets' natural host) at emergence. This would confound specificity testing since their behaviour would not be indicative of wild individuals.

The early adult experience of parasitoids with cues used during searching plays an important role in shaping later behaviour as is demonstrated by the behaviour of the aphelinid wasp *Aphytis melinus*. It can be reared on oleander scale, *Aspidiotus nerii*, feeding on squash, while its normal host is California red scale, *Aonidiella aurantii*, which feeds on citrus (Hare 1996). *A. melinus* utilises a kairomone *O*-caffeoyltyrosine (OCT) in the covers of *A. aurantii* to recognise its host. Those that emerge from oleander scale have a reduced preference for red scale because they have not experienced OCT at emergence (Hare 1996). *A. melinus* fails to learn to use OCT as a searching cue, and hence it may use inappropriate cues, possibly including plant cues, while searching for hosts due to rearing on an unnatural host plant and host insect. Thus the behaviour of these natural enemies is aberrant and specificity testing would not reflect their natural preferences in the field.

Case study: the parasitic wasp *Aphidius rosae*

A recent study of the behaviour of the parasitic wasp, *Aphidius rosae*, illustrates the potential for holistic evaluation of host selection behaviour in specificity testing (Kitt & Keller 1998). *A. rosae* was imported into Australia for control of the rose aphid, *Macrosiphum rosae*. Prior to its release, the behavioural responses to a range of plants and aphids was evaluated in a wind tunnel and several enclosures. Habitat selection was investigated by presenting female wasps with various plants in a wind tunnel. In the absence of aphids, female *A. rosae* chose to fly to roses, *Rosa* sp. var. Tea hybrid 'McGredy's Sunset' (Rosaceae), over five other non-rose species in paired choice tests. This suggests that *A. rosae* utilises the odour of roses as a filter in locating host habitats. As a result of these assays, aphids on roses were the primary focus of subsequent testing.

A. rosae also flew preferentially to roses infested with aphids compared to uninfested roses, but before landing did not distinguish between plants infested with their host and the non-host *Macrosiphum euphorbiae* which also feeds on roses. A noteworthy aspect of this choice assay was the spatial arrangement of the roses. When roses were spaced widely apart (25-30 cm), wasps did not distinguish between infested and

uninfested roses. Smoke plumes indicated that the odours from each rose stem were mixed at the point where wasps were released. Only when the roses were close together (separated by 5 cm) did the wasps consistently choose the infested plants. These observations illustrate the trade-off between reliability and detectability of cues used by foraging wasps. The wasps can perceive odours associated with aphids only from a short distance. When the roses were separated more widely, the wasps had to make a choice by flying to the left or right before they could perceive the odours associated with aphids and so flew to the equivalent rose odours of either plant. These results led to a focus on *M. rosae* and *M. euphorbiae* in subsequent testing. Other species were included in tests because it was feared that regulatory authorities would not accept tests with fewer species. *Rhodobium porosum* was included in tests because it occasionally occurs in mixed aggregations with *M. rosae* on roses in the field.

Additional assays were conducted to examine the behaviour of *A. rosae* when attacking 11 species of aphids. In no-choice tests, naive females attacked four species of aphid. *M. rosae* (attack index = 31.5) was attacked significantly more frequently than *M. euphorbiae* (4.5 - 4.8), *R. porosum* (0.2) and *Acyrthosiphon kondoi* (0.7). However, when they had prior oviposition experience of their target host, they only attacked the target. When given a choice of aphid species feeding together on roses, oviposition-experienced wasps attacked *M. rosae* (attack index = 17.7) significantly more frequently than *M. euphorbiae* (4.0) and *R. porosum* (0.2). It appears that stimulating cues from host aphids led the wasps to attack non-target aphids in this latter case. Although *A. rosae* may oviposit in the non-target *M. euphorbiae*, it does not develop in this species. Thus there is a physiological barrier, either host defence or unsuitable nutrition, that prevents development in this non-host. The series of tests summarised here indicated that the strain of *A. rosae* released in Australia can only develop in *M. rosae* and so is acceptably host-specific to it. Permission to release was granted on the basis of the known physiological host range, but a good argument against the validity of this assertion could have been made had only the short list of aphids tested been given. It was considered necessary to present the entire range of data on host selection behaviour when the case for specificity was made. If centrifugal phylogentic testing (Wapshere 1974) had been used, then it would have been necessary to test a much wider range of potential host species. The centrifugal phylogentic approach recommends testing of limited species beyond the same family and implies that other insect species sharing the same habitat preferences should also be tested, for instance whiteflies utilising roses. The approach utilised in our laboratory meant this extra and questionable experimental work was not required.

