CHOICE OR NO-CHOICE TESTS? EFFECTS OF EXPERIMENTAL DESIGN ON THE EXPRESSION OF HOST RANGE

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ABSTRACT

Estimation of the host range of entomophagous biological control agents (parasitoids and predators) is complex. It is not always possible to inoculate all test organisms with eggs or neonates to determine "physiological suitability". We argue that, for the host range testing of parasitoids, it is important to initially employ test procedures that will maximize the probability that the test species will be accepted for oviposition. This is vital to ensure that our testing methods do not generate data with a false impression of host specificity. No-choice tests are generally thought to maximize the expression of host range. The main reason for this may be increases in readiness to oviposit induced by host deprivation per se and/or associated changes in egg load, which has the potential to counteract any effects of prior experience. Sequential no-choice tests should only be used with caution as they have the potential to produce false negative results if the period of access to the lower ranked host is insufficient to allow time dependent changes in responsiveness of the parasitoid to become apparent, or if insufficient controls are utilized. Choice tests including the target host have the potential to mask the acceptability of lower ranked hosts, thereby producing false negative results. Examples where wider host ranges have been expressed in no-choice tests than in choice tests, and vice versa are presented. Sufficient variation exists that we recommend that researchers routinely use both assay methods for host range testing of parasitoids and predators.

INTRODUCTION

The most common methodologies employed for host range estimation are no-choice and choice tests (Van Driesche and Murray 2004). The way that scientists decide on the appropriate laboratory-based methodologies for the accurate estimation of field host range of proposed biological control agents however is an interesting issue. The accurate assessment of field host range of parasitoids and predators is complex because of the relationships the target and test organisms invariably have with their food plant. It is critical therefore that all potential non-target impacts are elucidated by the methodologies selected.

The assessment of host range in endoparasitoids is complicated as it is usually not possible to inoculate all test organisms with eggs or neonates to determine "suitability" (although

exceptions do exist, Fuester *et al.* 2001; Morehead and Feener 2000;). Such inoculation tests require an experimental separation between the act of oviposition and subsequent larval development. This is commonly achievable for herbivorous insects but is generally impossible for endoparasitoids. Thus, a testing regime to determine the host range of endoparasitoids is usually denied a useful tool: the so-called physiological host range test.

Whether it is parasitoids or predators that are under consideration as potential biological control agents, it is important to employ test procedures that will maximize the probability that the test species will be accepted for feeding or oviposition (Withers and Barton Browne 2004). Unless acceptance of at least one of the offered hosts occurs, there is a danger that a lower ranked but potential host may be left out of further experimental analysis. Without this acceptance being revealed, a realistic risk assessment process cannot proceed. We believe some test designs can definitely produce false negative results, and it is this we want to eliminate in host testing. In this paper, we discuss the potential implications of choice and no-choice test designs on maximizing the expression of host acceptance. This will focus primarily on oviposition in parasitoids, although most of the concepts are also relevant to predators.

Behavioural and physiological factors. In the chapter by Withers and Barton Browne (2004), the potential influences of various factors on the expression of host range in parasitoids and predators was reviewed. In theory, factors such as the physiology of the parasitoid and aspects of the test design such as the proportion of target to non-target species have the potential to impact on the outcomes of host range assays by altering the probability the parasitoid will attack non-target species. Withers and Barton Browne (2004) concluded that prior experience and time-dependent state of the parasitoid could alter the test outcomes, and the impact of these factors on the test outcomes could differ with different test types. We will briefly discuss three of these factors and then examine the test designs in more detail.

EFFECTS OF EXPERIENCE

Thanks to the high quality of the literature (e.g., Turlings *et al.* 1993; Vet *et al.* 1995), we now have a good appreciation of the complexity of experience effects on host-related behaviour in parasitoids (Withers and Barton Browne 2004). Significantly altered behaviour has been demonstrated in relation to experience by the adult parasitoid of the host it was reared in or on (rearing host), the complete plant-host complex and/or some of its components. This behaviour modification can occur with or without oviposition into hosts. There is strong but indirect evidence that any enhancement in responsiveness to a familiar host or plant-host complex is generally greater than any enhancement in responsiveness to an unfamiliar (novel) non-target or its plant-host complex (Fujiwara *et al.* 2000; Petitt *et al.* 1992).

It is commonly expected that an experienced parasitoid will be biased towards the host or plant-host complex that it experienced during rearing or previous laboratory trials. What influence this has on host range tests depends (i) upon the history of the parasitoids used in the tests, (ii) how the target and non-targets are presented in the tests, and (iii) the magnitude and nature of the effects of the previous experience. For example, the experience gained by a parasitoid of the rearing host and its host plant during larval development and subsequent adult emergence is likely to result in enhanced responsiveness to cues from this plant-host complex. Such an effect would be reinforced by continued contact with, and possibly ovipo-

sition experience on, the same plant-host complex, especially if the parasitoids were not removed from the rearing colony before or shortly after eclosion.

There are ways that experience-induced bias towards the target species can be reduced. The most difficult effect to avoid is any enhanced responsiveness towards the rearing host (which is usually the target pest) as a result of experience acquired at eclosion or shortly afterwards. For crucial tests, methods such as dissecting the parasitoid pupae out of the host (for endoparasitoids) or removing it from the host (ectoparasitoids) and washing the exterior of the parasitoid pupal case prior to eclosion can be used. This is probably the only method that can be applied to reduce experience effects in oligophagous parasitoids that have no high quality alternative host for rearing. The presentation of target and non-target species to the parasitoid on a neutral or "inert" substrate such as artificial diet or glass is a valuable means of avoiding a possible bias towards the parasitoid host's plant that was used during rearing. However, this is often impossible wherever test species are inseparable from their plants, such as with internally placed eggs, internally feeding larvae or when test species require the presence of the food plant for the duration of the assay. The most practical solution to minimize bias as a result of prior experience is collecting the parasitoids immediately after they have eclosed from their pupae and storing them in the absence of hosts and plant material (unless this is also food for the parasitoid).

READINESS TO OVIPOSIT WITH HOST DEPRIVATION

Another significant influence on insect behaviour, and hence the outcome of host testing will be the impact of time-dependent changes in responsiveness (Barton Browne and Withers 2002). This has been defined as changes in threshold in relation to elapsed time since an insect last fed or oviposited. The behavioural threshold for the acceptance of hosts can be expected to decrease with increasing periods of deprivation. Therefore female parasitoids that have been deprived of oviposition will show greater responsiveness to cues associated with oviposition sites (Barton Browne and Withers 2002; Papaj and Rausher 1983).

The most important practical result of this is that the probability of a parasitoid attacking a non-target host species that induces a lower stimulation to oviposit (is "lower ranked") increases with the period of time since they last successfully oviposited. Increased acceptance of lower ranked hosts by *Holomelina lamae* Freeman as time elapses since they eclosed may be an example of this (Fig. 1).

Further evidence for this phenomenon in parasitoids comes from experimental work on superparasitism, as superparasitized hosts are known to be lower ranked. Hosts already parasitized by conspecific females are increasingly accepted for oviposition by female parasitoids as they become increasingly deprived (e.g., Hubbard *et al.* 1999; Klomp *et al.* 1980). Similarly parasitoids that have recently suffered from a low encounter frequency with unparasitized hosts (e.g., Babendreier and Hoffmeister 2002) subsequently show increased acceptance of parasitized hosts. So in conclusion, time-dependent increases in responsiveness will act to increase the probability that lower ranked or non-target hosts will be accepted for oviposition in test assays.

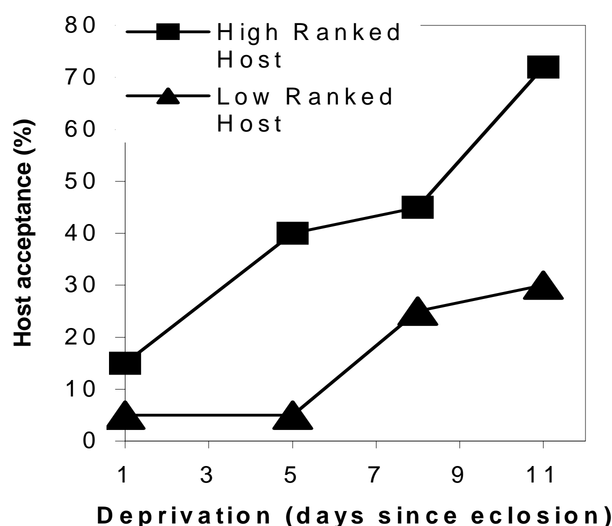

Figure 1. Probability of acceptance of higher ranked host, *Lymantria dispar* (L.) in no-choice tests, compared to the lower ranked host, pupae of *Holomelina lamae* Freeman by host-deprived *Brachymeria intermedia* (Nees). Adapted from Drost and Cardé (1992).

OVIGENY CHARACTERISTICS

Life history theory predicts that stimulation to oviposit is influenced, at least in part, by egg load (Mangel 1989). There is an abundance of empirical data that supports this prediction for parasitoids (Withers and Barton Browne 2004). However the effect of host-deprivation on egg load, and therefore the potential contribution of deprivation to any increased readiness to oviposit, is totally dependent on ovarian physiology. For example, a female of a pro-ovigenic species does not increase its egg load during host-deprivation so any increase in readiness to oviposit in a pro-ovigenic species cannot be attributed to egg load. Conversely, females of synovigenic species may increase their egg load, up to a point, during a period of host deprivation (e.g., Eliopoulos *et al.* 2003). The extent to which this happens is dependent on the nutritional reserves stored within the body and/or the availability of foods during the period of deprivation. This is particularly relevant in parasitoids that also feed on their hosts as host-deprivation will deprive the females of both nutrients for oogenesis as well as depriving them of the opportunity to oviposit. For example, when the host-feeding species, *Aphytis melinus* DeBach is maintained on honey but deprived of hosts, there is a reduction in egg load due to oosorption (Collier 1995). It is therefore vital that the ovarian physiology of the parasitoid is understood prior to the selection of host testing methodology, in order to understand its potential influence on the outcome of host tests.

No-choice tests. No-choice tests present the potential biological control agent with one non-target test species at a time. Thus if 10 non-target species are to be tested, there will be a series of ten cages (with replicates for each), plus appropriate controls (Van Driesche and Murray 2004). It is not usual for all tests to be undertaken at exactly the same time, due to the phenology and seasonality of the non-targets and availability of adult parasitoids, but this is acceptable if tests are sufficiently replicated with appropriate target species controls.

Potentially many factors could influence the outcome of no-choice tests. Time-dependent changes in responsiveness are likely to be significant factors acting upon parasitoids when subjected to tests with hosts that produce a lower stimulation to oviposit (are lower ranked). Encounter rates are likely to be lower if the test hosts are presented on plants/substrates that induce lower or no innate host searching preference. This, and the lower preference for the test host may lead to low oviposition rates. As discussed above both low encounter rates and low oviposition rates during the test have the potential to result in an increase in host-deprivation in the parasitoid (Barton Browne and Withers 2002).

In no-choice tests, if the parasitoid has had any experience of the target or aspects of the target's plant-host complex, this may act to reduce the probability of acceptance of unfamiliar hosts (non-targets). It is not known how long lasting the effects of experience are (Barton Browne and Withers 2002). What is likely however is that time-dependent effects have the potential to override the effects of experience if the duration of the no-choice test is long enough. This is why there are significant benefits in undertaking behavioural observations during host range tests. Only observation will elucidate whether temporal changes in attack behaviour are present that would indicate time-dependent changes in responsiveness are acting upon the parasitoid.

SEQUENTIAL NO-CHOICE TESTS

Although not commonly used, it is important we also consider the method of sequential no-choice tests in which insects are given no-choice access to a sequence of two or more test species, in which the target species is also presented at least once in the sequence. In parasitoid host testing, sequential no-choice tests are almost invariably used to assess host acceptance behaviours for oviposition. The sequence chosen for the presentation of target and non-target species can be varied according to the biology of the parasitoid, as can the duration of presentation and any "rest" durations between presentations.

A theoretical analysis of the potential outcomes of some sequential no-choice experimental designs in phytophagous insects has been undertaken (Barton Browne and Withers 2002). One of the most popular designs is the test sequence A - B - A (where A was the higher ranked host, and B a lower ranked, although acceptable host). Barton Browne and Withers (2002) concluded that the outcome of sequential no-choice tests varied according to the period of time for which the insects were given no-choice access, particularly access to host B. If the parasitoid oviposited during the first access to host A (which was often the aim – to ensure the parasitoid was physiologically and behaviourally ready to oviposit), it may not accept host B when it first entered its no-choice access to the non-target host B. Whether it does accept host B during the test depended on whether the test was run for a sufficient length of time for time-dependent processes to act upon the parasitoid to lower its acceptance threshold to a level whereby host B stimulated attack behaviour. Hence the chance that the lower ranked host was scored as unacceptable was negatively related to the duration of the period of access to this host. To help control for time-dependent effects, a control should be run at the same durations of presentation of the order A – A – A.

Another variation on the sequential no-choice test gives "rest" periods (deprivation) where no hosts are available, in between the periods of access to hosts. This allows time-

dependent effects to increase the stimulation to oviposit during the period of no access to hosts, and in theory should increase the probability that the parasitoids will oviposit in host B. This is, in effect, equivalent to prolonging the period of access to less preferred hosts in a sequential no-choice test (Barton Browne and Withers 2002).

Sequential no-choice tests of the design B – A – B – A – B – A for 2 hours each with no rest period between tests (where A is the target, and B the non-target species) were used to test oviposition responses of *Trichopoda giacomellii* (Blanchard) (Tachinidae) (Coombs 2004). This method was chosen instead of multiple choice tests where the authors were concerned false positive results might occur due to priming (i.e. central excitatory state caused by the presence of target species). The exposure duration was chosen “after observing oviposition patterns of the parasitoid on its target host”. It is likely the duration was appropriate to the biology of *T. giacomellii* because the non-target native species *Glaucias amyoti* (White) were attacked during their 2 hour presentation time and the test results have since been supported by post-release field studies showing *G. amyoti* is being parasitized at a comparable low level in the field (1%) (Coombs 2004).

Porter and Alonso (1999) used another variation of sequential no-choice oviposition testing. These experiments used a design of A – B and B – A, with presentation times of 60-90 mins with a variable duration of 30 mins or more between presentations to recapture flies. This method permits the comparison of what effect prior oviposition experience on a target (A) has on the acceptance of the non-target (B). This example is interesting in that it has the appearance of central excitation. The only instances where both parasitic flies *Pseudacteon tricuspis* Borgmeier and *Pseudacteon litoralis* Borgmeier attacked the non-target (B) native fire ant *Solenopsis geminata* Forel were when they were first presented some time after the no-choice test on the target A (imported fire ants). It is not known how long the effects of central excitation last, but they are generally considered to be short lived. The duration between presentations in this case therefore probably excludes central excitation as an explanation. Controls of the design A – A could also have been employed here to elucidate any temporal patterning of oviposition.

Sequential no-choice tests of the design A – B – A were used by Gilbert and colleagues (Porter and Gilbert 2004) with the aim being to screen the motivational status of field-caught flies, which were the only ones available for host specificity testing at the time. Only those individuals that successfully attacked the first presentation of the target host A (imported fire ants, *Solenopsis* spp.) were used in the following B – A tests. Seldom are the effects of oviposition experience effectively understood or controlled for in these sequential tests. But this is always the case when field-caught individual parasitoids are used in host range testing. This level of uncertainty may be taken into account to some extent with the use of non-parametric statistical tests appropriate to sequential, non-independent data sets.

Our conclusion on the use of sequential no-choice oviposition testing of parasitoids are that it should be attempted with caution, and only when the physiology and behaviour of the parasitoid is understood in terms of its temporal patterning of oviposition. This is due to the high risk that a test of the design A – B – A, where the duration of access to the non-target B is too short, will produce a false negative result.

