How Time-Dependent Processes Can Affect the Outcome of Assays

Toni M. Withers\textsuperscript{1,2}, Lindsay Barton Browne\textsuperscript{1,3} and John Stanley\textsuperscript{1,3}

\textsuperscript{1}CRC for Tropical Pest Management, Alan Fletcher Research Station
P.O. Box 36, Sherwood, Q 4075, Australia,
\textsuperscript{2}Current address: Forest Research, P.B. 3020
Rotorua, New Zealand
\textsuperscript{3}CSIRO Entomology, PMB 3
Indooroopilly, Q 4068 Australia

Abstract
In an insect, the level of responsiveness to sensory cues varies throughout its life and this variation affects the probability that a response to any given cue will occur at a particular time. Important sources of variation in responsiveness to sensory cues associated with food or oviposition sites are changes induced by food or oviposition-site deprivation. Such changes, which have been termed time-dependent, have the potential to affect the outcome of host specificity assays of various designs. Groups of a biological control agent, the parthenium leaf-feeding beetle \textit{Zygogramma bicolorata} Pallister (Coleoptera: Chrysomelidae: Chrysomelinae), were tested in two different assays involving differently ranked plants. First, beetles differing in their time-dependent level of responsiveness were tested in two choice assays with plants in the subtribe Ambrosiinae of the Heliantheae. Second, groups of beetles were tested in no-choice sequential assays alternating exposure between the highest and lower ranked plants. These assays showed that time-dependent factors can influence the results of choice and no-choice feeding assays. In the choice test, \textit{Z. bicolorata} that had fasted for only 3 hours, consistently rejected the lower ranked host, \textit{Xanthium occidentale} Bertoloni (Noogoora burr), for feeding but accepted \textit{Parthenium hysterophorus} L. This situation produced the false impression that Noogoora burr is not an acceptable host plant for feeding. However, if beetles entered similar choice tests in a food-deprived state (i.e., having fasted for 6 days), many beetles fed on \textit{X. occidentale} when they encountered it first. The number of eggs laid on \textit{X. occidentale}, however, was consistently less than on parthenium, irrespective of the food-deprivation state of the beetles. Summed over the whole experiment, the 6-day food-deprived beetles laid fewer eggs per day than did less food-deprived beetles. In sequential no-choice assays, beetles initially did not feed or oviposit on \textit{X. occidentale} plants, but acceptance increased with time since the last exposure to parthenium. These data support predictions that choice tests using insects in a non-deprived state, and short duration sequential no-choice assays, will not adequately reveal the acceptability of lower ranked host plants.

Keywords: choice test, sequential test, behavior, biological control agent, host specificity testing, deprivation
Introduction

Ideally, host specificity testing and risk assessment methodologies should both prevent the release of any organism that is likely to have an unacceptable economic and/or environmental impact and minimize the likelihood that safe and potentially useful agents will be rejected. Thus, the challenge for the practitioner is to identify and use host specificity testing methods that will provide a realistic estimate of the field host range of a proposed biological control agent (Withers et al., 1999).

In the last decade there has been increasing interest in the design and interpretation of the laboratory assays used to assess the host range of phytophagous insects (Cullen, 1990; Harris and McEvoy, 1992; McEvoy, 1996; Blows, 1999; Marohasy, 1998). Debate over the virtues and shortcomings of the various assay methods has continued (Withers, 1997; Marohasy, 1998; Withers et al., 1999).

There is a range of assay designs that are commonly used for the host range estimation of biological control agents (Heard, 1997; Heard and van Klinken, 1998). In a recent review, Sheppard (1999) found that for the most commonly used groups of weed biological control agents, namely Coleoptera, Lepidoptera, and Diptera, feeding assays were dominated by no-choice tests, with choice tests that included the target weed in the array being used less often. In contrast, for oviposition tests, choice assays were used as commonly as no-choice assays (Sheppard, 1999). A common variation of the traditional no-choice assay (in which non-target plant species are presented separately to test insects using the same conditions as those for testing responses to the target weed) and choice assays, is the sequential no-choice design. Here non-target plants are presented to a group of insects one at a time, in a serial order alternating with the target plant. It is envisaged that host specificity testing programs will continue to be dominated by these types of assays - no-choice (sequential and parallel) and simple choice assays. But how much do we really know about the ability of these assay types to accurately predict the host range of a biological control agent in the field?

Marohasy (1998) discusses the useful concepts of false positives and false negatives in the context of host specificity testing. False positives occur when a test indicates that a plant species will be fed or oviposited on, when in reality it would not be attacked in the field. False negatives occur when a test indicates that a plant species is outside the host range of the insect species, when in reality it would be attacked in the field (Marohasy, 1998). One source of both false negatives and false positives is that the responsiveness of insects to sensory cues from a potential host can change over their lives. The phenomena responsible for these changes in responsiveness fall into three general categories: reversible changes resulting from food or oviposition-site deprivation (termed time-dependent changes by Papaj and Rausher, 1983), changes induced by experience (Szentesi and Jermy, 1990; Bernays, 1995) and ontogenetic changes (Barton Browne, 1993, 1995).

Of all the assay methods, the no-choice test is deemed to be the testing method least likely to produce false negative results (Cullen, 1990; Heard, 1997). It is widely believed, however, that no-choice tests of extended duration tend to over-estimate the field host range of insects (i.e., cause false positives). This is because increased acceptance often occurs as a result of effects of extreme deprivation and experience. Because of this perceived drawback, the choice test is frequently used for revealing the preference ranking for the target weed relative to other plants (Marohasy, 1998; Edwards, 1999), and/or for reducing the list of test plants required to be tested in further assays (especially when larvae are immobile and oviposition specificity decides the host range). Because of this, choice tests have been widely used and will continue to be used to measure the risk that test plants will be damaged in the presence of the target weed. The main concern with choice tests is their inability to reveal the acceptability of lower ranked host plants (Heard, 1997) because the insects can be expected to be in a low state of responsiveness due to their ready access to the highly ranked target species (Marohasy, 1998; Edwards, 1999). Also choice tests do not adequately predict the outcome of events in cases in which insects occur in localized areas where the target host is absent.

