FHP TECHNOLOGY DEVELOPMENT PROGRESS REPORT

PROJECT STATUS: Complete.

PROJECT NUMBER: R8-99-03

PROJECT TITLE: Development of a mass rearing scheme for the bark beetle natural enemy *Roptrocerus xylophagorum.*

SUBJECT: Biological control, bark beetles, *Dendroctonus*, *Ips, Roptrocerus xylophagorum.*

PROJECT OBJECTIVES: The core objective of this project is the development of a cost-efficient mass rearing procedure for the bark beetle larval/pupal parasitoid, *Roptrocerus xylophagorum*, capable of producing adequate numbers of insects for possible use in augmentative release programs aimed at controlling bark beetle infestations. No methods currently exist to rear *R. xylophagorum* in a cost-effective manner on its natural hosts (*Ips* or *Dendroctonus* beetles), because large quantities of tree tissue are required to rear the beetles themselves. We propose to develop methods for rearing *R. xylophagorum* on an artificial diet devoid of insect components. To meet this goal the project must address the following two component objectives:

(1) *Identification and synthesis of an oviposition stimulant for* R. xylophagorum. This parasitoid normally oviposits only on their natural hosts when these are enveloped in tree tissue. Collection and transfer of eggs from such "natural" oviposition sites to artificial media would be prohibitively labor intensive and too costly for large-scale implementation. To overcome this problem, we must characterize oviposition behavior and identify the natural cues (chemical and physical) involved in this process, and then develop a synthetic oviposition stimulant. This stimulant would be used to induce the parasitoids to deposit eggs directly on the diet or onto surfaces where the eggs might be easily recovered and manipulated.

(2) *Optimization of an artificial diet for rearing* R. xylophagorum. Previous work with Dr. M. Guadalupe Rojas (USDA-ARS) has resulted in a functional artificial diet capable of rearing *R. xylophagorum* from egg to adult, one that has no insect-derived components. However, further tests are to needed to measure the quality of the individuals produced on the artificial diet. We will compare the fecundity, longevity, size, etc. of adult parasitoids reared on the diet with those reared on natural hosts, and if necessary enrich or modify the diet to obtain similar performance.

BRIEF DESCRIPTION OF PROJECT: Studies will be conducted to meet component objectives 1 and 2 above.

(1) *Studies will be undertaken to characterize and then artificially reproduce the chemistry of* R. xylophagorum's *natural oviposition stimulant(s).* Oviposition behavior will be observed and categorized. Volatile and non-volatile compounds associated with

R. xylophagorum oviposition sites in host-infested bark will be isolated by a variety of techniques and identified using coupled gas chromatography-mass spectrometry. Compounds distinguishing the oviposition sites from other locations will be obtained in pure form, and synthetic blends of candidate oviposition stimulants will be bioassayed to check for oviposition-stimulating activity. Methods for presenting the stimulants to ovipositing parasitoids will be refined in order to maximize the facility with which eggs may be collected and transferred to media. Optimally, it will be possible to present the stimulants in such a way that oviposition occurs directly on media cells (see below), hence eliminating the need for manual or automated transfer.

(2) *Studies will be conducted to fully evaluate the fitness of* R. xylophagorum *reared on artificial diet.* Trays with multiple cells will be partially filled with the diet for *R. xylophagorum* and then eggs will be placed within each cell near the diet, as in our preliminary studies. Survivorship from egg to emerging adult will be recorded, and then measurements made of the fecundity, longevity, and size of the resulting adult wasps. Performance of *R. xylophagorum* on the diet will then be compared with those reared on natural hosts, and if necessary the diet will be modified/enriched until similar performance is obtained.

FHP PEOPLE WHO WILL LEAD PROJECT: Steve Clarke & Don Duerr, R8

- **CO-PRINCIPAL INVESTIGATORS:** Dr. Brian T. Sullivan and Dr. C. Wayne Berisford, Department of Entomology, University of Georgia, Athens, GA 30602; Dr. John D. Reeve, Southern Research Station, Pineville, LA 71360 (now located at: Dept. of Zoology, Southern Illinois Univ., Carbondale, IL 62901-6501, 618-453-6670, jreeve@zoology.siu.edu); Dr. M. Guadalupe Rojas, USDA ARS, New Orleans, LA 71360

Contributions: Dr. Sullivan will work on developing a synthetic oviposition stimulant for *R. xylophagorum* (see Methods, part 1), while Dr. Berisford will provide the necessary analytical equipment, oversight, and salary for Dr. Sullivan's work. Dr. Reeve will work on optimizing the diet for *R. xylophagorum* (see Methods, part 2), in collaboration with Dr. Rojas. All rearing and quality testing will be done at the Southern Research Station, with diet preparation and modification conducted at an ARS facility in New Orleans.