Choice tests. In choice tests, two or more host species are presented to the test insect simultaneously and thus the response is a measure of preference for one species in the presence of another species (Van Driesche and Murray 2004). Tests that offer more than two choices pose several challenges for experimental design as well as for statistical analysis (Hoffmeister 2005; Mansfield and Mills 2004). In the context of non-target risk assessment for biological control, the comparison between the target host and a single non-target host is usually more straight forward than a multiple choice situation.

It is generally expected that host preferences will be more clearly expressed by parasitoids in choice tests compared to in no-choice tests. This is because the impacts of time-dependent changes in responsiveness (that increase host acceptance of lower ranked hosts), as discussed above, will not occur when high ranked hosts are available for oviposition (Van Driesche and Murray 2004). For example, when a parasitoid enters a choice test containing two species of host (one high ranked, the other low ranked in relative acceptability) and each host is offered on its own food plant (Barton Browne and Withers 2002), we assume that the high ranked hosts will be contacted and accepted for oviposition first due to an inherent preference in the parasitoid for searching the food plant of the high ranked host first. Therefore when the lower ranked hosts are eventually located in the cage, they are less likely to be attacked, as they shouldn't stimulate the parasitoid sufficiently to oviposit. The outcome of choice tests therefore are expected to be a greater difference in parasitism (or attack rate, searching time, proportion of parasitoids produced) between the target and lower ranked host than would be expressed in a no-choice tests.

There are a number of other aspects of a choice test that have the potential to alter the outcome of the test (e.g., ratio of host abundance, the duration of the test permitting all target hosts to become parasitized). The effects of experience either with or without access to the plant-host complex may increase responsiveness towards the experienced host species or decrease responsiveness away from the novel non-target species. Any such experience-induced increases in responsiveness towards the target would in effect exaggerate the apparent difference between the rankings of the two hosts. This has implications for the interpretation of results from choice tests, particularly when (as is often the case) the target species and non-target species are presented on different host plants. In choice tests, increased contrast in ranking between the plant-host complexes would, in itself, increase the probability that attack on the non-target species will fail to be revealed.

COMPARING RESULTS OF NO-CHOICE TO CHOICE TESTS

While taking species-specific ovarian physiology into account as was discussed above, we can see that both time-dependent increases in responsiveness as well as effects of experience, many of which are unavoidable, are responsible for why we expect parasitoids to show a wider host range (greater acceptance of non-target species) when tested in no-choice tests than in choice tests that include their target host. This concept of greater acceptance in no-choice situations has also been clearly demonstrated with parasitoids expressing host acceptance behaviour for different developmental stages of the same host species. For instance Neveu *et al.* (2000) showed that in no-choice tests the parasitoid *Trybliographa rapae* Westwood (Figitidae) accepted and reproduced equally in first, second and third instars of the cabbage root fly, *Delia radicum* L

(this was not explained by any superparasitism). However when all larval stages were offered simultaneously to parasitoids in an equivalent choice test, an oviposition preference was clearly expressed towards the third instar (Neveu *et al.* 2000).

If we generally expect to see a wider host range expressed by parasitoids from no-choice tests than from choice tests, then this should be reflected in results from the literature. Some examples that support this conclusion have been summarized in Table 1. Note the majority of these examples are of quantitatively greater acceptance in no-choice than in choice tests.

It would be tempting to generalize that no-choice tests are the most suitable laboratory assay for revealing the maximal physiological host range of parasitoids. It is a well accepted notion in weed biological control that a no-choice test will seldom produce a false negative result (Hill 1999; Marohasy 1998; Van Driesche and Murray 2004). However, as mentioned above, parasitoids and predators bring a whole new level of complexity to laboratory assays. There are just as many examples in the literature where both no-choice and choice tests revealed extremely similar results in terms of the host acceptability (Table 2). This suggests both methods can be equally suitable for revealing attack on non-targets. Of more concern are examples of parasitoids where non-target attack has occurred in a choice test, which was not revealed in a no-choice test.

We are aware of only two unambiguous examples where parasitoids attacked a non-target species in choice tests but did not attack those same species in no-choice tests. The first example is of the parasitoid *Sphecophaga vesparum* Curtis (Ichneumonidae) being investigated as a biocontrol agent for *Vespula germanica* (F.) and *V. vulgaris* (L.) (Field and Darby 1991). *Sphecophaga vesparum* oviposited in (and then successfully developed in) two adjacent larvae within wax cells obtained from a hive of the non-target wasp *Ropalidia plebeiana* Richards. This occurred however, when the larvae had been presented in a choice test alongside cells of the target wasp, and were not guarded by adult wasps as would occur in the field. In the equivalent no-choice tests, no parasitism occurred on the unguarded non-target wasp larvae (Field and Darby 1991). The authors implied that *S. vesparum* may have been stimulated to oviposit in the nearby non-target cells because of the presence of their natural host and/or the preferred food source which is saliva of larval *Vespula* spp. (the target) in the choice test. One possible behavioural explanation for this observation may be that stimulation elicited by kairomones of the target species or the ingestion of the target saliva have generated an excitatory state in the female parasitoids central nervous system leading her to accept non-target species ("central excitation" *sensu* Dethier *et al.* 1965). In the field these species are unlikely to nest in such close proximity (*V. germanica* nests are subterranean and *R. plebeiana* nests are arboreal), leading to the conclusion that the result of the no-choice test is likely to reflect the field situation in this case.

In the second example, *Aphidius rosae* Haliday (Braconidae) showed complete rejection of the non-target *Macrosiphum euphorbiae* (Thomas) when presented on the same host plant (rose) as their target host *Macrosiphum rosae* (L.) but only when the parasitoid had prior oviposition experience acquired after being held with *M. rosae* during the preceding two days (Kitt and Keller 1998). Naïve *A. rosae,* in comparison, showed similar attack rates on the non-target *M. euphorbiae* in no-choice tests but a direct comparison was not available (see Table 3 in Kitt and Keller 1998). One possible behavioural explanation for this outcome is that host

Table 1. Examples of non-target species that received greater attack in no-choice tests than in choice tests that included the target.

Parasitoid Species	Parasitoid Family	Target	Non-target accepted more in no-choice test	Conclusion	Reference
Pelidnoptera nigripennis (F.)	Diptera: Sciomyzidae	*Ommatoiulus moreleti* (Lucas)	Two species of Paradoxosomatidae	Eggs dislodged so no development on non-targets	Bailey (1989)
Thripobius semiluteus Boucek	Hymenoptera: Eulophidae	*Heliothrips haemorrhoidalis* Bouché	*Hercinothrips bicinctus* Bagnall	Species not attacked in the field	Froud and Stevens (2004)
Trichogramma platneri Nagarkatti	Hymenoptera: Trichogrammatidae	*Cydia pomonella* (L.)	*Sitotroga cerealella* (Olivier)	Sitotroga was not parasitized in multiple choice tests	Mansfield and Mills (2004)
Cotesia rubecula (Marshall)	Hymenoptera: Braconidae	*Pieris rapae* (L.)	*Pieris napi olearacea* (L.)	Never yet attacked in field, but is in both test types	Van Driesche *et al.* (2003)

Table 2. Examples of non-target species being attacked at a similar rate in both choice tests with target and in no-choice tests.

Parasitoid Species	Parasitoid Family	Target	Non-target accepted similarly	Conclusion	Reference
Microctonus hyperodae Loan	Hymenoptera: Braconidae	*Listronotus bonariensis* (Kuschel)	*Nicaeana cervina* (Broun)	Pupal parasitism rates consistent	Goldson *et al.*, (1992); Barratt *et al.* (1997b)
Cotesia glomerata (L.)	Hymenoptera: Braconidae	*Pieris napi oleracea* (L.)	*Pieris rapae* (L.)	Parasitism rates consistent in both test types	Van Driesche *et al.* (2003)
Pseudacteon curvatus Borgmeier	Diptera: Phoridae	*Solenopsis invicta* Burden	*S. geminata* (F.), *S. xyloni* (MacCook)	Parasitism rates generally consistent	Porter (2000)
Laricobius nigrinus Fender	Coleoptera: Derodontidae	*Adelges tsugae* Annand	*Adelges piceae* (Ratzeburg), *Adelges abietis* (L.), *Pineus strobi* (Hartig)	Oviposition preferences (mean number of eggs laid in ovisacs) consistent	Zilahi-Balogh *et al.* (2002)
Dichasmimorpha kraussii (Fullaway)	Hymenoptera: Braconidae	*Bactrocera latifrons* (Hendel)	*Eutreta xanthochaeta* Aldrich	Probing responses and parasitism consistent	Duan and Messing (2000)

acceptance behaviour in favour of the target host was only modified in parasitoids with prior oviposition experience of the test host.

The example of attack of the non-target weevil *Sitona lepidus* Gyllenhal by *Microctonus aethiopoides* Loan (Barratt *et al.* 1997a) is sometimes quoted as being an example of a greater level of attack in choice than in no-choice tests (Van Driesche and Murray 2004). However the apparent difference in parasitism on *S. lepidus* (6% in choice c.f. 1% in no-choice) may be partially explained by a rapid host immune response. We cannot exclude the possibility, however, that a heightened excitatory state was induced in the parasitoid through being held in the presence of both target and non-target adult weevils within the choice test cage (Barratt *et al.* 1997a).

To summarize, particularly for polyphagous parasitoids, choice tests may be more suitable than no-choice tests for assessing the order of preference if the hosts are closely ranked (Mansfield and Mills 2004; Van Driesche and Murray 2004). Returning to the example from Kitt and Keller (1998), the use of naïve parasitoids (no prior oviposition experience with the target) produced the more useful data for the estimation of non-target species at risk using no-choice tests, whereas relying on oviposition-experienced parasitoids would have produced a false negative result. In parasitoids, Van Driesche and Murray (2004) suspect that false negative results in a choice test also containing the target species may be less likely than in herbivorous insects, and that the potential for false positives may in fact increase. Barratt (2004) similarly believes that choice tests can contribute different information but are probably less informative for insect rather than weed biological control agents. Our conclusion is that as the host range of parasitoids predicted by both methods differs, both methods should ideally be used in combination. Whether a wider host range is expressed in no-choice or choice tests depends on the species tested and on the relative strengths of any deprivation effects acting on the one hand, and the effects of experience and/or central excitation acting on the other. In many cases behavioural observations during both choice and no-choice tests could be instrumental in allowing us to make accurate interpretations of the data, and their value cannot be underestimated.

CONCLUSIONS

No-choice tests remain the most useful method for assessing host acceptance behaviour of parasitoids. As the duration of no-choice tests increases, the potential for time-dependent effects to act upon the parasitoid will increase. Similarly because of time-dependent effects, sequential no-choice tests should be attempted with caution as false negative results can occur when the period of exposure to non-targets is too short.

In choice tests, host experience may have a significant influence on the expression of host preference. Exposure to the host or plant-host complex at eclosion, even without actual oviposition experience, can bias host preference towards the natal host, obscuring acceptance of lower ranked hosts. This should be minimized by collecting the parasitoids immediately or soon after eclosion. The presentation of the hosts during the test itself (on an inert substrate, on the same host plant, or on different host plants) may overcome the potential effects of experience.

Finally, we believe that as the parasitoid host range predicted by the host range testing methods discussed in this paper have been shown to differ, ideally both no-choice and choice methods should be used in combination. In unusual cases where the results predicted by no-choice and choice tests differ significantly, further research will be required. The biology of the natural enemy involved will need to be examined and ideally the behavioural mechanism responsible for the discrepancy should be elucidated. Undoubtedly as more research is carried out on this topic, our understanding of how to interpret the results of different types of tests will increase.

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PARASITOID CASE HISTORY: AN EVALUATION OF METHODS USED TO ASSESS HOST RANGES OF FIRE ANT DECAPITATING FLIES

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ABSTRACT

The first three papers in this section have discussed factors that affect the efficiency and success of laboratory host range tests. This paper presents an evaluation of how well those factors applied to our investigations of host ranges of fire ant decapitating flies in the genus *Pseudacteon* (Diptera: Phoridae). We initially discuss the nature of the fire ant problem (Hymenoptera: Formicidae: *Solenopsis* spp.) and the need for effective self-sustaining biological control agents. We briefly review the biology of *Pseudacteon* decapitating flies, the overall results of our host range tests, and the current status of field releases of these biological control agents. We conclude by discussing how well the recommendations of the three initial papers about 1) statistical procedures, 2) biotypes and cryptic species, and 3) experimental design, plus a recent book on the subject of host range testing, apply to our experiences with fire ant decapitating flies.

BACKGROUND OF PARASITOID SYSTEM

THE FIRE ANT PROBLEM AND NEED FOR SELF-SUSTAINING BIOLOGICAL CONTROL

The major problem with invasive fire ants (Hymenoptera: Formicidae: *Solenopsis* spp.) is that there are so many of them. In north Florida pastures, fire ant densities average 1,800-3,500 ants per square meter or about 1.5-3.0 metric tons of fire ants per square kilometer (Macom and Porter 1996; converted from dry weight to wet weight). Economic damage to agriculture, electrical equipment, and human health in the United States is estimated at nearly 6 billion dollars per year (Lard *et al.* 2001; Pereira *et al.* 2002), not including environmental damage.

Fire ant populations in their South American homeland are about 1/5 as dense as populations normally found in North America (Porter *et al.* 1997). This intercontinental difference in fire ant densities was not explained by differences in climate, habitat, soil type, land use, plant cover, or sampling protocols (Porter *et al.* 1997). Escape from numerous natural enemies left behind in South America is the most apparent explanation for the intercontinental population differences. Classical or self-sustaining biological control agents are currently the only potential means for achieving permanent regional control of fire ants.

BIOLOGY OF *PSEUDACTEON* DECAPITATING FLIES

Information on the life history, phenology, and biogeography of South American *Pseudacteon species*, is accumulating (Porter 1998a; Folgarait, *et al.* 2002; 2003; 2005a; 2005b; Calcaterra *et al.* 2005). At least 20 species of *Pseudacteon* flies (Diptera: Phoridae) have been found attacking fire ants in South America (Porter & Pesquero 2001; Brown *et al.* 2003). Up to nine species of these flies have been found at a single site (Calcaterra *et al.* 2005). Each species has a distinctively shaped ovipositor that is presumably used in a lock-and-key fashion to lay eggs in a particular part of its host's body. Female flies usually contain a hundred or more eggs (Zacaro & Porter 2003). During oviposition, one egg is rapidly injected into the ant thorax with a short hypodermic shaped ovipositor (Fig 1A). Shortly after hatching, maggots of *Pseudacteon* flies move into the heads of their hosts where they develop slowly for two to three weeks (Porter *et al.* 1995a). Just prior to pupation, the third instar maggot appears to release an enzyme that dissolves the membranes holding the exoskeleton together. The maggot then proceeds to consume the entire contents of the ant's head, a process that usually results in rapid decapitation of the living host. The headless body is usually left with its legs still twitching (Fig. 1B).