Predictions

There are two key features of time-dependent changes in responsiveness for insect feeding or oviposition. Firstly, the responsiveness of an insect to food and oviposition-related sensory cues increases with elapsed time since the last meal or oviposition. Secondly, responsiveness to sensory cues decreases following a meal or laying of eggs (Dethier 1982; Miller and Strickler 1984). These features form the basis of models of Singer (1982), Singer et al. (1992), Courtney et al.
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(1989) and Courtney and Kibota (1990), which describe the increase in the number of hosts accepted for oviposition caused by such deprivations. In general terms, these conceptual frameworks predict (i) that an insect, upon completing a bout of feeding or oviposition on its most highly stimulating host (highest-ranked host sensu Courtney and Kibota, 1990), will, for a period, be unresponsive to sensory cues from this host (refractory phase) or lower ranked hosts (Simpson, 1982), (ii) that as time since feeding or ovipositing increases, the insect again becomes responsive to the higher ranked host but not to lower ranked hosts (discrimination phase), and (iii) that, if an insect is denied access to its highest ranked (or to any) host, it will progressively become more responsive so that it will, increasingly, come to accept food and oviposition sites providing lower and lower levels of stimulation (deprivation phase).

The consequences of time-dependent changes in responsiveness are that insects deprived of the opportunity to feed or oviposit for significant periods may feed or oviposit on hosts that are rejected by less deprived individuals. Evidence for this has been obtained in relation to feeding by the acridids Locusta migratoria (L.) (Bernays et al., 1976) and Chortoicetes terminifera Walker (Bernays and Chapman, 1973) and the psyllid Cacopsylla pyricola Foerster (Horton and Krysan, 1991). In the tephritid Bactrocera tryoni (Frogg.), it has been shown that host deprived individuals accept for oviposition, host species that are rejected by less deprived individuals (Fitt, 1986). When caged continuously in a no-choice situation with oviposition sites providing different levels of excitatory stimulation, female phytophagous insects may accept lower ranked hosts later than higher ranked hosts (Weston et al., 1992; Kostál, 1993). The relevance of such outcomes for host specificity testing is obvious.

In this paper we will examine the potential influence of time-dependent changes on the outcomes of two-choice assays and sequential tests. The example we use is one in which the higher ranked target weed is being compared with a non-target plant that is ranked lower than the target species for both oviposition and feeding. On the basis of the above conceptual framework, the following predictions can be made. If the insect is in a refractory or a discrimination phase when it enters a choice test that includes the two plant species, its first meal or oviposition will be on the higher ranked plant. Thereafter, it can be expected to fluctuate between the refractory and discrimination phases because of the continuous availability of the higher ranked plant. In this case, therefore, the insect would not be expected to feed or oviposit on the lower ranked plant over the course of the tests. In contrast, if the insect is in a highly deprived state when it enters the two-choice test, we predict that it will feed or oviposit initially on whichever plant species is encountered first. Thus, in this case, the expectation is that there will be some feeding or oviposition on the lower ranked plant early in a choice test, but that the incidence of this will decline to zero as the test proceeds.

For sequential no-choice tests, we predict that if the insect is in the refractory or discrimination phase when transferred from the higher ranked plant to the lower ranked plant, it will initially reject the lower ranked plant. It will, however, become progressively more responsive as the elapsed time since it last fed or oviposited on the higher ranked plant increases, until it reaches the state where it will accept the lower ranked plant. Thus, there will be little or no feeding or oviposition on the lower ranked plant for a period, but thereafter, it will increase with time. Our predictions require some simplifying assumptions, including that plant quality does not change during the assay, that no host-marking pheromone is deposited, that host selection is not influenced by long-range orientation behavior to the preferred host, and that the insect shows non-random movement in response to host plant cues, such that it will tend to remain and feed or oviposit on the higher ranked plant when it is located.

Our Model Insect-Plant System
The insect we used to test these predictions is a weed biocontrol agent that has caused controversy because adults have fed on non-target plants in areas where the target weed was rapidly defoliated (Jayanth and Visalakshy, 1994). This insect is the oligophagous leaf-feeding beetle Zygogramma bicolorata Pallister (Chrysomelidae), which has been released in Australia and India for the biological control of Parthenium hysterophorus L. (Heliantheae: Ambrosiinae) (Jayanth and Bali, 1994; Dhileepan and McFadyen, 1997), as well as for ragweed Ambrosia artemisiiifolia L. (Heliantheae: Ambrosiinae) in Australia. This insect has been subjected to host range testing in quarantine in Australia (R.E. McFadyen, unpublished data), field studies in Australia and India (Jayanth et al., 1993; Jayanth and Bali, 1994), and detailed behavioral observations (Withers, 1998, 1999).
Previous studies have shown that time-dependent processes play a part in the host acceptance behavior of *Z. bicolorata* (Withers, 1999). Adult beetles readily accept parthenium and ragweed for feeding. Noogoora burr (*Xanthium occidentale* Bertoloni, Heliantheae: Ambrosiinae), which is also a weed in Australia, is acceptable but generally only after prolonged periods of food deprivation. Noogoora burr often receives eggs in the field (as do other Heliantheae at times), and supports adult survival. However larval mortality is extremely high on Noogoora burr. Probably because of this mortality, population densities on this host rarely become high in the field (T. Withers, unpublished data). Sunflower (*Helianthus annuus* L., Heliantheae: Helianthinae) is accepted for feeding by only a small proportion of the adults in a *Z. bicolorata* population either under severe deprivation or when the plants’ acceptability has been increased by covering the leaves with parthenium pollen (Jayanth et al., 1993; Jayanth and Visalakshy, 1994; Withers, 1998). Sunflower is not a host for larvae.