This case study illustrates the potential for expanding host specificity beyond evaluation of host acceptance and suitability. Evaluation of habitat selection could limit the range of insect species that need to be tested. The experimental observations in the wind tunnel indicated that aphids feeding on roses should be the primary focus of specificity testing. Additional field research is warranted to validate this assertion. If such assays are to become more routine, then there is a need for practitioners to understand how volatile chemicals disperse in air and how insects respond to such chemicals. This was illustrated by flying *A. rosae* that did not choose between infested and uninfested plants when they were widely spaced in the wind tunnel. Observations like this highlight the problem of determining which factors affect choices among

alternatives. If a test species is not presented with appropriate cues, then it cannot make a biologically meaningful choice. The experiments with *A. rosae* also demonstrated that this wasp chooses hosts by a filtering process. The odours associated with aphids on rose plants lead searching wasps to land only on rose plants, but the final step in host selection among the aphid species present on roses occurs after landing. A complete understanding of host selection can only be gained by assaying the behaviour of both flying and walking wasps.

Conclusion

Behavioural assays have the potential to improve specificity testing by expanding consideration to a broader range of factors that limit host or prey utilisation. This could in many cases limit the number of species that must be tested. Even if it does not reduce the amount of work devoted to testing, it should provide greater insight into host selection by biological control agents and provide reassurance about the safety of exotic organisms. It would be reckless to advocate reliance on evaluation of habitat selection to limit the range of species to be tested until further experimental validation of this approach is undertaken. Nevertheless, there is potential for more effective host specificity testing by evaluation of host selection more broadly. Research in this area is warranted and aspects of such experimentation could be incorporated into host specificity testing immediately, as was shown by experiments with *A. rosae*. The ultimate goal must be achievement of balanced outcomes that ensure the ecological safety of exotic organisms while not unnecessarily restricting the range of species that could be released for biological pest control.

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Towards an integrated approach to predicting risk to non-target species

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Ideally, host specificity testing methodologies and assessment guidelines should together (1) prevent the release of any organism that is likely to have an unacceptable level of negative economic and/ or environmental impact, and (2) minimise the probability that safe and potentially useful agents will be rejected. It is recommended firstly, that the current testing methods take into account knowledge of insect behaviour, to ensure their applicability to individual species. Secondly, the assessment guidelines along with the approach to the order of testing method should be put into a context that fits into a best practice risk assessment framework.

Introduction

Together, the host specificity testing of biological control agents and legislation which assesses the benefits versus risks of releasing agents aim to provide an accurate estimate of the risk of unacceptable impact on valued non-target organisms. Ideally, host specificity testing methodologies, in conjunction with the assessment guidelines should both (1) prevent the release of any organism that is likely to have an unacceptable level of negative economic and/ or environmental impact, and (2) minimise the probability that safe and potentially useful agents will be rejected.

The legislation under which the results of host specificity tests are assessed will largely remain outside the influence of biological control practitioners. Thus, the challenge for the practitioner is to develop host specificity testing programs which will lead to outcomes that reflect a realistic estimate of risk, taking into account the nature of the assessment procedures required by the relevant legislation.

So how far have we come to being able to achieve this ideal balance ? The papers presented from this workshop indicate that, although considerable progress has been made, there is still further work required towards improving our ability to interpret results of host specificity tests in terms of predicting the probability of negative impacts on valued non-target organisms.

Heard and van Klinken have summarised the range of commonly utilised assay designs (Heard & van Klinken 1998) and it is unlikely this will change considerably, although there is scope for refinements that make better use of knowledge on the

biology of the candidate agent. It is envisaged that host specificity testing programs will continue to employ combinations of assays under quarantine conditions, such as no-choice tests (over parts of, or even multiple, generations) (Hill, Day, Barratt *et al.*, Sheppard this volume), choice tests (with or without the target organism present) (Edwards, Sheppard, Heard this volume), and various types of sequential testing. In addition, open field trials in the country of origin will become more widely utilised as the benefits of this approach are realised (Briese this volume).