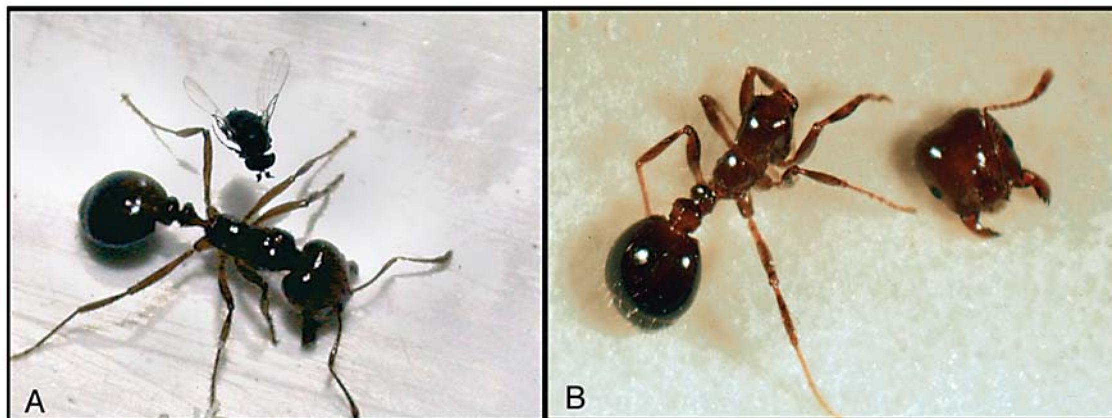

Figure 1. A) Female decapitating fly (*Pseudacteon*) preparing to inject an egg into the thorax of a fire ant worker (*Solenopsis*). B) Decapitated fire ant worker with a fly maggot consuming the contents of its head. UGA1390062, UGA1390063

The maggot then uses hydraulic extensions to push the ant's mouth parts aside, after which it pupates within the empty head capsule, positioned so that the anterior three segments harden to form a plate that precisely fills the ant's oral cavity (Porter 1998a). The rest of the puparium remains unsclerotized and is protected by the ant's head capsule, which functions as a pupal case. Pupal development requires two to three weeks depending on temperature.

Adult flies are generally mature and ready to mate and oviposit about three hours after emergence. Based on laboratory observations at 20 °C, adult *Pseudacteon* flies may live up to two weeks (Chen *et al.* 2005); however, higher temperatures and activity associated with oviposition will shorten their lives to one to three days (Porter 1998a). Once phorid attacks commence, fire ant workers become keenly aware of the presence of the flies. A single female fly usually stops or greatly reduces the foraging efforts of hundreds of fire ant workers in only a minute or two (Porter *et al.* 1995b). As soon as a fly appears, most workers rapidly retreat into exit holes or find cover. Other workers curl into a stereotypical c-shaped posture (Porter 1998a). Some fly species inhibit fire ant foraging as long as they are present, often for periods of several hours (Folgarait & Gilbert 1999; Wuellner *et al.* 2002). Reduced foraging activity appears to facilitate competition from ants that might otherwise be excluded from food sources in fire ant territories (Feener 1981; Orr *et al.* 1995; Morrison 1999; Mehdiabadi & Gilbert 2002). The overall impact of these flies on fire ant populations is unknown; however, it is clearly sufficient to have caused the evolution of a number of phorid-specific defense behaviors (Porter 1998a).

HOST SPECIFICITY OF *PSEUDACTEON* DECAPITATING FLIES

Based on the highly specialized behavior and life history of *Pseudacteon* flies, we conclude that they pose no threat to any arthropod except for ants (Porter 1998a). Based on the results of our host range tests (Porter & Gilbert 2004), we conclude that *Pseudacteon* decapitating flies are only a realistic threat to fire ants in the genus *Solenopsis*. None of the flies tested, to date, were attracted to other genera of ants in the field (Porter *et al.* 1995c, Morrison & Porter 2005c, Vazquez & Porter 2005) and the few attacks that occurred in the laboratory did not produce any parasitized workers (Porter & Gilbert 2004). It is theoretically possible for *Pseudacteon* phorids to switch to ant hosts in different genera because several species have done just that during the process of evolution (Disney 1994). However, this is only likely to occur in evolutionary time scales of hundreds of thousands of years. Even then, such switches would be limited to a small subset of ants of similar size (Porter 1998a). A major constraint on the evolution of host shifts and the broadening of host range is that phorids apparently use species-specific alarm pheromones to locate ant hosts (Vander Meer & Porter 2002). In almost eight decades of exposure to an expanding population of *S. invicta,* none of several species of *Pseudacteon* flies which attack native fire ants in North America have made the shift to the more abundant introduced species. All comparative and experimental evidence weighs heavily against the possibility that any of the fire ant decapitating flies from South America would ever become a generalist parasite of ants within ecological or microevolutionary timeframes.

Several of the *Pseudacteon* species proposed for release present a finite but acceptable risk to the native fire ants *Solenopsis geminata* (Forel) and *Solenopsis xyloni* MacCook (Porter & Gilbert 2004). The primary risk suggested by our specificity testing is that occasional attacks on these non-target native ants might occur. Several *Pseudacteon* species can also complete development in native fire ants. However, all of these species are much more successful at attacking imported fire ants than either of the native fire ant species tested. They also have a strong preference for imported fire ants over native fire ants when allowed to choose. These data justify a conclusion that *Pseudacteon* flies present a much greater risk to imported fire

ants than either of the native fire ants tested. This being the case, the likelihood is that these flies will actually benefit native fire ant species rather than harm them because imported fire ants are the primary enemy of native fire ants (Porter 2000). Furthermore, risks to native fire ants must be balanced against the possible benefits of these flies to hundreds of native arthropods and dozens of native vertebrates threatened by high densities of imported fire ants (Wojcik *et al.* 2001). This small risk is justified, in light of the benefit of finding an economic, self-sustaining, and target-specific biological control of imported fire ants.

RELEASE AND ESTABLISHMENT OF DECAPITATING FLIES IN THE UNITED STATES

Field introductions of South American fire ant decapitating flies in the United States began after careful analyses of risks and benefits as elaborated in three Environmental Assessments for field release which the authors separately prepared with and for officials at USDA/APHIS six, eight, and ten years ago. Three species of South American decapitating flies have been released in the United States. The first species was *Pseudacteon tricuspis* Borgmeier in Texas (Gilbert & Patrock 2002) and Florida (Porter *et al.* 1999). This fly attacks medium to medium-large fire ants and is especially abundant in the fall. A biotype of this species from near Campinas, Brazil is well established in eight states in the southeastern United States. Flies released in Florida have spread at least 180 km from their release sites (Porter *et al.* 2004). A second biotype of this species from northern Argentina has been released at several sites in Texas along with the first biotype, but its establishment, while likely, still needs to be confirmed by biochemical markers. Two biotypes of *Pseudacteon curvatus* Borgmeier have also been established in the United States, one on black and hybrid fire ants in Alabama, Mississippi, and Tennessee (Graham *et al.* 2003; Vogt & Streett 2003; Parkman *et al.* 2005) and the other on red fire ants in Florida (Vazquez *et al.* 2005), South Carolina (Davis & Horton 2005), and Texas (L.G. unpublished). This fly only attacks small fire ants and is especially abundant in the late summer. Impacts of this fly have yet to be assessed, but this fly often occurs in higher densities than *P. tricuspis*. A third species of decapitating fly, *Pseudacteon litoralis* Borgmeier, has been released at two sites in north Florida (Summer 2003, Fall 2004). First generation flies were recovered, but establishment has not been confirmed. This fly attacks medium-large to large fire ants and is most active in the morning and late afternoon until dark. A fourth species of decapitating fly, *Pseudacteon obtusus* Borgmeier, is being held in quarantine until permits can be obtained for its field release.

Studies of the impacts of these flies are ongoing, but field studies show that the impacts of a single species of fly (*P. tricuspis*) are not enough to rise above the 10-30% sensitivity of field tests (Morrison & Porter 2005a; 2005b). The introduction of additional species of decapitating flies and other natural enemies will increase the likelihood of permanently reducing imported fire ant populations in the United States.

EVALUATION OF RECOMMENDATIONS

The preceding authors in this section (Hoffmeister 2005; Hopper *et al.* 2005; Withers & Mansfield 2005) and those in a recent book (Van Driesche & Reardon 2004) have made a number of recommendations about procedures for assessing the host ranges of potential self-sustaining biological control agents from foreign countries. For the purposes of discussion,

we will divide these recommendations into six categories: 1) existing knowledge about the taxonomy and host specificity of potential biological control agents; 2) the importance of biotypes and cryptic species in host range tests; 3) selecting appropriate non-target organisms for testing; and 4) choosing the best ways to handle and select biological control agents for specificity tests; 5) experimental design for assessing host specificity; and 6) recommendations for proper statistical analysis of experimental data. We will proceed to discuss how well recommendations in each of these categories applied to our studies of the host ranges of fire ant decapitating flies.

EXISTING KNOWLEDGE

Explore literature. Generally, the first recommendation in assessing host ranges is to explore existing literature about identification and host records of potential biological control agents (Sands & Van Driesche 2004; Hoddle 2004). This is important advice. When we searched the literature, we found that all *Pseudacteon* species with host records had been collected attacking ants. We also found that more than 20 species of *Pseudacteon* flies had been described that attacked *Solenopsis* fire ants (Borgmeier 1925; 1962; 1969; Borgmeier & Prado 1975; Disney 1994). Indeed it appeared that *Pseudacteon* had diversified in a fire ant adaptive zone.

Contact experts. Hoddle (2004) recommended that taxonomists, museum curators, and other experts should be contacted for information. Contacting experts provided us with a wealth of information early in our programs. In particular, phorid specialist, Brian Brown shared his "*Pseudacteon* scrapbook" with us. This resource included references, descriptions, and illustrations for most of the species of flies that attacked fire ants. He also assisted with identifications when existing keys to the genus proved marginal and he provided taxonomic advise on numerous other occasions. David Williams and Don Feener provided additional literature about *Pseudacteon* flies as well as advise about their biology. Harold Fowler introduced SDP to these flies in the field. Roberto Brandão provided access to Thomas Borgmeier's collections at the Museum of Natural history in São Paulo. Roger Williams and Angelo Prado were also consulted about work they had done with these flies. In short, our colleagues provided an important foundation on which we were able to build.

Identification errors. Sands & Van Dreische (2004) warn that care must be taken to evaluate and validate old host records because some are not reliable. Indeed, we found two instances where improper identification of ant host records made it appear that three species of flies were less specific than they really are (Porter & Gilbert 2004). We also found evidence that a fourth species is likely more specific than generally reported (Porter & Gilbert 2004).

BIOTYPES AND CRYPTIC SPECIES

Hopper *et al.* (2005) caution that host range testing needs to be done on each new population of biological control agents being considered for field release. This is because cryptic species or biotypes can have different degrees of host specificity. We found this to be true with at least two species of *Pseudacteon* flies. In particular, we found that *P. tricuspis* appears to be two cryptic species, one of which attacks red fire ants and the other of which attacks black fire ants (Porter and Pesquero 2001). Similarly, we found that a biotype of *P. curvatus* collected from black fire ants in Buenos Aires, Argentina could not be established on red fire ants in the

United States while a biotype of *P. curvatus* originally from red fire ants in Formosa, Argentina was easily established on red fire ants in the United States (Vazquez *et al.* 2005). We also found that the two *P. curvatus* biotypes differed in their abilities to attack and develop in the two non-target native fire ants in North America (Porter 2000; Vazquez *et al.* 2004). These data indicate that each new population of a biological control agent needs to be screened for host specificity before field release, at least until the variability of host specificity is well understood within a particular species or genus. However, we do not think it appropriate to require separate permits for each new biotype of a species unless the new introduction falls outside of the host-specificity envelope already permitted for that species.

SELECTING NON-TARGETS FOR TESTING

Barratt *et al.* (1999) recommend that host range tests begin with closely related species in order to maximize the probability of identifying potential non-target host species. If closely related hosts are not suitable hosts, then additional testing with more distantly related organisms can often be greatly reduced because of the low probability that they would be suitable hosts. We generally agree with this line of reasoning. However, we initially tested more distantly related ant hosts to confirm literature observations that these flies were likely limited to ants in the genus *Solenopsis* (Porter *et al.* 1995c). If this screening test had shown broader than expected host ranges, further work with some or all of the fly species may have been abandoned. However, once we were convinced that *Pseudacteon* flies were likely very host specific, we focused our host range tests on the near native congener *S. geminata* and later on another native congener *S. xyloni* (Porter & Gilbert 2004). Two species of flies (*P. tricuspis* and *P. litoralis*) were not able to attack and develop in the native fire ants. Therefore, they were only tested with an abbreviated number of ants from other genera (Porter & Gilbert 2004). However, two species of flies (*P. curvatus* and *P. obtusus*) were capable of developing in one or more of the native congeners (Porter & Gilbert 2004) and as a result, they were both tested with a full battery of appropriately sized native ants from other genera (Porter 2000; Porter & Gilbert 2004).

HANDLING AND SELECTING BIOLOGICAL CONTROL AGENTS FOR TESTS

Withers & Browne (2004) and Withers & Mansfield (2005) make a number of suggestions for handling and selecting biological control agents for host range tests. Their suggestions are designed to "maximize the probability of attack on non-target species" in laboratory tests. Basically, their suggestions were to: 1) test biological control agents in groups, 2) use both naïve and experienced females, 3) select large females over small ones, 4) rear test agents on alternate hosts when possible, 5) deprive females of food prior to the test to increase motivation to oviposit, 6) use females deprived of oviposition opportunities for an appropriate amount of time, 7) test pro-ovigenic agents when young, and 8) use small test chambers. Several additional suggestions related to plant substrates, diet, and mating were generally not applicable to *Pseudacteon* flies.

1. **Test in groups.** This is a good recommendation for *Pseudacteon* flies. We have tested flies individually (Gilbert & Morrison 1997) but our preference is to test groups of 6-15 females when availability permits (Porter 2000; Folgarait *et al.* 2002; Vazquez *et al.* 2004; Porter & Gilbert 2004). A major benefit of groups is that a hundred or more flies can

easily be evaluated with only 8-12 test runs whereas individual testing would require a hundred or more test runs. Furthermore, tests with individual flies are often not dependable for many reasons including mating failures, ants killing flies, sick flies, no motivation to oviposit, etc. Finally, group testing is biologically normal because most *Pseudacteon* species attack gregariously in the field.

2. **Naïve and experienced females.** We used naïve females when using lab-reared flies and experienced females when using field-collected flies. We did not find evidence that prior experience in the field restricted subsequent host acceptability in lab trials. To the contrary, we actually have some evidence suggesting that flies attacking *S. invicta* in the lab are primed to approach non-target ants if exposed to them while they are still motivated. Specifically, tests with two species gave slightly higher rates of oviposition attempts (albeit unsuccessful) on non-target ants after having recently attacked the target species (Porter & Alonso 1999). Similarly, motivation to attack was generally short lived after Gilbert & Morrison (1997) transferred flies from target to non-target ants.

3. **Large females.** Withers & Browne (2004) recommended the use of large females on the assumption that they would have more eggs to lay and consequently be more motivated to oviposit. The relevance of this recommendation depends on details of an insect's life history. In the case of *Pseudacteon* females it is probably better to use a mixture of all sizes. This is because fire ant workers vary greatly in size and large and small female phorids attack different sizes of host workers (Porter 1998). Furthermore, small females could be more motivated to lay eggs because, under some circumstances, they do not live as long as large flies (Chen *et al.* 2005), thus canceling any benefits of small versus large.

4. **Rear on alternate hosts.** The suggestion about testing the host range of agents reared on alternate hosts has merit in some systems, but is largely impractical for most *Pseudacteon* species because their production rate is either very low or non-existent on alternate hosts. We know of no instance in which a *Pseudacteon* species from South American fire ants could be successfully cultured on North American fire ants or vise versa. Nevertheless, we were able run a small test to see if *P. curvatus* flies reared on the native fire ant *S. geminata* switched from their normal preference to *S. geminata*. We found that flies reared on the alternate host (*S. geminata*) showed little or no inclination to attack the alternate host indicating that host preferences in this fly were more genetic than facultative (Porter 2000).

5. **Deprive food.** This recommendation has little relevance for phorid flies that attack fire ants. Although we routinely deprived *Pseudacteon* flies of food in our tests, this is because they show little interest in feeding and the presence of food in oviposition chambers appears not to have much effect on fly health or parasitism rates. Also, most *Pseudacteon* species appear to be pro-ovigenic (Zacaro & Porter 2003) so feeding does not facilitate egg development.

6. **Deprive oviposition opportunities.** This recommendation applies best to insects with longer life spans. Depriving phorid flies of oviposition opportunities to improve motivation in host range tests is probably not necessary and could be counterproductive.