We predicted that, when introduced into choice tests containing both a higher ranked plant (parthenium), and a lower ranked plant (Noogoora burr), adult *Z. bicolorata* in a discrimination phase would exhibit little or no feeding on Noogoora burr over the course of the test. On the other hand, we predicted that insects contacting Noogoora burr first when introduced into the choice test in a deprived state would feed on Noogoora burr. We predicted that in sequential no-choice tests, adult *Z. bicolorata* would be in a discrimination phase when transferred from parthenium to Noogoora burr, and that Noogoora burr would not initially be accepted for feeding. With increasing duration of exposure to Noogoora burr, we predicted an increase in feeding during the test. We present the results of experiments designed primarily to examine the effects of food deprivation on the outcome of feeding assays. However, some data on number of eggs laid are also briefly presented.

**Materials and Methods**

**Experiment 1 – Two Choice Assays**

*Insects.* The *Z. bicolorata* population used in these experiments originated from adults collected from parthenium in Monterrey, Mexico in 1980 and reared in the laboratory on parthenium in Brisbane, Australia until release in 1983. In addition to its establishment on parthenium weed in central Queensland, the beetle also established on ragweed in Brisbane. For our tests, adult *Z. bicolorata* were collected in Brisbane in spring off ragweed, and their offspring reared for two to three generations on potted ragweed plants. As adults eclosed from pupation sites in the soil of these pots, they were collected and held in cages with ragweed plants in a greenhouse maintained at 26°C (±2°C) and 85-95 % RH. They were two to three weeks old (mean of 10 days old) at the time of experimentation (January 1998). Ragweed ranks as highly as parthenium for feeding by *Z. bicolorata*. Using ragweed to rear the insects meant beetles were naïve to those plant species used in experiments.

*Zygogramma bicolorata* adults of three different levels of feeding responsiveness were obtained by depriving them of the opportunity to feed for one of three time periods:

- **Recently-fed beetles** were obtained from beetles on ragweed that were continuously observed until they were seen to have just completed a meal. These individuals were collected and used in tests within 30 minutes of the end of their meal.
- **Three hours post-meal beetles** were ones held on ragweed and continuously observed between 08:30 and 09:30 hrs. Immediately after each beetle completed a meal, it was placed into a 5 x 10 x 20 cm plastic container with a mesh insert in the lid containing a moistened sand/bark mixture. They were held for approximately 3 hours before testing. This interval was chosen because it is almost one inter-meal interval for both adult male and female beetles (T. Withers, unpublished data).
- **Deprived six days beetles** were ones removed from ragweed plants at 11:00 hours and placed into a container as above and tested six days later.

The state of responsiveness of beetles in each of these three groups at the start of the test were expected to be for:

- Recently-fed beetles, initially unresponsive to both higher and lower ranked host plants because the time since the last meal had not exceeded the intermeal interval for either male or female beetles (refractory phase)
- Three hours post-meal beetles, responsive to the highest ranked plant for feeding, but not the lower ranked plant (discrimination phase)
- Six days deprived beetles, highly responsive to
both the higher and lower ranked plants due to severe food deprivation (deprivation phase).

**Responsiveness state of the beetles.** No-choice behavioral observations were undertaken concurrently with the two-choice tests to indicate the level of responsiveness of each group of beetles to plant cues. On each day on which experiments were set up, two beetles from each treatment group were chosen and held in a cotton mesh covered cage (40 x 40 x 85 cm h, with an open front through which the observations were made) containing either a parthenium or a Noogoora burr plant. Behaviors were recorded directly onto a portable computer programmed with the behavioral recording software "The Observer, version 3.0" (Noldus, 1990). The observations took place alongside the two choice tests. Timing, to the nearest second, and the location of the beetle in the cage or on the plant were recorded as all behaviors were occurring. Behaviors recorded were sample biting, feeding, walking, or pausing. Observations were made on each beetle until it completed a meal or until 30 minutes elapsed, whichever occurred first (see Withers [1998] for details of the behavioral recording protocol). Due to some difficulty obtaining recently-fed beetles, the final sample sizes were 10 recently-fed, 12 three hours post-meal and 16 six days deprived beetles.

**Procedure for choice tests.** Inside the cotton mesh covered cages used for the two choice tests (55 x 90 x 85 cm h) a wooden frame with 4 large holes (20 cm apart) was placed over the pots containing parthenium and Noogoora burr plants. Brown paper was then placed on top of the wooden frame with slits cut in the appropriate place to allow the plants to protrude and to prevent the beetles from escaping down the sides of the cage and into the plants pots.

Recently-fed, three hours post-meal, or six days deprived beetles were introduced (generally 10 per cage) into one of three identical test cages containing two plants each of the higher ranked parthenium and the lower ranked Noogoora burr. The cages were situated in a naturally lit greenhouse (28 - 32 °C). Half the beetles were marked on the elytra with a whitening fluid (Tipp-Ex Germany, Malaysia). The marked beetles were introduced into each cage onto the young leaves of one Noogoora burr plant. The other half of the beetles (unmarked) were introduced onto leaves of one parthenium plant. The position of each beetle was recorded at 2-5 minute intervals for the first 20 mins, half hourly for the rest of the day, and hourly for the next two days, between the hours of 0830 and 1730. Each morning at 0830 hours the number of eggs laid, and the number of meals taken from each plant was recorded. After the third day (70 hours), the beetles were captured and after freezing, dissected to obtain the sex ratio within that test.