Recommendations for an integrated approach to host specificity testing

The main refinements I would like to see put into place in all the above assays, is that more attention be given to the temporal dimension of attack by the agent during the tests. Increasingly, data are becoming available to show that temporal changes in responsiveness by insects over time, can have considerable influences on the outcome of assays (Bernays & Chapman 1973; Withers 1999). The benefits of continuous observations and sequential monitoring of behaviour at intervals during the life of the insect will provide crucial information on the insects natural patterning of host finding and acceptance behaviour. A better understanding of the temporal profile of oviposition or feeding on target hosts will help us to generate suitable assays to test for likelihood of acceptance of non-targets on a case-by-case basis. There are many examples of the usefulness of observations of host selection behaviour to understanding the host preferences of phytophagous insects (Degen & Städler 1997; Foster *et al.* 1997; Galanihe & Harris 1997; Harris & Miller 1988) and of parasitoids (Vet & Dicke 1992; Kitt & Keller 1998).

By routinely taking into account some well-established concepts from animal behaviour during the design of different types of test, there is great scope in being able to improve our ability to correctly interpret assay results. This will in turn enable us to make assessments of risks to non-targets based on sound scientific principles and methods.

Marohasy introduced the useful concepts of false positives and false negatives (Marohasy 1998) and discussed the wide range of possible behavioural processes capable of producing such outcomes from different assay methods. For instance, there is abundant evidence that responsiveness to sensory cues associated with food or oviposition sites may change in relation to time since an insect last fed or oviposited (Withers 1999). This is a likely basis for the common finding that insects apparently discriminate more clearly between hosts of different ranking (i.e., eliciting different levels of excitatory sensory input) in choice than in no-choice tests. Producing a false negative result, for instance, a test species might be apparently rejected in choice tests which include their target species (a high ranked host), but accepted in a no-choice test of even quite short duration compared to the life span of the relevant life-stage (i.e., not a long-duration starvation test) (Marohasy 1998; Withers 1999).

Another behavioural process that can influence the outcome of host specificity tests, through its effect on the responsiveness of the insect to host and/ or habitat cues, is the previous experience of the insect. For example, an insect that has had feeding or oviposition experience on a particular host (e.g., the target species) might for a time afterwards, show enhanced responsiveness to the previous experienced host, reduced

responsiveness towards novel hosts, or both (Cooley *et al.* 1986; Prokopy *et al.*1986). If any of these occur, an experienced insect may show stronger preferences than a naïve insect for the previous experienced host in a choice test. This would increase the likelihood of a choice test that includes the previous experienced host (often the target host) giving a false negative result for one or more of the non-target species. Similarly, in circumstances where substantially decreased responsiveness to novel hosts has been caused by previous experience of a different host (target) a false negative result in a no-choice test is possible. In parasitoids, experience can significantly influence the approach behaviour and subsequent host-searching intensity on novel substrates or substrates or plants associated with the previous experience (McAuslane *et al.*1991; Price *et al.*1980; Sheehan & Shelton 1989; Vet & Schoonman 1988), resulting in either false negative or false positive results. This adds a critical dimension to parasitoid host specificity testing, and suggests that preferences for substrates and non-target hosts should be tested on both naive and host-oviposition experienced females.

Because the methods utilised for gathering data for predicting host specificity of biological control agents remain at the discretion of the researcher, there is the opportunity to take greater account of existing knowledge of arthropod behaviour and physiology when designing and interpreting tests used in the host range estimation process (Marohasy 1998). By doing so, we can begin to generate data that better lend themselves to a more quantified risk assessment approach. The application of a generic risk assessment approach has been advocated by a number of researchers recently (Marohasy 1998; Wan & Harris 1997; Withers *et al.* 1999). The justification of this approach is that as biological control comes under increasing scrutiny for its environmental safety, it is important that the decision making procedures used (e.g., McFadyen & Heard 1997) are compliant with 'international best practice in risk assessment'.

Formal risk assessments comprise four distinct stages: risk identification, analysis, decision-making, and treatment of the risk (Withers *et al.* 1999). Briefly, identification of the risk involves identifying where the risks of biological control lie-through the careful selection of host test lists, whilst taking into account public concerns. Analysis of the risk is the stage involving host specificity testing. It requires the independent estimations of the probability of biological control agents establishing on non-target species, and the consequences of that event, namely the damage agents could cause those species. Decision making on the basis of risk analysis, and treatment of risks are bound up with the legislative processes that control the decisions of whether or not agents are permitted to be released, and the extent to which non-target impacts need to be monitored after releases.

The key concern for researchers of biological control is, therefore, how to best analyse the risks of biological control through the independent estimation of the probabilities of agent establishment, and damage. By providing the body responsible for making the decisions on the risks of biological control (to release or to not release) with such data, we will ensure the process runs as closely to a formal risk assessment as is possible at this stage. In order to achieve this, it is proposed that, by basing order of host specificity testing on the following approach, the process of host specificity testing will begin to meet those requirements.