Indeed, if anything, *Pseudacteon* females are more likely to approach novel hosts immediately after exposure to normal host ants. *Pseudacteon* flies are usually very short lived when ants are available to attack (1-4 days) and oviposit most vigorously when they are young.

7. **Test pro-ovigenic agents when young.** Withers & Browne (2004) stated that pro-ovigenic agents would likely be best tested when they were young because they are often short-lived while synovigenic agents needed to be tested after eggs have matured and are ready to be laid. This is good advice for *Pseudacteon* flies because they are both pro-ovigenic and short lived. Nevertheless, we prefer tests which run for the full adult life of the flies because it gives them full opportunity to oviposit across all age ranges.
8. **Small test chambers.** We used small test chambers (Porter & Gilbert 2004) mostly because of limited space in our quarantine facilities; nevertheless, the use of small chambers in our tests rather than large ones probably did improve the likelihood of oviposition because the females could simply use visual or other short-range cues to find their hosts. This was good because it maximized the probability that test flies would oviposit in both target and non-target hosts. The down side of the small chambers is that we were not able to evaluate host specificity associated with long-range host detection.

EXPERIMENTAL DESIGNS

Van Driesche & Murray (2004) discuss the strengths and weaknesses of a number of experimental designs that have been used with host range testing including no-choice tests, choice tests, sequential tests, open field tests, preference ranking tests, and post-release tests. Withers & Mansfield (2005) evaluate choice and no-choice tests and recommend the use of either no-choice tests or a combination of no-choice and choice tests. During the course of our host range studies, we have used almost all of the experimental designs just mentioned.

No-choice tests. As recommended, we agree that no-choice tests are the best design for determining host ranges of *Pseudacteon* flies in the laboratory, at least when test flies are available in sufficient numbers either from the field or from a laboratory colony. No-choice tests were run with groups of flies (Porter 2000; Vazquez *et al.* 2004; Folgarait *et al.* 2002) for the entire life of the test flies. This allowed us to measure attraction rates, oviposition rates and most importantly parasitization rates.

Choice tests. We conducted binary choice tests when female flies in no-choice tests had demonstrated some abilities to attack and develop in non-target native fire ants (Porter 2000; Porter & Gilbert 2004). The objective was to determine whether females had a preference for the target species over the non-target native species. Our results showed strong preferences for imported fire ants over native fire ants. This preference data together with poor rates of development on native fire ants strengthened the argument that release of these flies would most likely benefit the native ants because of their impacts on imported fire ants (see specificity discussion under Background section).

We also used binary choice tests to screen ants in non-*Solenopsis* genera (Porter & Alonso 1999; Porter 2000; Porter & Gilbert 2004). However, these tests functioned like no-

choice tests since test flies always showed little or no attraction to ants from other genera and no test flies were ever reared from ants in other genera. Testing 3-4 species of non-target ants simultaneously would have increased testing efficiency. The drawback is that if flies had been attracted to any of the species of ants, we would have needed to repeat the tests to make sure that attraction to one ant species was not masking attraction to another (Withers & Mansfield 2005).

Sequential no-choice tests. Sequential no-choice tests were used to investigate the host specificity of several groups of flies transported into U.S. quarantine facilities from South America. Because of the short lifespan of field collected flies (2-5 days) and the time and expense required to hand carry these flies up from South America (1-2 days) we had very few flies and a very short time to conduct as many tests as possible. Gilbert & Morrison (1997) and Morrison & Gilbert (1999) chose to use an A-B-A pattern where the motivation of individual flies was tested against target ants (A) for five minutes and then against non-target ants (B) for 20 min, and finally against target ants (A) again to reconfirm motivation. In these tests, attacking flies moved from trays of target *S. invicta* (A) to trays of non-target *S. geminata* (B) initially approached, and sometimes attempted to oviposit in *S. geminata* workers. Typically however, motivation to attack carrying over from exposure to *S. invicta* was short lived and waned quickly after exposure to *S. geminata*. Porter & Alonso (1999) chose to test small groups of three flies in an A-B and a B-A pattern where some flies were first exposed to the target host while others were exposed first to the non-target host (each for periods of 60-90 minutes). This pattern controlled for any effects of recent exposure to the target host.

These sequential tests had two weaknesses: first all of the flies had been collected after they had prior experience with the target host and secondly test times (20 min. or 60-90 min.) could have been too short to overcome the effects of prior experience. Nevertheless, these limitations were largely unavoidable because of transport times, short life spans, and the fact that, at the time, the flies could not be cultured in the laboratory. Fortunately, results from these tests were equivalent to larger no-choice tests run later indicating that prior experience as wild flies is not a major factor affecting host range tests with *Pseudacteon.*

Withers & Mansfield (2005) recommend that Gilbert & Morrison (1997) could have used an A-A-A pattern to control for time dependant effects and similarly that Porter & Alonso (1999) could have used an A-A pattern. We agree that this suggestion could have provided some useful information. However, since the numbers of flies were very limited and many of them only survived one test cycle, we do not feel that the value of this information would have justified using 1/3 of the available flies. In the case of Gilbert & Morrison (1997), the second exposure to the target host in the A-B-A cycle provided most of the information that would have been provided by an A-A-A cycle. In our opinion, activity in an A-A-A cycle would not have been directly comparable to activity in an A-B-A cycle because the presence of the target host caused greatly increased activity that generally sapped the vigor and longevity of test flies. Our challenge was to keep flies alive and vigorous through even a short A-B-A cycle. In the case of Porter & Alonso (1999), an A-A test would have proved that flies exposed first to the target host (A) retained sufficient vigor to attack the non-target host (B). However, in keeping with the behavioral observations noted above for the A-B-A tests, the data showed that test flies were actually slightly more likely to attack the non-target

ant after being exposed to the target ant than vise versa (3/36 versus 0/79 attacking flies, P=0.029, Fisher's exact test, data for two species of flies combined). Thus, for *Pseudacteon*, we consider the sequential no-choice test to be conservative in that it tends to over-estimate the tendency of these flies to attack non-targets.

Open field pre-release and post-release tests. We conducted several pre-release and post-release open field tests with *Pseudacteon* flies. The major advantage of open field tests is that they take into account the long-range search and discovery abilities of test organisms. The major disadvantages of open-field tests are that the selection of potential hosts in pre-release tests are limited to what is available in the country of origin while in post-release tests, the biological control agent has already been released and can rarely be recalled. For the first open field test Porter *et al.* (1995c) used an $AB_1B_2B_3B_n$ design where target ants (A) were presented simultaneously with a menu of non-target ants (B). In subsequent papers (Porter 1998b; Morrison & Porter 2005c; Vazquez & Porter 2005), authors used a sequential B-A-B design where non-target ants (B) were presented for 30 minutes followed by target ants (A) and finally by non-target ants again (B). The advantage of this sequential design is that it allowed us to first determine if flies were attracted to non-target ants when no target ants were present and then it allowed us to determine if the flies would attack non-target ants after large numbers of flies had been attracted to the immediate area by the target ants. Van Driesche & Murray (2004) call post-release tests a "necessary step" in evaluating the accuracy of pre-release predictions. Results from our post-release tests confirmed that our pre-release predictions of host specificity were accurate for both species of flies that are currently established in the United States (Morrison & Porter 2005c; Vazquez & Porter 2005).

Statistical analyses. Hoffmeister (2005) discusses a number of important aspects of statistical design that apply to host range testing including proper controls, randomization, and pseudoreplication. He also discusses the potential importance of using power analyses to describe the power of statistical procedures to resolve differences between effects of interest.

Controls. Proper controls are vital to most kinds of statistical tests, but they are especially important to simple no-choice tests because the failure of a parasitoid to attack a potential non-target host could be due to poor test conditions or unhealthy parasitoids. To control for these possibilities, we randomly assigned test flies to simultaneous controls and treatments. On several occasions, we had to discard a run because the controls failed due to improper handling of the flies. Zilahi-Balogh *et al*. (2005) mention that the use of negative controls (tests without both a parasite and a host together) could have helped with interpretation of their oviposition tests. We did not use negative controls in any of our tests. Negative controls using ants that were not exposed to flies might have been useful in identifying ant mortality caused by parasitism prior to pupation of the parasite. However, based on random dissections of dead workers, we felt that pre-pupation mortality of host ants was not sufficiently large to justify the extra effort needed to quantify it.

Pseudoreplication and randomization. We attempted to avoid pseudoreplication in our tests by randomly assigning subjects to treatments and using experimental units that were independent of one another. However, in practice, flies were usually assigned to test groups using "haphazard randomization" and the locations of test trays were usually rotated sequentially among test groups so that whatever effect tray location might have would be uniformly

distributed across treatments. Finally, our host range data are from specific populations of flies; consequently, our results can only be safely applied to those specific populations. Extrapolating host range results from a single population to all populations of a species is a form of pseudoreplication that can lead to failures in host range predictions (Hopper *et al.* 2005)

Power analyses. We did not use power analyses as discussed by Hoffmeister (2005) in our host range tests. An *a priori* power analysis is useful for predicting the necessary sample size for a test if variability is known (Zilahi-Balogh *et al.* 2005). However, since we rarely knew variability beforehand, we simply continued to increase sample sizes in our tests until standard errors of the means dropped to reasonable levels.

Hoffmeister's (2005) recommendations concerning the use of power analyses to assess the probability of falsely accepting the null hypothesis of "no effect" were not particularly applicable to the kinds of host range tests we did with phorid flies– this was because rates of attraction and parasitism were always very different between target and non-target hosts. Furthermore, if critical aspects of host specificity had been similar enough that they could not be easily resolved statistically, then we would have simply accepted the null hypothesis that no difference existed. We would not have worried whether parasitization rates may have actually been slightly different because they would still have been similar enough to have caused serious concern about the safety of releasing a particular biological control agent in the field.

Hoffmeister's (2005) recommendations concerning power analyses, however, are highly applicable to the assessment of impacts of biological control agents on field populations of target and non-target organisms. In the case of field impacts, it is important to know what power the statistical tests had to resolve differences when no statistical difference was found. This is exactly the problem faced when evaluating the field impacts of *P. tricuspis* on imported fire ants and other ant competitors (Morrison & Porter 2005a). Morrison & Porter (2005a) dealt with the problem by reporting what percent of the mean that two standard errors were. This was done on the assumption that means two standard errors apart would normally be statistically detectable. Power analyses probably provide a more effective way of providing this information.

CONCLUDING REMARKS

The model systems around which many of the general ideas about biological control are framed depart substantially from the phorid–fire ant system in terms both of the enemy and the victim. Conceptually, the decapitating fly – fire ant system resembles host-specific leaf miners and a woody plant host. However, *Pseudacteon* flies are likely to be more host specific than their herbivorous counterparts because the chemical cues they use for host discovery are under selection to be highly distinct among ants for reasons of close physical competition. Ants are mobile, dangerous targets for an attacking fly and the behavior and mechanics of inserting an egg into an armored predaceous host surrounded by aggressive sisters adds additional potential causes for specialized behaviors and morphology in these phorids. Add to these features the likelihood of internal defenses against phorid larvae and it is not surprising that *Pseudacteon* flies exhibit striking host specificity. By contrast the parasitoids of the eggs, larvae and pupae of Lepidoptera, for example, face many fewer challenges that might be solved

by evolving increased specialization. Many practical and theoretical similarities and distinctions of this system and other systems need to be further explored.

CONCLUSIONS

Host range testing is essential because it allows scientists to predict the potential target and non-target impacts of new biological control agents prior to their release in the field. Information about potential impacts, both positive and negative, permits a reasoned decision about whether the likely benefits of releasing a particular agent clearly outweigh the potential problems. The papers in this session and recent books on the subject have set out a number of important procedures and principles that applied to our work with fire ant decapitating flies and to host range testing generally. We would like to emphasize how important it is to do a thorough review of the literature concerning the biology of a prospective agent, the target host, and organisms related to the agent and hosts. We found that biotypes and cryptic species can have different host ranges both as related to target and non-target species; consequently, it is important that biological control practitioners consider this when conducting their tests. We agree that host range tests should be conducted using methods that initially maximize the probability of attack on non-target species. These methods will vary depending on the agent being tested. We attempted to maximize this probability by testing congeners, using small test chambers, using no-choice tests, testing flies of all ages, testing flies in groups, and using both experienced and naïve flies. Good experimental design that uses appropriate controls, randomization, and replication allows valid interpretations to be drawn. Finally, we want to emphasize the need for post-release host range monitoring. Post-release monitoring is important because it verifies the validity of the prerelease testing procedures and provides data that facilitate the release of future biological control agents.

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A PREDATOR CASE HISTORY: *LARICOBIUS NIGRINUS*, A DERODONTID BEETLE INTRODUCED AGAINST THE HEMLOCK WOOLLY ADELGID

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ABSTRACT

The hemlock woolly adelgid, *Adelges tsugae* Annand (Homoptera: Adelgidae) is an invasive alien pest of eastern North American hemlocks (*Tsuga* spp.) and is the target of a classical biological control program in the eastern United States. Host range testing conducted under quarantine in Blacksburg, Virginia determined the suitability of *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) a predatory beetle, as a biological control agent of this pest. Members of the genus *Laricobius* are known to feed on adelgids. *Laricobius nigrinus*, native to western North America, was tested on three other adelgid and three non-adelgid species of Homoptera in three families. Host acceptance and host suitability tests were conducted on test prey. In paired-choice and no-choice oviposition tests, *L. nigrinus* females preferred to oviposit in HWA ovisacs over the other test species. Feeding tests showed that *L. nigrinus* consumed more eggs of HWA than eggs of *Adelges piceae* (Ratzeburg) and *Pineus strobi* (Hartig), but not of *Adelges abietis* (L.). In larval development tests, *L. nigrinus* only completed development on HWA. These results suggest that *L. nigrinus* has a narrow host range and that it has potential for biological control of HWA. *Laricobius nigrinus* was cleared for field release by USDA APHIS in 2000 based on these findings and NAPPO Guidelines for 'Petition for Release of Exotic Entomophagous Agents for the Biological Control of Pests'. Test design will be discussed in a retrospective analysis in relation to the practical realities of host range testing in this system and compared with what might be the ideal.

INTRODUCTION

HEMLOCK WOOLLY ADELGID

The hemlock woolly adelgid (HWA), *Adelges tsugae* Annand is an invasive alien pest of native hemlocks (*Tsuga* sp.) in eastern North America (McClure 1996). This insect was first observed in North America in the Pacific Northwest in the early 1920's where it was described from specimens collected on western hemlock, *T. heterophylla* (Raf.) Sargent (Annand 1924). Since its introduction into the eastern United States in the early 1950's (Souto *et al.*

1996), HWA has spread along the eastern seaboard in parts of 13 states on the eastern sea board (USDA FS 2004).

Eastern hemlock is an important ornamental and forest tree that is very susceptible to HWA attack. Infested trees exhibit poor crown condition, reduced terminal branch growth and needle loss, and have been reported to die within four years after initial attack (McClure 1991). HWA populations in the eastern United States are not regulated by effective natural enemies (McClure 1987; Montgomery and Lyon 1996; Wallace and Hain 2000). In contrast, HWA has little impact on Asian and western North American species of hemlock. Tree resistance and natural enemies have been reported as playing a role in maintaining HWA below injurious levels in these regions (Cheah and McClure 1996; Montgomery and Lyon 1996).

LARICOBIUS NIGRINUS

Members of the genus *Laricobius* are predacious on woolly adelgids (Homoptera: Adelgidae) (Lawrence and Hlavac 1979; Lawrence 1989). *Laricobius nigrinus* Fender is native to western North America (Fender 1945; Hatch 1962; Lawrence 1989). It was found in close association with HWA on western hemlock in British Columbia, Canada (Zilahi-Balogh *et al.* 2003) where HWA is not considered a forest pest. We hypothesized that *L. nigrinus* may play a role in regulating HWA abundance in the Pacific Northwest and therefore warranted investigation as a candidate biological control agent of HWA in the eastern United States.