This procedure was repeated three times during three consecutive weeks in January 1998. The only difference between repetitions involved the sample size of beetles in the recently-fed treatment, i.e., sample size in this treatment was dependent upon the number of beetles observed taking a meal within the 30 minute period preceding the tests. This resulted in 6, 10 and 8 beetles per repetition, respectively, for the recently-fed treatments. The tests all began at 11:30 hrs on the first day and finished at 08:30 hrs on the third day. During this time, daily counts were made of the number of meals consumed from leaves (estimated by counting each scalloped area removed from a leaf edge), and the number of eggs laid, both without disturbing the beetles on the plants.

Data were expressed as the number of meals taken per beetle per day or eggs laid per female beetle per day. These data were tested for homogeneity of variances across treatments using a Bartlett's test. Where heteroscedasticity remained, median values were compared between treatments using appropriate non-parametric tests at $P < 0.05$.

The location of beetles (marked versus unmarked individuals) was recorded at various times following their introduction, and analysed as follows, using combined data from the three repeats. If the proportion of those beetles originally released on plant species $a$ which have remained there is $p_{aa}$, while the proportion of those beetles originally released on plant species $b$ which have moved to species $a$ is $p_{ab}$, then the total proportion of beetles on species $a$ will be:

$$P_a = \frac{(p_{aa} + p_{ab})}{2}.$$

Similarly,

$$P_b = \frac{(p_{ba} + p_{ab})}{2}.$$

The difference between $P_a$ and $P_b$ was then used as a measure of the preferential movement by the beetles between the two plant species,

$$P_a - P_b = \frac{(p_{aa} + p_{ba} - P_{ab} - P_{ba})}{2}.$$
Using variances and covariances from the multinomial distribution, an approximate standard error for this difference was obtained as follows:

\[ s.e.(p_a - p_b) = \sqrt{\frac{\hat{p}_a(1-\hat{p}_a) + \hat{p}_b(1-\hat{p}_b) + \hat{p}_{ab}(1-\hat{p}_{ab}) + \hat{p}_{ba}(1-\hat{p}_{ba}) + 2\hat{p}_a\hat{p}_{ab} + 2\hat{p}_b\hat{p}_{ba}}{4n}} \]

where \( n \) is the number of beetles originally released on each species. A z-score was then used as an approximate test of the statistical significance of the difference from zero,

\[ Z = \frac{(p_a - p_b)}{s.e.(p_a - p_b)} \]

This procedure was used to test the hypothesis that the beetles expressed a preference for parthenium over Noogoora burr as a substrate, reflected in their location in the cage, at each assessment period. Similar procedures were used to compare the effects of treatments on plant species preferences, and on the proportion of beetles that were not located on either plant species.

**Experiment 2: No-Choice Sequential Assays**

Sequential no-choice trials were conducted to examine the potential effects of time-dependent changes in responsiveness as well as feeding experience on the acceptance of non-target plants. Knowing that acceptance of Noogoora burr, and also of sunflower, increases with extreme food deprivation, we tested *Z. bicolorata* in a no-choice sequential assay with a long exposure time (5 days) on non-target plants to see whether we could induce either false negative or false positive results.

Adult *Z. bicolorata* were collected from annual ragweed in Brisbane and maintained on parthenium for two weeks prior to testing. Tests were conducted under glasshouse conditions (25±3°C) with supplementary halogen lighting used to create a photoperiod of 14:10 L:D.

Sixteen clear plastic cylinders (25 cm diam x 32 cm H) were filled with soil to a depth of 25 cm. A black gauze sleeve (25 cm diam x 110 cm L) was suspended above each cylinder and fitted tightly to prevent the escape of the insects. A single, potted, vegetative-to-early flowering parthenium plant was placed into each enclosure. The pots were buried to give a smooth soil surface up to the base of the plant stem.

Five mating pairs of adult *Z. bicolorata* were introduced into each cage of the 16 cages between 08:30 and 09:30 hrs on the first experimental day (March 1999). For the next two days at 09:00 hrs, the number of eggs were counted and feeding level noted on each plant. On the third day the 16 sets of insects were randomly allocated to 4 treatments (each with 4 cages): Noogoora burr, sunflower, bean (*Phaseolus vulgaris* L., Fabaceae) or parthenium. For the next 5 days the number of feeding sites (each scalloped area removed from a leaf edge) as well as eggs laid were recorded as accurately as possible, e.g., on parthenium scoring of feeding sites was impaired by the extensively lobed leaf margins and the extensive feeding. After 5 days the insects were transferred to new enclosures containing parthenium plants where feeding and oviposition were again recorded for a 5-day period. This produced a sequential, no-choice trial in which the insects were monitored for two days on the target host (parthenium), then 5 days on one of three test plants or the target-control, and then returned to parthenium for a further 5 days. A clean enclosure sleeve was fitted at the time of each insect transfer to reduce the possibility of cross-contamination of host plant cues.