Selection of assay type in a risk assessment approach to predicting risk of non-target attack by phytophagous insects.

The first stage requires determining the probability that the agent will establish on non-target species (encounter and colonise the plant). What is measured as establishment will vary depending on the dispersal and host colonisation mechanisms used by the insect, and should focus on the damaging life stages. One example involves the case of relatively immobile larvae, hatching from eggs laid on a plant that cannot leave unsuitable hosts (e.g., internal feeders) to locate other host plants (e.g., Withers *et al.* 1999). The relevant first stage of testing will be no-choice and choice oviposition trials with the adult female to determine likelihood of the larva establishing on the plant. This reflects the approach suggested by Sheppard (this volume).

The second stage of the host specificity testing process for risk analysis purposes involves the independent determination of consequences of damage to non-target plants. In order to avoid the confounding (dependent) variable of oviposition behaviour, in the case of the internal-feeding habit larvae, that cannot leave unsuitable hosts (as above), separate egg or larval transfer no-choice trials that ascertain development in, and resultant damage to, non-target plants is preferable.

It has been suggested (Withers *et al.* 1999) that an estimate of risk to each non-target species, can be obtained by multiplying probabilities of establishment from the first stage of testing, and the degree of damage seen in the second stage of testing. In order to do so, zero values (resulting from, for example, no oviposition on plants or zero survival in larval feeding assays) need to be assigned with non-zero values, such as 0.001. In a similar approach, Wan (1997) multiplied the probability of acceptance of non-target plants at each of the crucial stages in the host finding and acceptance sequence to obtain risk figures for host suitability. Undoubtedly further research and critical examination of this risk analysis stage of the risk assessment is required, in order to ensure such an approach is applicable to weed biological control.

Note that in order to consistently carry out a formal risk assessment, risk needs to be analysed equally for each non-target plant that has been identified as being theoretically at risk (by its inclusion on the host test list). This means undertaking both stages one (probability of establishment) and two (consequences of establishment) for each non-target plant. A formal risk assessment approach therefore can not advocate any reduction in the test plant list following an initial series of experiments, but requires that both stages be carried out on all non-targets. For instance, for mobile adults that oviposit selectively with immobile larvae, no-choice egg transfer trials should still be conducted on all non-target plants, independent of the number of non-target plants which receive eggs in choice oviposition trials. It is recognised that this approach may not be welcomed, as the reduction in test plants chosen for no-choice development trials following initial oviposition assays has been common practice recently (Sheppard this volume). My recommendation for no reduction in test plant list for both stages of testing, is based upon the best practice risk assessment approach, and is validated by acceptance that false negative results can be obtained, especially in choice oviposition or feeding trials that include the target weed.

Selection of assay type in a risk assessment approach to predicting risk of non-target attack by parasitoids.

A risk analysis system for parasitoids comparable to that proposed above for phytophagous insects is not yet available. This is because internal oviposition, and hence the ability to inoculate all test species, limits the range of species that can be assessed for suitability for development to those which elicit oviposition from the parasitoid (Barratt *et al.* this volume). Thus the probability of establishment of a parasitoid on a non-target and the consequences of that establishment cannot be determined independently of one another.

I propose that a risk analysis for non-target insects from attack from a parasitoid needs to be based upon determining the risk to non-targets from assays that maximise the likelihood of oviposition. Techniques for maximising attack from parasitoids will differ from species to species, but useful methods may include depriving the parasitoid of access to the target host, thus increasing its responsiveness, by placing test insects on the target insects' normal host plant, through by-passing the early cues in habitat and host finding sequence, or by presenting non-target and target hosts together in close contact to attempt to generate a central excitatory state.

The critical stage of such a risk analysis for non-target insects then involves ascertaining the most likely behaviour of the parasitoid when released into the field. Such assays (see Keller this volume) will need to demonstrate the natural host habitat and host location behavioural mechanisms utilised by that particular parasitoid, in order to ascertain its host preferences. Useful approaches may include testing oviposition preferences between target and non-target hosts in choice situations, testing naïve compared to oviposition-experienced parasitoids, testing undeprived compared to deprived parasitoids, and by introducing the target and non-target hosts in contact with their usual field host plant, compared to on novel host plant cues.

Conclusion

A best practice risk assessment approach is advocated for the host specificity testing stage of the biological control process. This involves the independent determination, where possible, of non-target species that are likely to be attacked, and the consequences of that attack, by all damaging life-stages of the potential biological control agent. The practical difficulties in applying such an approach to some types of insects, particularly parasitoids, is acknowledged, and this approach would undoubtedly benefit from further refinement.

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