We studied the life history of *L. nigrinus* over two years in British Columbia (Zilahi-Balogh *et al.* 2003). This beetle is univoltine. Females lay eggs singly within the woolly ovisacs of HWA from January to May. Onset of oviposition by *L. nigrinus* coincides with oviposition by the over-wintering (sistens) generation of HWA. After hatching, larvae feed preferentially on the eggs of HWA. On completion of feeding, mature larvae migrate to the soil to pupate. After eclosion, adults remain in the soil in an aestival diapause resuming activity in late September to early October at about the same time that aestivating first instar HWA sistens resume development (Zilahi-Balogh *et al.* 2003). Adult feeding by *L. nigrinus* in the winter contributes significantly to adelgid mortality (Lamb *et al.* 2005a). The phenology of *L. nigrinus* in Virginia (Lamb *et al.* 2005a) is similar to that in British Columbia (Zilahi-Balogh *et al.* 2003).

A summary of host specificity tests on *L. nigrinus* followed by a retrospective analysis of host range testing procedures addressing issues presented in this symposium are discussed. The issues are: 1) test design (Withers and Mansfield 2005), 2) statistical design (Hoffmeister 2005), and 3) genetics: relation of local populations to whole species (Hopper *et al.* 2005).

MATERIALS AND METHODS

Laricobius nigrinus adults used in this study were field collected from HWA infested western hemlock from coastal British Columbia, and imported to Virginia for quarantine evaluation (Zilahi-Balogh *et al.* 2002). Field collection and testing of adults coincided with the ovipositional period (peak oviposition is early to mid-March) of *L. nigrinus* (Zilahi-Balogh *et al.* 2003). Immature stages tested were progeny of field collected adults. Insects were main-

tained on field collected HWA infested eastern hemlock twig cuttings in environmental chambers at 15°C, 12:12 (L:D) h, and 75-87% RH.

Six species of test prey in the order Homoptera in three families (Adelgidae, Aphididae, Diaspididae) were used in host specificity tests. They were selected based on taxonomic or ecological similarity to HWA as well as availability. Test prey species are listed in Table 1. With the exception of *M. persicae*, all test prey could be encountered by *L. nigrinus* in the natural forest setting in southeast United States.

Table 1. Test prey on associated host plants used in host range tests conducted between February and April 2000 (from Zilahi-Balogh *et al.* 2002).

Test Prey	Distribution	Host Plant
	Family Adelgidae	
Adelges tsugae Annand (HWA)	Asia, North America[a] (Target insect)	*Tsuga canadensis* (L.) Carrière
Adelges piceae (Ratzeburg)	Europe, North Americaa	*Abies fraseri* (Pursh) Poir
Adelges abietis (L.)	Europe, North America, North Africa, India[a]	*Picea abies* (L.) Karst.
Pineus strobi (Hartig)	North America, Europe[a]	*Pinus strobus* L.
	Family Aphididae	
Cinara pilicornis (Hartig)	Europe, Australia, New Zealand, North and South America[a]	*Picea abies* (L.)Karst.
Myzus persicae (Sulzer)	World wide[b]	*Capsicum frutescens* L. var. *grossum* Bailey
	Family Diaspidae	
Chionaspis pinifoliae (Fitch)	North America[c]	*Pinus cembra* L.

[a]Blackman and Eastop 1994; [b]Blackman and Eastop 1984; [c]Kosztarab 1996

The egg stage was used in all tests for members in the family Adelgidae and Diaspididae. Eggs of adelgids are typically laid in a mass by a sessile female and surrounded by flocculence (waxy/woolly filaments). This stage was selected because we found *L. nigrinus* females laying eggs in the woolly ovisacs of HWA (Zilahi-Balogh *et al.* 2003). *Chionaspis pinifoliae* (Diaspididae) over-winters in the egg stage underneath the female scale. In May, these hatch into crawlers which move over the needles for a few days and then settle down to feed (Kosztarab 1996). Host plant material infested with *C. pinifoliae* were field collected in the early spring and held at 4°C until used in tests. HWA differs from the other adelgids tested in that it breaks aestival diapause in late September/October, develops throughout the winter and begins to lay progrediens and sexuparae eggs in February (McClure 1987). In contrast, *A. piceae*, *A. abietis* and *P. strobi* over-winter as early instar nymphs and begin to lay eggs in the spring when buds begin to break (April or May) (Arthur and Hain 1984; Craighead 1950; Friend and Wilford 1933; Gambrell 1931; Johnson and Lyon 1991; USDA 1985). The challenge was synchronizing development of the various adelgid species with that of HWA. This was achieved by moving adelgid infested potted saplings (Table 1) from an outdoor nursery

into a greenhouse (~ 24°C) beginning in January to accelerate development before being used in tests. Test prey in the family Adelgidae and Diaspididae remain attached to their host plant once crawlers settle. Excess individuals were removed from the host plant with fine forceps when numbers exceeded those required for a particular test. Test prey in the family Aphididae were tested at the early instar nymphal stage as adult females exhibited vivipary. Individuals within the family Aphididae were transferred onto or removed from their respective host plant with a fine brush to attain the appropriate number on the host plant cutting.

Host specificity tests (Zilahi-Balogh *et al.* 2002) were of two types – host acceptance and host suitability. Host acceptance tests determine whether a candidate biological control agent will feed and/or oviposit on a host. Host suitability tests determine whether the agent is able to complete development to the adult stage and produce viable offspring on a particular host (Browne and Withers 2002; Kok *et al.* 1992). Host suitability tests therefore are more crucial in determining potential host range.

HOST ACCEPTANCE

Oviposition tests. Both no-choice (single-prey) and paired-choice oviposition tests were conducted to evaluate the effect of prey type on acceptance and preference by *L. nigrinus* females for oviposition. All tests were conducted in 14 x 2.5 cm plastic petri dishes. One male-female pair was placed in a petri dish with either one bouquet of associated host plant twigs housing test prey (no-choice test) or two adjacent bouquets of host plant with associated prey (paired-choice test). A bouquet was made up of two to four terminal tip branches (10-12 cm length) of prey infested host plant held together by wrapping the cut end with parafilm to prevent the twigs from drying out. In the paired-choice tests, HWA was paired with each of the six test prey. The same numbers of prey (~60 individuals per bouquet) were used in each test. Duration of each test was three days. The number of *L. nigrinus* eggs deposited on each plant bouquet was counted at the end of each test (Zilahi-Balogh *et al.* 2002). A 3-day test was selected based on preliminary trials that showed that three days was a long enough interval to get a treatment effect without resulting in host plant desiccation or having to add additional prey.

Adult feeding test. Prey acceptance by adult *L. nigrinus* was examined in a single-prey feeding experiment using eggs of the four adelgid species, HWA, *A. abietis*, *A. piceae*, and *Pineus strobi*. Even though *L. nigrinus* adults preferentially feed on nymphs and adult stages of adelgids, eggs were selected to test because they are uniform in size within and between adelgid species. Adult *L. nigrinus* starved for 12 h, were placed individually in 50 x 9 mm petri dishes containing one of four prey types attached to sections (< 5 cm) of host plant. Egg numbers of test prey were estimated before introduction of the predator. After 3 d, adult beetles were removed and the number of eggs that remained were counted (see Zilahi-Balogh *et al.* 2002 for details).

Host Suitability. Development and survivorship of *L. nigrinus* were followed from the egg to adult stage on all test prey except *M. persicae*. We did not evaluate *M. persicae* because it was the only test prey that *L. nigrinus* females did not oviposit on during the oviposition tests. *Laricobius nigrinus* eggs (d•24 h old) were transferred individually onto test prey in petri dishes as described above in the adult single-prey feeding test. The stage of test prey

used was similar to that described for the oviposition tests. Egg hatch was followed daily. Other stages were examined daily or every other day for survivorship until adult emergence. Fresh prey was added each time an individual larva was examined. Larval molt was determined by recording the presence of an exuvium. Once the pre-pupal stage was reached, moistened sterilized peat was placed at the base of each petri dish and acted as a pupation medium. The pre-pupal stage was determined to be the stage that mature larvae left the twig with abundant prey and appeared to be actively searching for a suitable pupation site (Zilahi-Balogh *et al.* 2002).

RESULTS AND DISCUSSION

HOST ACCEPTANCE

Oviposition tests. In both the no-choice and paired-choice oviposition tests, *L. nigrinus* females laid significantly more eggs in HWA ovisacs ($P < 0.0001$ to 0.02) over the other test prey (Zilahi-Balogh *et al.* 2002). In the paired-choice test, no eggs were laid on host plants housing non-adelgid prey (*C. pilicornis, C. pinifoliae*, and *M. persicae*). Oviposition was more than five times greater on HWA than on adelgid test prey (*A. piceae*, *A. abietis*, *Pineus strobi*) in the paired-choice tests. These differences indicate an ovipositional preference for HWA over these other adelgids (Zilahi-Balogh *et al.* 2002). In no-choice tests, no eggs were laid on sweet pepper housing *M. persicae*, and very few eggs (mean: $d \leq 0.2$ eggs) were laid on host plants housing the other non-adelgid Homoptera (*C. pilicornis* and *C. pinifoliae*). In no-choice tests, *L. nigrinus* laid ~ 2 to 12 times more eggs in HWA ovisacs over the other adelgid non-target prey.

Adult feeding test. In this no-choice feeding test, eggs of all the test adelgids were fed on by adult *L. nigrinus*. Significantly more eggs of HWA were consumed than eggs of the *A. piceae* and *Pineus strobi*, but not *A. abietis*. Though not statistically significant, *L. nigrinus* adults consumed on average 2x more eggs of HWA (48.4) than *A. abietis* (24.7) (Zilahi-Balogh *et al.* 2002).

HOST SUITABILITY

Laricobius nigrinus only completed development to the adult stage on a diet of HWA. *Adelges piceae* and *P. strobi* supported larval development to the fourth instar, providing evidence of larval feeding, but did not support further development. Larvae provided with *A. abietis*, *C. pilicornis* or *C. pinifoliae* did not survive beyond the first instar (see Zilahi-Balogh *et al.* 2002 for details).

RETROSPECTIVE ANALYSIS

TEST DESIGN

Host specificity tests are designed to determine host acceptance and host suitability (defined earlier) (Kok *et al.* 1992). No-choice and choice tests have been used widely to evaluate host

ranges for both weed and arthropod biological control (Sands and Van Driesche 2003; Van Driesche and Hoddle 1997; Van Driesche and Murray 2004a).

No-choice tests combine the biological control agent with a single test species for a set period of time (Van Driesche and Murray 2004a; Withers and Mansfield 2005). Sequential no-choice tests involve the presentation of target and non-target hosts in a sequence. Choice tests utilize two or more test species with the biological control agent simultaneously (Withers and Mansfield 2005). The paired-choice test includes two treatments (i.e., hosts or prey) being offered simultaneously to the biological control agent. In our tests, the target prey (HWA) was always paired with a non-target prey. We used both no-choice and paired-choice for ovipositional preference and no-choice tests for adult feeding and larval development. Both no-choice and choice tests contribute to information on possible ecological host range of the biological control agent and ideally both should be used in combination (Withers and Mansfield 2005).

Estimation of physiological host range examines the suitability of a candidate biological control agent to survive and complete development on a test host/prey. No-choice larval development tests are able to determine physiological host range and may be more restrictive than no-choice oviposition tests. Physiological host range testing can be challenging when assessing endoparasitoids as it requires observing whether the parasitoid develops and emerges from a test species that has been previously accepted by a female in an oviposition test (Van Driesche and Murray 2004a; Withers and Mansfield 2005). However with a predator, eggs can be transferred easily onto test prey and assessed for feeding and development (Zilahi-Balogh *et al.* 2003). We were able to assess host suitability for larval development to the adult stage. In our case, even though *L. nigrinus* developed to the fourth instar on several non-target hosts, it was only on HWA that this predator developed to the adult stage.

No-choice tests are important in host range testing because negative results can provide good evidence that a test species is not likely to be a field host. Host acceptance in a no-choice test can identify low ranked hosts missed in choice tests. Choice tests are useful in ranking order of preference within a list of possible hosts (Van Driesche and Murray 2004a). With choice tests, we expect a bigger difference in predation or oviposition between target and non-target (lower ranked hosts) (Withers and Mansfield 2005). In our oviposition tests, *L. nigrinus* accepted more non-target hosts than in the paired-choice tests. In the paired choice tests, none of the non-adelgid test prey were accepted as hosts for oviposition. This is consistent with what we expect.

Physiological and behavioral factors can influence the outcome of host range lab assays whether they are choice or no-choice (Withers and Mansfield 2005). Several relevant to our study system are discussed.

Prior experience. A confounding factor in interpretation of results from no-choice and choice tests is prior experience to host or prey (Withers and Mansfield 2005). Studies on both parasitoids and predators have shown there is an enhanced responsiveness in foraging behavior with prior experience to that host (prey) or volatile (Van Driesche and Murray 2004a; Withers *et al.* 2000; Withers and Browne 2004; Withers and Mansfield 2005).

A weakness in our test design is prior experience of adult *L. nigrinus* to HWA prior to tests. *Laricobius nigrinus* adults used in host specificity tests were field collected and therefore were preconditioned to the target prey. This has introduced bias in favor of the target prey (HWA). Though not ideal because of preconditioning of *L. nigrinus* to HWA, it was a practical reality in our system. *Laricobius nigrinus* is a difficult species to rear in the laboratory because of the obligatory aestival diapause exhibited by adults. We initially experienced high mortality in aestivating adults in laboratory culture. A mass rearing protocol has subsequently been developed for *L. nigrinus* (Lamb *et al.* 2005b), but it can only be kept in culture if reared on HWA. No artificial diet has been developed for this species yet. Withers and Browne (2004) suggested that predators and parasitoids should be reared and maintained on species other than the target host (prey) or on artificial diet if possible in order to minimize any experience-induced bias in favor of the target species, especially in the context of choice tests. The use of artificial diet to rear insects can creates some inherent problems because such diets are seldom optimal for development.

Time dependent effects. The period of food or oviposition site deprivation can have major effects on the acceptance threshold of a biological control agent to host cues (Browne and Withers 2002). The consequence of host deprivation is that deprived insects may accept a wider range of hosts than non-deprived individuals (Browne and Withers 2002; Withers and Mansfield 2005). In our studies, beetles were deprived of prey for 12 h prior to feeding tests, but were not deprived prior to oviposition tests. Had females been deprived of host prior to oviposition, would the outcome of the tests be different? We do not think so because of the longevity of *L. nigrinus*. Long-lived species are more likely to resorb eggs in the absence of suitable oviposition sites, as is typical of synovigenic species.

Physiological state of test insects. An important consideration in all bioassays with insects is ensuring that all test insects are of a similar physiological age and have been exposed to the same conditions. When doing oviposition bioassays, it is important to have an understanding of the life history and reproductive biology of the biological control agent. In our case, we were dealing with a predator that is univoltine, and undergoes an obligatory aestival diapause for ~ 4 months of the year (Zilahi-Balogh *et al.* 2003). As mentioned earlier, practical considerations necessitated the use of field collected beetles. Beetles were collected in February, within the ovipositional period of *L. nigrinus* (Zilahi-Balogh *et al.* 2003).

Negative controls. Though not discussed by Withers and Mansfield (2005), the use of negative controls (arenas with no predators) in no-choice feeding tests and controls (with no prey) in oviposition tests are useful for interpretation of results (Van Driesche and Murray 2004b). Negative controls in a feeding test account for any mortality in prey not attributed by the biological control agent, while a no-prey control in an oviposition test can account for the potential of prey dumping in the absence of prey-related cues (Van Driesche and Murray 2004b). We did not include negative controls in our feeding tests or a no-prey control in our oviposition tests. In retrospect, we should have considered these controls, but do not think that it would change our findings. Had we used a no-prey control, and oviposition in this treatment was not significantly different from non-adelgid homopteran hosts, we might have been able to conclude that oviposition on these non-target hosts may be due to egg dumping

rather than host acceptance. In our feeding tests, we were not assessing mortality. We assessed the difference between the number of test prey eggs present before predator introduction and number of test prey eggs present after the predator was removed three days later.