**Results**

**Experiment 1 – Two Choice Assays**

*Responsiveness state of the beetles*. The results obtained from observations of individual beetles in this study agreed with the findings of earlier experiments (Withers, 1999), on which basis the treatments had been chosen. The sampled beetles were in the expected time-dependent states, with the exception that both the recently-fed beetles and the three hours post-meal beetles responded similarly to host plants. Most recently-fed and three hours post-meal beetles were responsive to parthenium (10/11 fed) but not to Noogoora burr (1/11 fed). This suggested that the recently-fed beetles were best described as being in a discrimination phase rather than a refractory phase. This result has two possible explanations. Either the experimental parthenium plants ranked higher than the ragweed plants on which the beetles had taken their previous meal, or for some beetles, being removed from ragweed following a meal and being held in a container for up to 30 mins in some cases was sufficient to increase their responsiveness when introduced onto parthenium. For this reason we have combined the results of the recently-fed and three hours post-meal categories for all further analyses.
Half (3/6) of the 6-day deprived beetles accepted Noogoora burr. Acceptance of Noogoora burr only occurred after a greater number of test bites were taken (mean of 23) than were taken preceding acceptance of parthenium (mean of 3). This confirms that the 6-day deprived beetles were in the deprivation phase. However, even in this state, the lower ranked plant was not accepted as readily for feeding as the higher ranked plant.

** Movements of beetles between plants. **

In the two-choice tests, almost all recently-fed and 3 hour post-meal beetles released onto parthenium remained on parthenium plants for the entire test. Only in seven instances was a beetle, released onto parthenium, found later for a short time on a Noogoora burr plant. In all deprivation treatments, beetles released onto a Noogoora burr plant moved off the plant and ended up on a parthenium plant. The differences between treatments were in how rapidly this movement from parthenium to Noogoora burr occurred. Over half of the recently-fed and 3 hour post-meal beetles released onto Noogoora burr had left within the first 30 mins. In contrast, half of the 6-day deprived beetles had left Noogoora burr after between 5 and 24 hours (Fig. 1). The proportion of beetles found elsewhere in the cage (on the netting walls, and paper floor) was consistently lower in the 6 day deprived treatment than in the recently-fed/ 3 hour post-meal treatment (Fig. 1).

The difference in the proportion of beetles remaining on parthenium was compared with the proportion of beetles remaining on Noogoora burr at a number of times throughout the test. Significant differences in beetle location, on the basis of a difference in the proportion of beetles leaving the plant species that they were introduced on to, occurred virtually immediately in the recently-fed/ 3 hours deprived treatment. However, in the 6-day deprived treatment a difference in the proportion of beetles showing a location preference for parthenium over Noogoora burr was significant only two hours into the test. The plant preferences shown by beetles on the basis of location were compared directly between treatments and visualised on a logarithmic scale, where movement of beetles within the first 10 hours could be seen more clearly (Fig. 2). Beetles in the 6-day deprived treatment were slower to leave Noogoora burr and move onto parthenium (revealed as a smaller difference), as well as slower to move elsewhere in the cage, in comparison to the recently-fed/3 hours post-meal treatment. The differences between the time-dependent treatments in the proportion of beetles remaining on the plant species onto which they had been introduced, were significant between 30 minutes and 28 hours into the test.

**Fig. 1.** The influence of initial state of food deprivation on the location of adult *Zygogramma bicolorata* Pallister over the course of two-choice cage tests. The proportion of beetles on a parthenium (*Parthenium hysterophorus* L.) plant, a Noogoora burr (*Xanthium occidentale* Bertol.) plant, or elsewhere in the cage, when beetles were introduced (a) recently-fed or 3 hours post-meal (combined data *n* = 54), or (b) after 6 days of food deprivation (*n* = 30).
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Fig. 2(a-b). The influence of initial state of food deprivation (recently-fed/3 hours post-meal compared to 6 days of food deprivation) on the location of adult *Zygogramma bicolorata* Pallister over the course of two-choice cage tests. (a) The difference in the proportion of beetles showing a preference for *Parthenium hysterophorus* L. over *Noogoora burr* (*Xanthium occidentale* Bertol.) or (b) proportion of beetles found elsewhere in the cage. Note the logarithmic scale. Significant differences in location according to treatment at each time period are indicated (* at $P < 0.1$, and ** at $P < 0.05$).

From the second day (28 hours after introduction) initial treatment was no longer influencing location of beetles in the cage (Fig. 2).

**Plant consumption.** Overall, significantly more meals were taken per beetle from *parthenium* than from *Noogoora burr* throughout the two-choice tests (Fig. 3) (Mann-Whitney test $W = 378, P < 0.0001$). In the recently-fed/3 hours post-meal treatments, virtually no meals were taken from *Noogoora burr*, whereas a significant number of meals were taken from this plant when beetles had been deprived for 6 days (Fig. 3).

The number of meals taken from *Noogoora burr* plants was significantly affected by deprivation state of the beetles at the start of the test (Mann-Whitney test $W = 182.5, P < 0.0001$) (Fig. 3). Most meals taken from *Noogoora burr* were in the 6-day deprived treatment (mean of 0.7 meals/bettle/day), and the least in the recently-fed/three hour post-meal treatments (mean of 0.03 meals/bettle/day). There was no significant effect of day of the test on the number of meals per day taken from *Noogoora burr* (Kruskal-Wallis test $H = 1.3, df = 2, P > 0.51$). Overall the number of meals taken on the *Noogoora burr* plant onto which beetles had been introduced was positively correlated (+0.64) with the mean proportion of beetles located on that plant on that day. The same correlation was not obtained when the data from both *Noogoora burr* plants were combined.

The number of meals from both *parthenium* plants was not significantly influenced by the deprivation state of the beetles at the start of the choice tests (Mann-Whitney test $W = 283.0, P > 0.12$). Over all three days, the mean number of meals taken from the *parthenium* plants by beetles that began the tests in a 6-day deprived state appeared lower (6.5 meals/bettle/day) than for beetles in the less deprived states (mean of 9.4 meals/bettle/day) (Fig. 3). The number of meals from a *parthenium* plant overall was positively correlated (+0.36) to the mean proportion of beetles located on both *parthenium* plants that day.