STATISTICAL DESIGN

Hoffmeister (2005) argued that the problem in host range testing is assigning a probability of accepting the null hypothesis of no effect, i.e. that the biological control agent does not include a given non-target host into its host range. This may be impossible to prove with certainty, but what is required is utilizing an experimental design that aims at achieving accuracy and precision from the sample population that is tested. This requires a robust experimental design and decision by researchers on the magnitude of an effect that is desirable to be detected, appropriate sample size to use, and knowledge of the power of the statistics used (Hoffmeister 2005).

Statistical power. The power of a statistical test, defined as $1-\beta$ is the probability of rejecting the null hypothesis when the null hypothesis is false and should be rejected (Zar 1984). Power is dependent on the α-level, variance, sample size (n) and effect size (Quinn and Keough 2002). Power analysis can be done *a priori*, for a given level of variability, sample size and power (0.80 is common) to determine how big the change (i.e., effect size) is needed before it would be detected as significant (Hoffmeister 2005; Quinn and Keough 2002).

In our study, preliminary no-choice and paired-choice oviposition tests were done to determine an appropriate length of time to use for a bioassay that allowed for adequate oviposition to occur without host plant material desiccating or having to add additional host material. The number of replicates used for these preliminary tests were n=12 and n=20. Using the variance from the preliminary tests, we could have conducted power analysis to calculate the minimum detectible effect size for a given level of power, or calculate sample size to decide on how much replication is necessary given a level of power, variability, effect size and α (α=0.05 is standard) (Quinn and Keough 2002). Instead, after appropriate analyses of the preliminary tests, we determined that n=12 was a reasonable sample size to get a significant treatment effect. Sample sizes in oviposition tests ranged between n=11 and n=20. We used n=7 in the no-choice feeding test. The limited sample size is this case was due to the limited availability of test predators. Even with this limited number of replications, when we compared number of eggs eaten by *L. nigrinus* when adult predators were presented with eggs of either target prey (HWA) or non-target prey, the predator consumed significantly more HWA eggs than two of the three non-target prey. Though not statistically significant, *L. nigrinus* adults consumed ~ 51% fewer non-target *A. abietis* eggs than target HWA eggs (Zilahi-Balogh *et al.* 2002). A larger sample size might have shown a significant difference in predator consumption between HWA and *A. abietis* eggs.

Statistical analysis. Both paired-choice and no-choice tests were used in our study. The response variable in these tests is quantitative (i.e., number of eggs laid, number of prey consumed). Therefore ANOVA and paired-t tests are an appropriate choice as long as data are normally distributed and there is homogeneity of variance (Horton 1995; Zar 1984). Prior to analysis, data were examined for normality using the Shapiro-Wilk W Test and for homogeneity of variance using Levene's test for Equality of Variance (SAS 1989). The Shapiro-Wilk

test was done on the difference between paired observations in the paired-choice tests. Transformation of data using log (x+1) prior to analysis was done as necessary to correct for heterogeneity of variance and/or non-normal sample distributions. Parametric tests on transformed data were selected over non-parametric tests as they are more powerful than non-parametric tests.

Pseudoreplication. Pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent (Hurlbert 1984). If treatments are spatially or temporally segregated, if replicates of a treatment are interconnected somehow, or if replicates are only samples from a single experimental unit, then replicates are not independent (Hurlbert 1984). It is important to determine the experimental unit. Steel and Torrie (1980) define the experimental unit as the unit to which one application of a treatment is applied. The treatment is the procedure whose effect is to be measured and compared with other treatments (Steel and Torrie 1980). For all experiments in this study, the experimental unit was an individual *L. nigrinus* adult, male-female pair, or egg (larva) in a petri dish. The treatment was the host/prey material (host plant with associated homopteran prey) in which the predator was exposed. Pseudoreplication did not apply to our study.

GENETICS: RELATION OF LOCAL POPULATIONS TO WHOLE SPECIES – IMPLICATIONS FOR HOST RANGE TESTS

In classical biological control it has been common practice to introduce natural enemies from many geographic locations (Unruh and Woolley 1999). However, it has been well documented that different populations have shown differences in host affinities and behavior (Hopper *et al.* 2005, and references within). The term biotype has commonly been used for populations that display differences in some biological attributes (Unruh and Woolley 1999). Diehl and Bush (1984) categorized insect biotypes by their genetic polymorphisms, non-genetic polyphenisms, geographic variation and host races. Molecular genetics provides tools to unraveling this variation. Hopper *et al.* (2005) discussed the implications of using distinct populations in host range testing.

All collections of *L. nigrinus* evaluated under quarantine were collected from the same site in a HWA infested western hemlock seed orchard near Victoria, British Columbia, Canada and thus would be considered the same 'local' population. Although this may not represent all existing populations of the species, it allowed for the elimination of inter-population variations.

CONCLUSIONS

A summary and interpretation of our test results is shown in Table 2. Although adult feeding tests indicated feeding acceptance on other adelgid species in addition to HWA, no-choice larval development tests showed that *L. nigrinus* only completed development to the adult stage on HWA. Based on the larval development tests, we concluded that these adelgid species are not suitable hosts for completion of larval development of HWA. If we solely based our conclusions on the paired-choice and no-choice oviposition and no-choice adult feeding

test, our interpretation would be that the other test adelgids would be inside the host range of *L. nigrinus* (see Table 4, Sands and Van Driesche 2000). Oviposition and feeding tests are concordant with larval development tests. We consistently see HWA ranked as the most preferred host. Non-host adelgids rank second, while non-adelgid hosts rank at the bottom.

Table 2. Summary of results of acceptance and suitability tests of Homoptera prey screened as hosts of *Laricobius nigrinus* (from Zilahi-Balogh *et al.* 2002).

	Acceptance[a]		Suitability[a]	
Test species	**Oviposition**	**Adult feeding**	**Larval development**	**Final host status[b]**
Adelgidae				
Adelges tsugae Annand	+	+	+	Yes
Adelges piceae (Ratzeburg)	+	+	-	No
Adelges abietis (L.)	+	+	-	No
Pineus strobe (Hartig)	+	+	-	No
Aphididae				
Cinara pilicornis (Hartig)	+	x	-	No
Myzus persicae (Sulzer)	-	x	x	No
Diaspididae				
Chionaspis pinifoliae (Fitch)	+	x	-	No

[a] +, positive response on test prey; -, negative response on test prey; x, test not conducted;
[b] Whether the species could serve as a host to L. nigrinus.

Laboratory host range tests are further strengthened by the synchrony between ovipositional period of *L. nigrinus* and presence of suitable oviposition sites (i.e., HWA ovisacs) in the field (Zilahi-Balogh *et al.* 2003). There is poor synchrony between ovipositional period of *L. nigrinus* and availability of suitable oviposition sites with the non-target adelgids tested (Arthur and Hain 1984; Craighead 1950; Friend and Wilford 1933; Gambrell 1931; Johnson and Lyon 1991; USDA 1985). When this information is combined with the larval development tests, we predict that these adelgids are outside of the ecological host range of *L. nigrinus*. We conclude that adult feeding by *L. nigrinus* may occur under natural field conditions on the other test adelgids, but that these hosts are phenologically and/or physiologically unsuitable for larval development.

Though not without flaws, we believe our host specificity tests provide a consistent pattern in regards to the predicted ecological host range of *L. nigrinus*.

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GENETICS: RELATION OF LOCAL POPULATIONS TO THE WHOLE "SPECIES" – IMPLICATIONS FOR HOST RANGE TESTS

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ABSTRACT

Populations of parasitoids collected from different host species or geographical regions can differ in host specificity. Where the necessary research has been done, such populations have usually been found to represent various stages of speciation. Here, we review the literature on variation in host specificity among populations and sibling species of parasitoids. We then summarize our results on the evolution and genetics of host specificity in *Aphelinus varipes* Foerster and *Aphelinus albipodus* Hayat and Fatima (Hymenoptera: Aphelinidae). Populations of *A. varipes/albipodus* from *Diuraphis noxia* (Mordvilko), *Ropalosiphum padi* (L.), and *Aphis glycines* Matsumura (Homoptera: Aphididae) collected in France, Georgia, Israel, China, Korea, and Japan differed in parasitism of seven aphid species in five genera and two tribes on four host plant species in no-choice laboratory experiments. Some populations showed narrow to monospecific host use, others attacked most or all host species tested. Most populations were reproductively isolated by pre-zygotic, behavioral barriers involving female choice. However, some allopatric populations where partially or completely reproductively compatible in laboratory crosses, although they differed in host specificity. A molecular phylogeny based on three nuclear and two mitochondrial genes indicated that these compatible, allopatric populations are distinct lineages, and morphometric analyses showed subtle differences

between them. Our conclusion is that *Aphelinus varipes/albipodus* is a rich complex, with populations in various stages of speciation. Although there was some concordance between phylogenetic affinities of host species and parasitoid species, other cases showed flips in host use between closely related taxa in the complex. We have been able to introgress genes for use of a novel aphid species from one parasitoid species to another in laboratory crosses, and we are using these crosses to map genes involved in host specificity. The take-home lessons for biological control are: (1) parasitoids in what appears to be a single species, but collected from widely different geographical regions or from different host species, may differ greatly in host specificity and thus should be tested separately, and (2) allopatric sibling species with different patterns of host use may introgress if placed in sympatry, which could lead to evolutionary changes in host use.

INTRODUCTION

Populations of parasitoids collected from different host species or geographical regions can differ in host specificity. Parasitoid species may consist of distinct host races that switch little between host species in the field (Cameron *et al.* 1984; Henter *et al.* 1996; Hufbauer 2002; Nemec and Stary 1983; Powell and Wright 1988; Stary 1983). Differences in host use among populations may often be explained by unrecognized sibling species. Evidence accumulated during the last decade suggests that sibling species of parasitoids may be far more common than previously realized (Campbell *et al.* 1993; Clarke and Walter 1995; Gauld and Janzen 2004; Kazmer *et al.* 1996; Pinto *et al.* 2003). Here, we review some of the literature on variation in host specificity among populations and sibling species of parasitoids, summarize our results on this issue, and draw conclusions concerning biological control introductions.

LITERATURE REVIEW

Microctonus aethiopoides (Hymenoptera: Braconidae) from different regions and host species differ in parasitism of *Hypera postica* versus *Sitona* spp. (Coleoptera: Curculionidae) (Sundaralingam *et al.* 2001) and also in parasitism of different *Sitona* spp. (Loan and Holdaway 1961; Phillips *et al.* 2002; Sundaralingam *et al.* 2001). Some of the differences parasitism result from differences in encapsulation by the host (Phillips *et al.* 2002). *Microctonus aethiopoides* from different sources differ in nuclear and mitochondrial DNA sequences (Vink *et al.* 2003). Although Vink *et al.* (2003) found no morphological differences among sources, Sundaralingam (1986) was able to discriminate between parasitoids from *H. postica* in France and those from *Sitona discoideus* in Morocco using eight quantitative traits. Furthermore, parasitoids from *H. positica* in France and *S. discoideus* in Morocco were partially reproductively isolated, with much lower frequences of males courting and females accepting insects from the other source (Sundaralingam *et al.* 2001). These results suggest that some of the differences in host use among populations of *Microctonus aethiopoides* can be explained by confounding of cryptic, sibling species.

Aphidius ervi (Hymenoptera: Braconidae) comprises a complex of populations, some of which have been recognized as host races or sibling species based on patterns in parasitism of

their aphid hosts, reproductive compatibility, morphology, and molecular markers (Atanassova *et al.* 1998; Pennacchio *et al.* 1994). Stary (1975) synonomized many species in a morphology-based revision of *Aphidius colemani* (Hymenoptera: Braconidae), another major parasitoid of aphids. But subsequent research has shown that *A. colemani* is a complex of reproductively isolated sibling species with different patterns in host use (Messing and Rabasse 1995; Ode and Hopper, unpublished data).

Populations of *Apocephalus paraponerae* (Diptera: Phoridae), a parasitoid ants in Central and South America, show differences in morphology, molecular markers, and host specificity sufficient to consider them cryptic species (Morehead *et al.* 2001). Populations of *Pseudacteon tricuspis* (Diptera : Phoridae) appear to be cryptic species with different host ranges (Porter and Gilbert 2005). Populations of *Pseudacteon curvatus* (Diptera : Phoridae), which are being introduced to control imported fire ants in North America, also show differences in host specificity which may affect their potential for impact on non-target native ants (Porter and Gilbert 2005; Vazquez *et al.* 2004;).

Leptopilina boulardi (Hymenoptera: Figitidae), a parasitoid of *Drosophila* spp., shows geographical variation with a genetic basis in responses to different host-associated odors (Campan *et al.* 2002) and ability to avoid encapsulation by its hosts (Dupas *et al.* 2003). *Asobara tabida* and its sibling species *Asobara rufescens* (Hymenoptera: Braconidae) also show geographical variation in ability to overcome encapsulation by their hosts (Kraaijeveld and Godfray 1999; Kraaijeveld *et al.* 1994).

HOST USE IN *APHELINUS VARIPES* COMPLEX

Although *Aphelinus varipes* has been reported from 40 host species across several genera of aphids (Kalina and Stary 1976), we found distinct patterns of host use among *A. varipes* from different hosts and regions (Fig. 1) as well as different populations within a region (Fig. 2). We measured host use in single-host-species laboratory experiments, where female parasitoids had the choice of whether to oviposit or not in a particular host species. This is frequently the choice parasitoids make in the field. “Choice” tests in the laboratory provide different species in close spatial and temporal proximity, but the behavior on encountering a particular host is still whether to parasitize in or not. Our goal was to determine host acceptance/suitability in an environment that appears to harbor only one aphid species on only one plant species and where parasitoid females re-encounter this combination repeatedly with a full egg complement after a relatively long period without encountering other host species. In these experiments, we exposed 100 aphids (mixed stages) on host plant to individual, naïve, mated female wasps for 1 day, with 10-20 replicates per host-species/parasitoid-source combination. We measured parasitism as the number of mummified aphids produced during this exposure.

Most of these populations in the *A. varipes* complex had fixed differences in DNA sequences, subtle but highly significant differences in morphology, and were reproductively incompatible. It appears that *Aphelinus varipes/albipodus* is a rich complex, with populations in various stages of speciation. Thus, the host range reported in the literature for *A. varipes* is incorrect because sibling species have been confounded.