In order to test for a significant difference in preference for *parthenium* over *Noogoora burr* caused by degree of time-dependent responsiveness at the start of the two-choice test, the following analyses were carried out. A coefficient of preference (Heard, 1995) for *parthenium* over *Noogoora burr* (CP) was calculated using the formula:

$$CP = \frac{(P-NB)}{(P+NB)}$$

where $P =$ mean number of meals taken per beetle from *parthenium*, and $NB =$ mean number of meals taken per beetle on *Noogoora burr*. This index varies from -1 (when all meals are taken from *Noogoora burr*), to 0 (when equal meals are taken from both *parthenium* and *Noogoora burr*), to +1 (when all meals are taken from *parthenium*).

The Coefficient of Preference calculated daily over the combined replicates indicated that two-choice tests with beetles initially 6-day food deprived revealed the lowest preference for *parthenium* over *Noogoora burr* on the first 24 hours data. The preference for *parthenium* over *Noogoora burr* increased as the days of the two-choice test passed, however it never reached the same level of virtually complete preference for *parthenium*, as occurred when the test beetles were recently-fed and 3 hours post-meal (Fig. 4).
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Fig. 3(a-b). The influence of initial state of food deprivation on consumption by adult Zygogramma bicolorata Pallister beetles of foliage over the course of two-choice cage tests. The mean number of meals taken per beetle on each of three days of the test from parthenium (Parthenium hysterophorus L.) or Noogoora burr (Xanthium occidentale Bertol.) when beetles were introduced (a) recently-fed or 3 hours post-meal (combined data n = 54), or (b) after 6 days of food deprivation (n= 30).

Oviposition results. Significantly more eggs were laid on parthenium plants (mean of 6.8 eggs/female/day) than on Noogoora burr plants (mean of 0.15 eggs/female/day) over all the tests (Mann-Whitney test W = 489.5, P < 0.001). This was the case in both the recently-fed/3 hours post-meal states, as well as the 6 days deprived state.

The state of deprivation of the beetles significantly influenced the number of eggs laid per female on parthenium plants (Mann-Whitney test W = 331.0, P < 0.0001) and this was not significantly influenced by the day of the test (Kruskal-Wallis test H = 2.55, df = 2, P > 0.28). On parthenium, the least eggs were laid when the beetles began the test 6-day deprived (mean of 0.3 eggs per female/day), and the most eggs were laid when the beetles were in the recently-fed and 3 hours post-meal states (mean 13 eggs per female/day).

Neither state of deprivation of the beetles (Kruskal-Wallis test H = 3.6, df = 1, 26, P > 0.06), nor day of the test (Kruskal-Wallis test H = 0.89, df = 2, P > 0.64), significantly influenced the number of eggs laid on Noogoora burr plants, probably because so few eggs were ever laid on Noogoora burr throughout the tests (range of 0 to 0.3 eggs per female/day).

**Experiment 2: No-Choice Sequential Assays**

Feeding was extensive at all times on parthenium, and continuous throughout the sequence used as a control (Fig. 5). Feeding sites were scarce (mean of 0.1 meals per beetle) on the first day of no-choice exposure to Noogoora burr, but increased steadily each day that the no-choice test continued (Fig. 5). There was a significant effect of the day of exposure to Noogoora burr in the no-choice test on the number of meals taken from Noogoora burr (Kruskal-Wallis test H = 16.5, df = 4, P < 0.002). In three cases, feeding sites on Noogoora burr were too numerous to be counted accurately after the fifth day of the no-choice trial, so were assigned the score of 100. There was no feeding at all on the non-target plants of sunflower and bean (Fig. 5). In all cases, consumption of parthenium after the 5 days exposure to the non-target, returned immediately to pre-non-target levels and continued for the last 5 days of the test (again feeding sites on parthenium were too numerous to be counted).

Oviposition results. During the testing sequence of no-choice exposure to a test plant or a control plant, significantly more eggs were laid per day (Fig. 6) on parthenium (mean of 8 eggs/female) than on Noogoora burr (mean of 0.72 eggs/female) (Mann-Whitney test W = 332.5, P < 0.0001). Oviposition in the control cages (continuous access to parthenium plants) differed significantly between days (Kruskal-Wallis test by day: H = 28.5, df = 11, P < 0.003). In particular, egg laying was significantly reduced (oviposition dropped to a mean of 0.7 eggs/female) on the day that beetles had been handled and moved to another plant (day 3).

A small number of eggs were laid apparently indiscriminately (on the cage walls) in all tests during the no-choice trials. On parthenium significantly more eggs were laid on parthenium.
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In this paper, we have focused on how food or oviposition-site deprivation experienced by an insect (i.e., changes designated as time-dependent by Papaj and Rausher [1983]) might influence the outcome of host specificity tests. Typically, in cage-based choice and no-choice tests, feeding or oviposition is scored at the end of an often arbitrarily selected assay period, with results from non-target plants compared to the target weed. Such host specificity assays usually do not provide an opportunity for examining the behavioral mechanisms responsible for observed outcomes.

Theoretical models of host acceptance, particularly the hierarchy-threshold model of individual insect diet (Courtney et al., 1989) and the rolling fulcrum model (Miller and Strickler, 1984), helped us to formulate specific predictions about the influence of time-dependent changes in responsiveness upon the endpoints of common types of host range assays.

In relation to choice tests, our predictions included the following outcomes. Commonly, choice tests include both one higher ranked plant species (e.g., the target weed) and at least one lower ranked but acceptable plant species (which may be taxonomically-related or chemically similar to the target weed) (Heard and van Klinken, 1998). We predicted that a non-deprived insect introduced into a test will always find, or be in contact with, the target weed, well before becoming sufficiently deprived to ever accept the lower ranked plant. Thus, we predict that choice tests including the target weed are particularly prone to producing false negative results. However, should the same insect enter the same choice test as described above when deprived

Discussion

Time-Dependence Influencing the Outcome of Host Specificity Assays

In this paper, we have focused on how food or oviposition-site deprivation experienced by an insect (i.e., changes designated as time-dependent by Papaj and Rausher [1983]) might influence the outcome of

Fig. 4. The influence of initial state of food deprivation on the coefficient of preference shown by adult Zygogramma bicolorata Pallister for parthenium (Parthenium hysterophorus L.) over Noogoora burr (Xanthium occidentale Bertol.) in two-choice tests. The coefficient of preference when beetles were introduced recently-fed and 3 hours post-meal (n = 54) or after 6 days of food deprivation (n = 30). The coefficient of preference varies from +1 to −1, with +1 indicating that all feeding was on parthenium, −1 indicating that all feeding was on Noogoora burr, and 0 indicating no preference.

(mean of 8 eggs/ female) than on the cage (mean of 1.1 eggs/ female) (Mann-Whitney test $W = 530, P < 0.016$). However, on Noogoora burr (on which more eggs were laid than on the other test plant 3.7 versus 0.7 eggs/female) ($W = 537.5, P < 0.0003$). There were no eggs laid on Noogoora burr on the first day of the test. Despite this, there was no significant effect of day of testing on Noogoora burr on eggs laid on a Noogoora burr plant per day (Kruskal-Wallis test $H = 5.7, df = 4, P > 0.2$). On sunflower, more eggs were laid on the cage structures (mean of 1.2 eggs/female) than on the plant itself (mean of 0.16 eggs/female) ($W = 546, P < 0.0001$). However, in bean tests, very few eggs were laid (Fig. 6), even on the cage (mean of 0.1 eggs/ female) so there was no significant difference in where the eggs were laid ($W = 422, P > 0.3$).

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Fig. 5. The mean number of meals taken by five pairs of adult Zygogramma bicolorata Pallister per day over a sequential no-choice trial (n = 4 replicates). The first two days were no-choice exposure to the target Parthenium hysterophorus L., followed by five days on one of three non-target plants or P. hysterophorus (control), followed by another five days on P. hysterophorus. A score of 100 meals was assigned when the number of meals was too large to be accurately counted.
of food, its responsiveness to plant cues will be much higher. In this case, we predict that the lower ranked non-target plant may receive eggs or be fed upon early in the choice test, but that the incidence of this will decline as the test proceeds, reducing the likelihood of a false negative result.

Time-dependent changes in responsiveness are also likely to have significant impacts on the measurable outcomes of no-choice tests, particularly when high-ranked and lower ranked plants are presented sequentially to the insect. Insects which have had unlimited access to higher ranked plants (such as is normal when rearing procedures for the insect require continual access to the target weed) before being put in a no-choice assay with non-target plants, will be in a state of low responsiveness. Whether or not the non-target plant is ever accepted for feeding or oviposition, will depend in part upon the duration of the no-choice assay. Only if the duration exceeds that required for responsiveness to reach the acceptance level will feeding or oviposition on the non-target take place.

We predicted that short duration no-choice assays have a high potential for producing false negative results.

**Predictions on the Outcome of Assays with *Z. bicolorata***

We were able to design the assays testing the impacts of time-dependence on host acceptance with substantially more knowledge of *Z. bicolorata* than is usually available for biological control agents. Previous experiments (T. Withers, unpublished data) had shown that adult female beetles take almost twice as many meals per day (6-7), as males (3-4). Meals on parthenium take 4-6 minutes to complete, and eggs are laid singly on parthenium leaves, generally away from the sites of feeding. This means that beetles that have just completed a meal are unresponsive to plants for feeding. Thereafter, responsiveness to host plant cues increases. Following the normal inter-meal interval of approximately 2.3 hours for female beetles and 4 hours for males, feeding resumes on parthenium (Withers, 1999). If deprived of host plants, adult *Z. bicolorata* become progressively more responsive until lower ranked and non-target plants are accepted for feeding (McFadyen and Heard, 1997) and oviposition (Withers, unpublished data). For instance, after 6 days of deprivation, over 50% of adult beetles accept the less acceptable host, Noogoora burr, for feeding (Withers, 1999), whereas none accept it when in a discrimination phase, after they have fed on either parthenium or ragweed. Although a poor host for physiological development for larvae, Noogoora burr has been shown to be a host in the field and under no-choice conditions in the laboratory. Therefore it is important to reiterate that a lack of feeding or a lack of oviposition on Noogoora burr by adult *Z. bicolorata* in cage assays can be considered to be a genuine false negative result (sensu Marohasy, 1998).

It is fortunate that all host plants (Heliantheae: Ambrosiinae) that support development to the adult stage of *Z. bicolorata* in Australia as well as in India are weeds, and not beneficial or native plants. The controversy surrounding adult *Z. bicolorata* causing feeding damage on the leaves of sunflower plants in the field in India (Jayanth et al., 1993; Jayanth and Visalakshy, 1994) can be attributed at least partly to the same behavioral mechanism, i.e., a deprivation-induced increase in responsiveness to plant cues. We did not focus strongly on sunflower in these experiments because this plant does not support larval development, and because of that, some biological control workers would not consider a lack of acceptance of sunflower in laboratory assays necessarily as a false negative result.

Did the results of choice and no-choice sequential assays with *Z. bicolorata* agree with our predictions of their potential to induce false negative results? We predicted that, in relation to feeding, when adult *Z. bicolorata* were in a refractory (satiated) or...
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The importance of such a result is obvious. If no-choice sequential assays were used when the duration of exposure to the non-target were only one day, or possibly two days, any slight amount of feeding on Noogoora burr may be deemed insignificant or perhaps misinterpreted as "exploratory feeding", thereby producing a false negative result. Whereas a no-choice sequential assay with a duration of exposure to the non-target of four or five days, as was run in this experiment, more accurately predicted the field host range of Z. bicolorata. Therefore sequential no-choice assays are capable of producing both genuine and false negative results, according to the duration for which they are run.