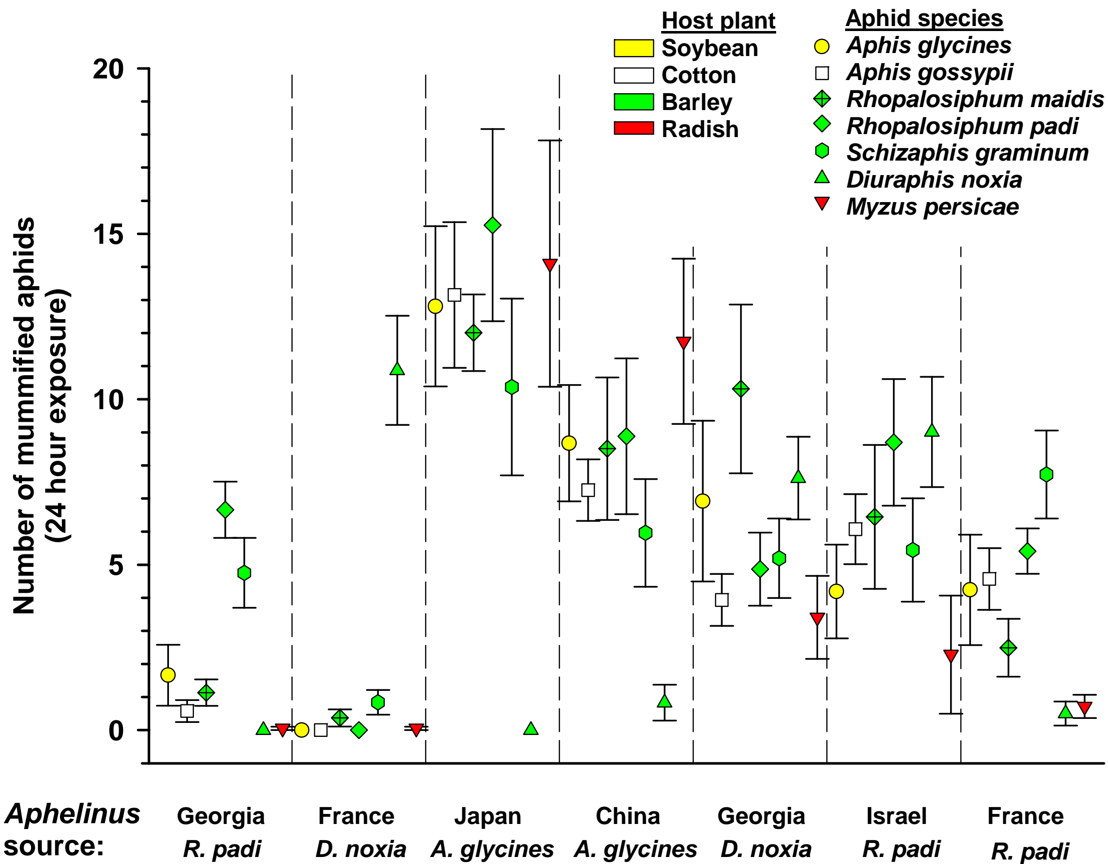

Figure 1. Host specificity in *Aphelinus varipes* complex: differences among host and regional sources.

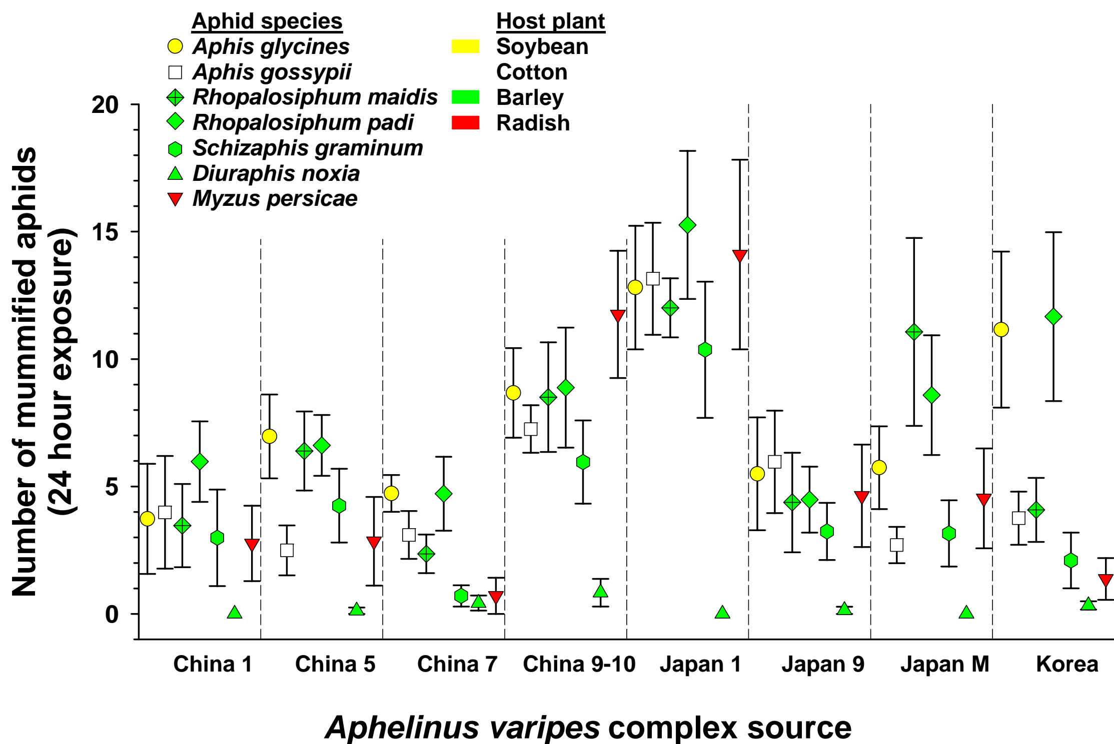

Figure 2. Host specificity in *Aphelinus varipes* complex: differences among populations from *Aphis glycines* in the Far East.

Although closely related species sometimes show similar patterns of host specificity, phylogenetic affinity was not a reliable indicator of host specificity. Even among the rather closely related species and populations in the *A. varipes* complex, use of some host species roughly maps onto the parasitoid phylogeny, but use of other species does not.

Therefore, we need to examine the genetic basis of host switches if we are to predict when they will occur. Two populations in the *A.varipes* complex, one from *D. noxia* in Georgia ('Georgia-*D. noxia*') and the other from *A. glycines* in Japan ('Japan-*A. glycines*') were reproductively compatible, despite differences in DNA sequences, morphology, and host use. 'Japan-*A. glycines*' parasitoids do not parasitize *D. noxia*, whereas 'Georgia-*D. noxia*' parasitoids readily parasitize this host (Fig. 1). By crossing and backcrossing, we have introgressed genes from 'Georgia-*D. noxia*' into the 'Japan-*A. glycines*' background and produced hybrids segregating for parasitism of *D. noxia*.

CONCLUSIONS

The take-home lessons for biological control are: (1) parasitoids in what appears to be a single species, but collected from widely different geographical regions or from different host species, may differ greatly in host specificity and thus should be tested separately, and (2) allopatric sibling species with different patterns of host use may introgress if placed in sympatry, which could lead to evolutionary changes in host use.

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FROM DESIGN TO ANALYSIS: EFFECTIVE STATISTICAL APPROACHES FOR HOST RANGE TESTING

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ABSTRACT

The major goal of host range testing in biological control is to minimize the probability that released biological control agents have unwanted effects on populations of non-target hosts. This leads to a non-trivial problem in statistical hypothesis testing, since the standard approach in statistical tests is to ask whether or not an effect – in this case acceptance of a non-target host – exists and to attribute a precise probability to err only with rejecting the null hypothesis that assumes no effect. The problem is that it is difficult to assign a probability with accepting the null hypothesis of no effect, i.e., that the biological control agent does not include a given non-target insect into its host range. Yet, this piece of information is exactly what we need for high precision and confidence. Confidence in this respect increases with sample size and the statistical effect size, i.e., the difference from the null hypothesis that is considered biologically meaningful. However, sample size is often limited due to limitations in test subjects, research money, and space for testing arenas. Consequently, there is a high premium on using a very good experimental design and employing the most powerful statistical approach available. This paper discusses common problems with experimental designs, emphasizes the necessity to decide on the statistical effect size that is biologically meaningful, points towards the need to determine the statistical power of the host range test employed, and provides an overview about powerful statistical approaches for analyzing experiments on the host range of potential biological control agents.

INTRODUCTION

Over the last two decades ecologists have become increasingly aware of novel and powerful statistical approaches. This trend can be witnessed by a number of recent textbooks on design and statistical approaches in the life sciences (e.g., Crawley 1993; Crawley 2002; Grafen and Hails 2002; Hilborn and Mangel 1997; Quinn and Keough 2002; Ruxton and Colegrave 2003) and changes in approaches used in more recent publications. This reflects both the increased awareness that conclusions in ecological studies need to be drawn in a quantitative manner with high precision and confidence, and that, for a number of reasons, large sample sizes are often difficult to obtain. This is especially so for studies on the host range of agents for bio-

logical control, since these animals have to be tested on a number of non-target hosts. Thus, the need for powerful statistical tools that allow precise analysis from limited sample sizes is especially evident in this field of research. Formerly, the statistical analysis of data in ecological investigations has been fraught with the difficulty that many if not most of the data sampled in these cases are not normally distributed and are thus not suitable for the parametric 'standard' approaches of Analysis of Variance (ANOVA) and Student *t*-tests. Instead, non-parametric statistics like, e.g. Kruskal-Wallis and Mann-Whitney U-Tests have been used that are known to be less powerful. In theory, the lack of power of non-parametric statistics may be compensated by larger sample sizes. However, an increase in sample size is often not feasible for agricultural entomologists who are usually limited by the time that can be invested, the money that can be spent on experiments, and/or the number of replicates that can be obtained through a shortage of either experimental fields or insects to work with.

In this paper, I want to make 4 points: Firstly, that in many experiments of host range testing it becomes most interesting when we do not find a statistical effect, e.g., no effect on non-target hosts, a situation that is inherently difficult to interpret in statistical testing. Secondly, and following from the first point, that it is generally important to determine and to report on the precision with which we can conclude that no effect exists when no statistically significant effect has been found, i.e., the Power of the statistical test. Thirdly, that it is usually advisable to carefully consider the distribution of the data and find the most powerful means of analyzing them. And fourthly, that as yet, not all research questions in insect host range testing can be analyzed with easily accessible powerful statistical methods and that further progress in this field is clearly needed.

Throughout, I will use verbal examples or computer generated (fake) data sets to elucidate my arguments.

β-ERRORS AND THEIR IMPORTANCE FOR INSECT HOST RANGE TESTING

The very basis of statistical testing is that, by performing an experiment, it remains impossible to prove, for example, that a natural enemy will never attack a non-target host or prey. Using a sound experimental design, we can only aim at achieving high accuracy and precision in what we conclude from the sample that we tested. Yet, using standard statistical procedures, there is always some possibility that our interpretation of the data is wrong. This is due to the fact that all the measurement variables we are interested in are usually subject to random variation (i.e., variation between sample units that cannot account for a treatment factor considered) and that our conclusion is based on a sample rather than the entire population. In general there are two ways to err: 1) based on test results we may either conclude that there is an effect when in fact there is none, or 2) we may conclude that there is no effect when in fact there is an effect (Fig. 1). Standard statistical testing is much concerned with the first kind of error, the so called α-error or Type I error, which is returned as P-value in test results. However, in insect host range testing, it is often much more important to know the probability of committing a β-error: let us assume that we have tested the mortality of non-target hosts in field cages with and without the presence of a biological control agent, have found 10 and 17 % mortality in control and treatment cages, and have not found a statistically significant deviation from the null hypothesis that states in our case that no difference exists in mortality

of the non-target prey in control cages without and treatment cages with the biological control agent present. Assume further that our statistical test returns a *P*-value of $P = 0.167$. Is it safe to conclude that we cannot reject the null-hypothesis? In this case we would usually state – using words rather than statistical jargon – that in our test the biological control agent did not cause significant mortality of the non-target prey. However, we do not know the β-error (that an effect exists that we did not detect). If we decide to release an exotic natural enemy for biological control based on such results, and if in fact we committed a β-error, i.e. the natural enemy in fact causes mortality of the non-target prey, unwanted non-target effects may be the consequence. This seems much more problematic than committing an α-error, i.e. rejecting a natural enemy for biological control based on tests that falsely led to the conclusion that the biological control agent would cause mortality of non-target prey. Therefore, in non-target testing, it seems fundamental to obtain information about the β-error. This is where power analysis comes into play.

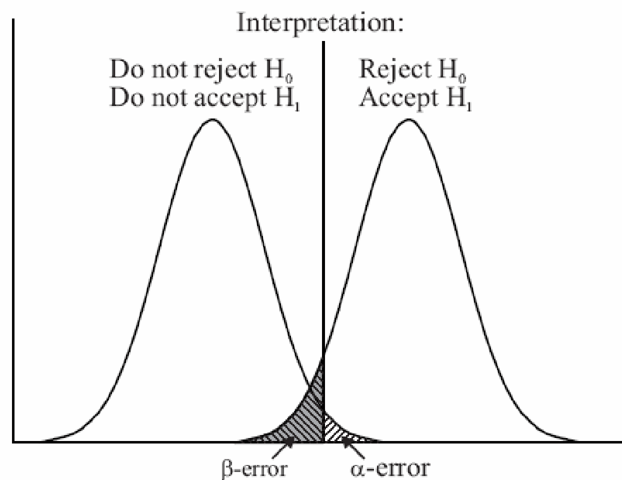

Figure 1. Graphical representation of α-error (area hatched in white and black) and β-error (area hatched in grey and black) probabilities, using a one-sided *t*-test, comparing, e.g., encounter rates of biological control agents with non-target hosts. The curves on the left (for the null hypothesis) and right (for a specified alternative hypothesis) represent the probability sampling distribution of the statistical test done. Note that usually, the alternative hypothesis is not specified, i.e. H_1 is just different from H_0, and the probability distribution of the statistical test done for H_1 is unknown (modified from Quinn and Keough 2002).

REPLICATE NUMBER, EFFECT SIZE, AND POWER OF STATISTICAL TESTS

While the β-error is defined by the probability of not finding an effect when in fact there is an effect, statistical power is the probability of a given statistical test finding an effect (rejecting the null hypothesis) when in fact there is an effect. Hence, power = 1 - β. For any particular test, power is dependent on the α-level, the sample size, the sampling variance and the so called effect size. The effect size can be regarded as the magnitude of the departure from the null hypothesis (observed effect size) or the difference between the values considered in the null and the alternative hypothesis (Fig. 1). Sample size is positively related to power, i.e., with increasing sample size does the power of a statistical test increase. However, this rela-

tionship is not linear, thus a twofold increase in power requires more than a twofold increase in sample size. Power analysis can follow three different routes, it might: 1) be used *a-priori* to define the sample size necessary to detect an effect with a predefined precision, 2) be used *a-posteriori* to calculate the Power of a test that has not detected a significant effect, or 3) to find compromise levels for α- and β-errors when sample size is fixed. The latter is a consequence of the fact that α- and β-errors are closely related. As can be seen in Fig. 1 a decrease in the α-error leads to an increase in the β-error and vice versa (e.g., imagine to shift the interpretation borderline in Fig. 1 between not rejecting H_0 and accepting H_1 to the left; the shaded areas for α- and β-errors would increase and decrease, respectively). Thus, if sample size cannot be increased, and β-errors are of concern one may compromise the α-error in the interpretation of test results, e.g., stating that a significant effect exists up to a P-value of 0.2, to use a somewhat extreme example. If sample size can be increased, i.e. before an experiment is carried out, *a-priori* power analysis can be used to define the necessary sample size. However, the effect of size needs to be determined in advance. While there are conventions for small, medium, or large effect size for different tests (Cohen 1998), in non-target tests, one may simply use the deviance from the null hypothesis of no effect as being biologically meaningful.

Let us use the above mentioned example of a field cage test on non-target effects of a biological control agent. If we would consider a mortality of 5 % induced by the biological control agent as the maximum that is acceptable and we know that in such experiments we have a background mortality rate of 10 % with a known standard deviation, we can use the arcsine-transformed proportional values (to allow for parametric tests like t-tests) to calculate the effect size. With transformed means of 0.322 and 0.398 and a standard deviation of 0.22, the effect size is 0.341 and thus falls between the values of 0.2 for small and 0.5 for medium effects that are conventionally considered. An *a-priori* power analysis for a one-tailed t-test (we are not interested whether mortality in the treatment is lower than in the control) for an α-error of 0.05 and power of 0.8 (note that this allows a β-error of 20 %) suggests a required sample size of 216, a replicate number that is often unachievable in host range testing. Allowing for 10 % mortality induced by the biological control agent would increase the effect size to 0.645 and reduce the total sample size needed to 78.