Based upon time-dependent changes in responsiveness we made similar predictions in relation to oviposition by Z. bicolorata, as those that were made for feeding. We know less about the temporal patterning of oviposition of Z. bicolorata than we do about feeding. The time-dependent treatments were designed for replicating food deprivation, and the effect of these treatments on responsiveness for oviposition were uncertain. Nevertheless we counted eggs laid in all experiments. The oviposition data do not agree closely with all time-dependent based predictions. More eggs were laid than was expected on Noogoora burr in the just-fed and 3 hours post-meal treatments in the two-choice tests, although the level was less than one egg per female per day. We had predicted no oviposition on the lower ranked host in the presence of the higher ranked target weed. When beetles were introduced into the two-choice test after six days of food deprivation, oviposition had ceased completely and the first eggs were first laid on parthenium only on day three. Previous experiments have indicated that oocytes are resorbed by female Z. bicolorata following 2-3 days of food deprivation (Withers, unpublished data). This conclusion has been reinforced by dissection of deprived females (Withers, unpublished data).

We obtained further information about the oviposition behavior of Z. bicolorata from the no-choice sequential test with parthenium as the control, and eggs counted daily for the full 12 days. This revealed an overall mean egg laying rate of 8 eggs per female per day. Oviposition was apparently affected by external conditions such as handling, e.g., the egg laying rate noticeably reduced following handling of the beetles on days 3 and 8 of the 12 day experiment (Fig. 6). Oviposition was also significantly reduced when beetles were transferred to lower ranked plants. Oviposition on Noogoora burr...
followed an almost identical pattern to feeding, with no oviposition on day 1, although egg laying gradually increased over the next 4 days. On all the lower ranked plants some egg laying continued throughout the no-choice test, but more so on the cage, than on the plants themselves. Oviposition steadily increased towards pre-non-target levels, following the return of beetles to parthenium plants. This pattern is explained by the resorption of oocytes by deprived female beetles, and the resumption in oocyte production following two days of feeding on parthenium.

The oviposition results suggest that oviposition behavior by *Z. bicolorata* on cage structures in no-choice tests may occur because the insects ranked some non-target plants even lower than some neutral surfaces (Withers and Barton Browne, 1998). In conclusion, with *Z. bicolorata*, the results of oviposition trials of a duration greater than 24 hours would indicate that Noogoora burr is within the fundamental host range for oviposition (van Klinken, this volume), although it ranks considerably lower than parthenium weed. This conclusion would not be considered as a false result, but an accurate reflection of the situation in the field.

**Implications and Recommendations for Host Specificity Testing Protocols**

An important issue in host specificity testing is the ability for different tests to accurately predict the likely field host range of an insect. We were fortunate to be able to use hindsight to allow us to compare the outcomes of laboratory assays with field data. In the field, we know that very high population levels of *Z. bicolorata* sometimes occur in combination with a virtual collapse in the availability of their target plant, parthenium weed. This sometimes has a predictable seasonal component, with parthenium weed (and beetles) rapidly appearing following the onset of the rainy season, while parthenium dies soon after the onset of drought. Such was the case with *Z. bicolorata* in India (Narendra, 1990). In this case parthenium weed was completely defoliated and destroyed by large populations of *Z. bicolorata*. This resulted in beetles becoming severely food deprived and some adults accepted sunflower foliage for feeding. A more common event in Australia is oviposition on, and defoliation of, the acceptable but lower ranked weed, Noogoora burr. The ability for biological control researchers to predict such an event from laboratory based assays will always be limited. Our results indicated that, as predicted, it was only when deprived *Z. bicolorata* were placed into choice tests, or sequential no-choice tests were run for greater than two days, that the acceptability of lower ranked non-target plants was revealed.

No-choice tests will always be more effective than choice tests to reveal the acceptability of lower ranked hosts because of the action of time-dependent increases in responsiveness following deprivation from higher ranked hosts. Thus, if a plant is ever to become acceptable to an insect in a naturally occurring time-dependent state, then it is more likely to be expressed during a no-choice test than in any other test. In order to maximize the safety of an introduction of an exotic insect, we recommend therefore that no-choice tests be used to ascertain the maximum range of acceptable hosts. In order to avoid the potentially frustrating occurrence of false positive results (which may be caused by an unrealistic excessive period of deprivation in a no-choice test), choosing an appropriate duration for the no-choice assay is very important. This can only be done after additional information is gathered on the insects natural temporal patterning of feeding and oviposition, their biology, and the effects of time-dependent changes in responsiveness. For instance, significant acceptance of Noogoora burr for feeding occurs after two to three days of food deprivation. This duration is equivalent to the loss of between eight and eighteen normal meals (based on the observation of 4-6 meals per day, depending on beetle age and sex).

Choice tests will continue to be an important test method for ascertaining the relative acceptability of different hosts and to predict which plants will be acceptable under a range of field scenarios (Marohasy, 1998). Our findings reveal that choice tests which include the target do not always reveal the acceptability of lower ranked hosts. On this basis it would be unwise to use multiple choice tests that include any high ranking host plants as the first method to ascertain non-target plants under risk of attack. Reducing the host testing list of plants for subsequent no-choice feeding assays on the basis of such results would be risky. Such an order of testing has a very high potential for producing potentially dangerous, false negative results. In most cases we would recommend that a reduction in the host testing list of plants for more stringent tests be only made on the basis of results from appropriately-designed and run no-choice feeding or oviposition assays.
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