While it is usually advisable to conduct *a-priori* power analyses before conducting experiments, often some needed values like the variation around means or, in our example background mortality rates are unknown. Thus, in many cases, power analysis only comes into play, after researchers have not found a statistically significant effect and need to know the confidence with which they can decide not to reject the null hypothesis of no effect. For those *a-posteriori* power analyses, a critical parameter is the effect size assumed. Generally there are two possibilities to determine the effect size. First, the effect size may be computed from the data. However, this does not add new information about the data (see Thomas 1997 for a valuable discussion why this is so). Rather, the effect size should be either determined by using conventions or should – and I would consider this more sensible – be calculated from a biological meaningful effect that we wish to detect.

If, for example, one would have carried out the above mentioned experiments with 10 field cages each for control and treatment and would have found on average 10 % mortality in the control cages and 17 % mortality in the cages with biological control agent and non-target prey, and we would have found no significant effect of the biological control agent on the

mortality of non-target prey ($P = 0.167$), the power would be 0.277 if the effect size would reflect that we accept a maximum of 10 % mortality induced by the biological control agent. This value is unacceptably low.

Programmes to conduct power analyses are either available for free in the internet or are increasingly often included as modules in current statistical software packages (see Thomas and Krebs 1997 for a list of programs and comprehensive review on this topic and Hoffmeister *et al.* 2006 for a recent discussion and alternative ways of achieving power estimates). However, not all tests are covered yet. For example, to my knowledge, no power analysis is as yet available for Generalized Linear Models (see below).

PSEUDOREPLICATION AND DATA INTERDEPENDENCE, A CLASSICAL ISSUE, UNFORTUNATELY

One of the central assumptions of almost all statistical tests is that data points are independent from each other (one exception is planned dependencies in paired data designs). This said, we might wonder why this assumption is so often violated in experiments (see e.g., Hurlbert 1984). One of the most frequent reasons for data interdependence is pseudoreplication. It occurs whenever inferential statistics are used to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent (Hurlbert 1984). Statistical independence means that each individual data point might positively or negatively deviate from the population average due to random variation not related to the deviation of another point. Although the awareness of researchers to avoid pseudoreplication has increased and fewer studies contain analyses with pseudoreplicated samples (Heffner *et al*. 1996), an alarmingly 46% of 105 studies were found to be pseudoreplicated in a recent study on pseudoreplication in experiments on the olfactory response of insects (Ramirez *et al*. 2000) Thus, pseudoreplication still is an issue in the design of experiments, and much care has to be taken to avoid any spatial or temporal segregation of samples from different treatments. For example, when testing the host range of biological control agents, it is essential that insects for the tests on non-target hosts do not come from one rearing container or incubator and control animals (for the test on target hosts) come from another, or that non-target hosts are always tested in the same container or field cage or on the same plant and target hosts are tested in another cage or on another plant. Equally, positions of experimental units within an experimental chamber or on a field plot need to be switched between treatments to avoid confounding effects of differences in temperature and light conditions etc. In the same manner, the full set of trials on non-target hosts should not be conducted before tests with target hosts are carried out. Randomization of testing order or random assignment to plants or test cages assures that pseudoreplication can be avoided. For further reading, I encourage the reader to take a look at the section on pseudoreplication in Ruxton and Colegrave (2003).

GENERALIZED LINEAR MODELS, POWERFUL STATISTICAL APPROACHES FOR INSECT HOST-RANGE TESTING

Many of the traits to be analysed in biological investigations do not follow a Gaussian (also called "Normal") distribution, and thus standard *t*-tests, analyses of variance (ANOVA) or regression analyses cannot be used to statistically test the effect of a treatment. All these different "classical" methods assume that the distribution of residuals around the fitted model (i.e., the error distribution) is normal (Gaussian). Thus data need to be transformed to achieve a Gaussian distribution or different approaches have to be used. While transformation is often possible, it changes the relationships between parameters in the model. For example, log-transformation of data would make the relationship between parameters in the statistical model multiplicative that has been additive for untransformed values. Thus approaches should be favoured that do not make it necessary to transform values to achieve a Gaussian distribution of data. While non-parametric tests like Mann-Whitney U tests or Kruskal-Wallis tests lack statistical power, Generalized Linear Models can be used to predict responses both for dependent variables that are not normally distributed and for dependent variables which are nonlinearly related to the predictors. They are a generalization of general linear models that underlie classical statistical tests like ANOVA and regression. While in general linear models, the data distribution is Gaussian and the link function is identity, various types of data distribution and link functions (see McCullagh and Nelder 1989) can be chosen, depending on the assumed distribution of the y variable values. Table 1 gives the list for the four main generalized linear models that can be used in experiments done to estimate host range of biological control agents.

To give an example, imagine a large arena choice test as suggested in van Lenteren *et al.* 2006. Three different treatments are used, with 10 field cages each: (1) with the target prey (or host which is used synonymously here) and non-target prey present in the same field cage together with the natural enemy, (2) with only the non-target prey and the natural enemy in the same field cage, and (3) with only the target prey and the natural enemy in the same field cage. We are interested in whether the target prey is killed at a higher rate than the non-target prey and whether the mortality of the non-target prey depends upon the fact whether the target prey is available to the natural enemy or not. To achieve independent data, one should not compare whether mortality rates of target and non-target prey are equal within a single treatment. Rather, one should test whether the mortality of non-target prey in treatment (1) is equal to the mortality of non-target prey in treatments (2) and equal to the target prey in treatment (3) (this is our null hypothesis). Again, I use computer-generated data. Given the mortality rates found were 4.1 %, 10.6 % and 50.5 % in (1), (2) and (3), respectively, a Generalized Linear Model with binomial distribution and logit link finds a significant effect overall and also between treatments (Table 2). Thus, in this example, the non-target prey is attacked at relatively low rate and even less so, when target prey are available. This result is visible from the estimates in Table 2, where the estimate for mortality is positive and thus higher in treatment (2) than in treatment (1), and much higher (more than 3 times higher) in treatment (3) than in treatment (1).

Table 1. List of the main generalized linear models that can be used in experiments done to estimate the host range of biological control agents. Link functions indicated are the most frequently used ones. Other can be used in particular cases (see McCullagh and Nelder 1989, for an exhaustive description).

Distribution	Model description	Appropriate link function	Example for data type
Gaussian	General linear model	identity: $f(y) = y$	Morphological data
Binomial	Logistic regression	logit: $f(y) = \log\{y/(1-y)\}$	Proportions like parasitism
Poisson	Log-linear model	log: $f(y) = \log(y)$	Counts like egg load or number of prey consumed
Gamma	Gamma model	inverse: $f(y) = 1/y$	Time durations like survivorship

Table 2. Results of a Generalized Linear Model on computer-generated data for the mortality rates of target and non-target prey in large arena choice tests (for details, see text).

Parameter	Treatment	Estimate	DF	χ^2	Pr > ChiSq
Intercept		-3.2591	1	378.47	<.0001
Target host	(3)	3.2511	1	329.63	<.0001
Non-target prey in no-choice test	(2)	1.0839	1	30.14	<.0001
Non-target prey in choice test *	(1)	0	0	0.0000	

* In the SAS statistics package, which was used here, the last treatment [in this case (1)] is set to zero by convention and the difference between the last and all other treatments [(2) and (3)] is tested.

A special case of Generalized Linear Models exists if measurements are taken repeatedly. If, for example one plans to monitor the mortality induced by the natural enemy on the target and non-target host across a time period after the release of the natural enemy, several data points from the same treatments will be taken. In this case, A GEE model can be specified with the Generalized Linear Model (see e.g., Quinn and Keough 2002) that adequately deals with such data.

TIME DURATIONS AND CENSORED DATA

Time duration data like survival times or latency until attack usually follow an exponential distribution, because the probability λ to die or to become attacked in each time unit is constant. While generally such data can be analysed with Generalized Linear Models with gamma distribution and inverse link function, they cannot if data points are censored, i.e., when we were unable to measure a quantifiable value. Right-censored data origin, for example, from host range experiments in which we measure the latency until attack of target and non-target prey in small arenas with behavioural observation, when a predator did not attacked the prey until the end of the observation (in this case we just know that the latency is larger than the

time of observation, but cannot quantify it properly). If we just ignore those censored values, the interpretation of the test might be wrong. A Cox regression model (= proportional hazards model) can adequately deal with censored time duration data (Cox 1972). Recently, a plethora of different studies have used such an statistical analysis for ecological investigations on insects (e.g., van Alphen *et al*. 2003). Besides using this sort of analysis to study changes in survival time, a Cox survival analysis can also be used when it comes, for example, to testing residence times or giving up times of natural enemies on patches with target and non-target prey, or when testing the latency until a natural enemy attacks a host or prey.

A POWERFUL STATISTIC FOR EVERY PROBLEM? – UNFORTUNATELY NOT

Recent advancements in statistical methods may give the impression that almost every biological problem imaginable in insect host range testing could be analysed with one of the powerful methods described above. Unfortunately this is not so. Besides the banality that good statistics cannot cure poor experimental designs, some of the research questions one will often address in insect host range testing cannot be easily analyzed with powerful statistical methods. For example imagine a no choice test with a natural enemy on target and non-target host. It is statistically not problematic to test the null hypothesis that acceptance of target and non-target prey does not differ. However, this test is not the most interesting research question we might have in mind. If we are to decide whether or not to introduce an exotic natural enemy, we need to know whether the natural enemy will accept the non-target host at all. One approach would be to assume that host acceptance does not vary and, given that we have found in say, 10 replicates on non-target hosts, that they are not accepted while the target host has invariably been accepted. No statistical test would be needed in this case. However, host acceptance usually is variable. Host acceptance experiments with biological control agents of different degrees of host deprivation clearly show increasing acceptance rates with increasing host deprivation (Withers and Mansfield 2005, this issue).

One possibility to solve the problem using statistical methods would be to decide on a threshold of acceptance that can be tolerated, and given one has found no acceptance of non-target hosts in n replicate trials, one can compute the probability to obtain a series of n host rejections given the threshold level (see Porter *et al*. 1995 for a published example). Alternatively, we might use an exact test based on a binomial distribution. Here, we need to define a null hypothesis (H_0) about the likelihood that a biological control agent accepts the non-target host and an alternative hypothesis (H_A) about a threshold level of this probability that we believe would be crucial to detect. For example, let us assume the H_0 that the biological control agent would have an inherent probability of $\lambda_0 = 0.01$ to accept the non-target host (thus on average, 1 out of 1000 parasitoids would accept the non-target host). Let us further assume that we wish to detect if the true acceptance rate of the parasitoid, our H_A, is $\lambda_A = 0.05$ (the dotted line in Fig. 2). In this case, we would need 32 replicates to obtain a power of > 80 % (Fig. 2). Critical values to detect a significant deviation ($P < 0.05$) from the null hypothesis of 0.1 % acceptance rate of non-target hosts are detected if at least r non-target hosts are accepted ($r = 1$ for sample sizes of $1 \leq n \leq 51$ and $r = 2$ for $52 \leq n \leq 100$).

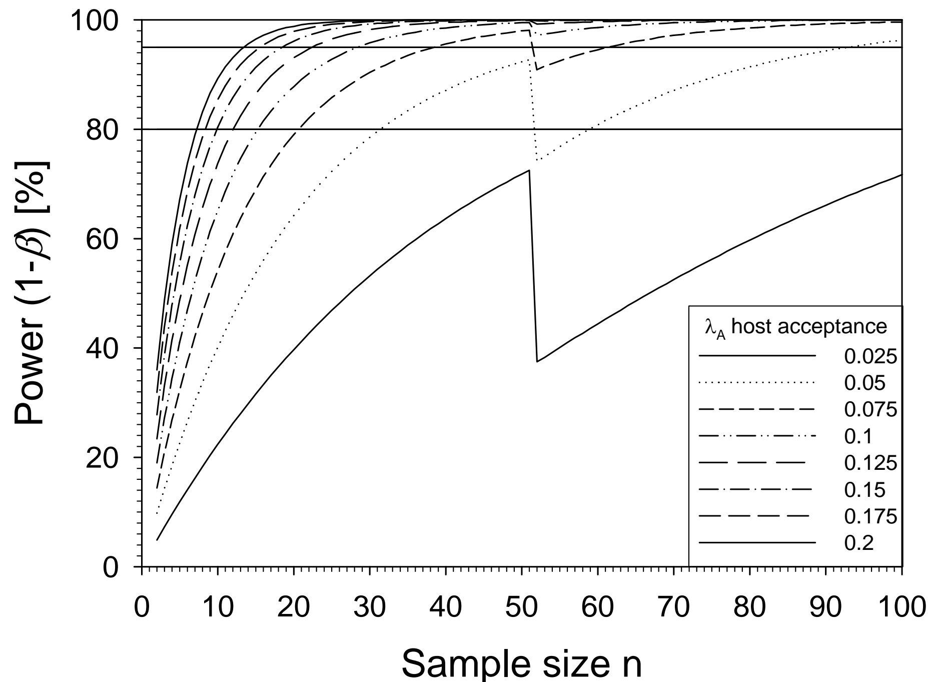

Figure 2. Statistical power for a non-target test based upon an exact binomial test under the null hypothesis H_0 of an acceptance rate of non-target hosts of $\lambda_0 = 0.001$. The tests specifies the Power, given one does not accept (P 0.05) the alternative hypothesis H_A that assumes an acceptance rate of λ_A given in the figure legend, and given fewer than r host were accepted by the parasitoid, with $r = 1$ for $n < 52$ and $r = 2$ for $n \leq 52$. Horizontal lines mark 80 and 95 % power. See text for details.

A second issue that cannot be solved statistically are optimal designs for choice or no-choice tests. As Withers and Mansfield (2005) point out, there are different benefits associated with no-choice and choice tests. If we think about the statistical analysis of such tests, choice tests can be problematic. Since the same animal will be confronted with target and non-target hosts, we usually wish to obtain more than a single data point for each animal, i.e. for example acceptance rates of target *and* non-target hosts. Thus, some sort of repeated measurement design has to be used in this case (alternatively, only the acceptance rate of non-target hosts is analyzed; see the above example). While analysis of such dependent data is generally possible (see, e.g., GEE models in Generalized Linear Models), an additional problem exists, if target and non-target hosts or prey are exposed to the natural enemy simultaneously. The acceptance of non-target hosts or prey may well depend upon the frequency of target and non-target hosts within the experimental arena. If this is so, every target prey that is removed or every target host that is accepted and that is not replaced alters the experimental conditions of the experiment, and the acceptance of any given host or prey may depend on the current availability of alternative hosts or prey. If exploited hosts or prey cannot be replaced immediately, simultaneous choice test may become almost impossible to interpret. Thus, from a statistical point of view, sequential no-choice tests may be favourable (see Singer 1986 for a discussion), where all effects like the sequence of species presented, the motivational status of the tested insect can be statistically controlled for. Yet, these two tests may lead to very different outcomes biologically (Withers and Mansfield 2005) and thus both tests have their merit, despite the problems associates with simultaneous choice tests.

CONCLUSIONS

In the past, decisions to use or reject a species as biological control agent were more often based on gut feeling than exact scientific methods. Today, sound host range tests are a prerequisite in the evaluation of biological control agents. However, despite great advances in the field (Van Driesche and Murray 2004; van Lenteren *et al.* 2006; Withers and Mansfield 2005), some issues on the interpretation of data are still unsolved. This paper advocates for a rigorous use of Power analyses to obtain a measure of confidence if one does not find significant deviations from the null hypothesis of no effect. Further, the most powerful statistical methods should be used when sample sizes are a limiting factor in insect host range studies. Despite the introduction of a number of new statistical tools, some of the basic statistical problems in host range testing are still unresolved. For example, no standard test is available to calculate a measure of confidence for an experiment where one has not found acceptance of the non-target host in n replicates (but see above for a possible method). Until now, researchers working in biological control are largely dependent on educated guesses with respect to how many replicates would be necessary to decide that an insect does not accept a given non-target host (D. Sands, J. van Lenteren, pers. comm.). Thus, further advances in statistical techniques are clearly needed.

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