ACCOMPLISHMENTS AND RESULTS:

(1) *Identification and synthesis of an oviposition stimulant for* R. xylophagorum.

Description of Oviposition Behavior

Oviposition by *R. xylophagorum* is the end result of a well-defined sequence of behaviors. In nature, *R. xylophagorum* females enter bark beetle gallery galleries to locate hosts which they parasitise by drilling through the gallery walls with their

ovipositor. The behavioral steps leading to oviposition (as observed during the course of this study) can be categorized into the following: 1) semi-random search of a substrate, 2) intense, localized search of the substrate, 3) probing and drilling through the substrate with the ovipositor, and 4) egg deposition. These steps can be defined as follows. Semi-random search is characterized by rapid walking accompanied by frequent, sinuous turns. This behavior is normally confined to the surfaces of host-infested substrates. Intense localized search is distinguished by the complete arrestment of the insect and the prolonged antennal palpation of a small area (1-3 mm diam) on the substrate surface. This palpation involves only the antennal tips, which are alternately swept in circles that gradually decrease in size until they are brought together over a particular spot. To initiate probing and drilling, the female moves forward, gradually arching her body so that the tip of her ovipositor sheath is dragged anteriorly over the substrate until it reaches the spot localized with the antennae. Once the ovipositor sheath reaches this spot, the female moves backwards to begin forcing her ovipositor into the substrate. The female continues to move her abdomen posteriorly and ventrally as her ovipositor penetrates ("drills") deeper into the substrate. Egg deposition occurs when an egg is extruded from the tip of the ovipositor buried within the substrate. Eggs are apparently laid only in cavities within the substrate (typically larval galleries or pupal chambers) and are never deposited on an exposed surface. As mentioned previously, *R. xylophagorum* will not lay eggs on or near exposed hosts.

This complete behavioral sequence appears to be inflexible: each step is never observed without the previous steps having occurred in their stereotypic order. The process may also be terminated during any of the steps leading to egg deposition. We believe that stimulation of egg deposition for the artificial mass rearing of *R. xylophagorum* likely will require the orchestration of this same sequence of behaviors in the presence of synthetic "hosts" or media. Hence our studies focused on identifying stimuli necessary to induce one or more of the behavioral stages leading to egg deposition.

<u>Probing/drilling stimulants</u>

Through a process of trial and error, we found that female *R. xylophagorum* would drill into pieces of moistened filter paper that had been pressed onto the inner surface of excised, host-infested bark for several seconds and then removed. A treated paper's activity could persist for as long as an hour, and over this period individual females were observed to drill multiple times and at several locations on the paper's surface. Although drilling behavior was strongly stimulated, eggs were never found to have been deposited beneath the paper, even when the paper was creased to provide small, "gallery-like" cavities beneath the paper's surface. The strong drilling response observed simultaneously with the absence of egg deposition suggested that these two behaviors are triggered by a distinct set of cues, with the egg deposition cues apparently being detected by sensilla on the ovipositor. We therefore devised a small series of tests to examine some of the possible stimuli required for egg deposition (see below).

Several pertinent observations were made concerning the activity of bark-pressed filter paper. The paper would lose its ability to stimulate drilling if allowed to dry out, but this activity could be revived if the paper was carefully re-moistened. In addition, dry

filter paper did not appear to be as effective at picking up the drilling cues from bark to which it was pressed. These observations suggested that the stimulant(s) were water-soluble and probably required liquid water medium to be conducted to sensilla on the female's antennae. The first conclusion was supported when it was discovered that a water extract of host larval frass could induce intense search behaviors and ovipositor probing when presented in an atmosphere of the concentrated odors of infested bark. In contrast, organic solvent extracts of frass exhibited little or no activity. In addition, the water extract of frass appeared to have activity when dried and then rehydrated several days later.

The solubility of the probing/drilling stimulant in water and its apparent lack of volatility indicated that GC-MS was not an appropriate tool for identifying the stimulant's chemical composition. The physical properties of the stimulant suggested that it might be an excretory product of the host. Since the excretory products for at least one host of *R. xylophagorum* have been identified (Barras, 1976) and were readily available commercially, we tested four of these compounds for stimulant activity. These compounds proved inactive both separately and in combination.

Analysis of the active constituents in larval frass will almost certainly require fractionation of the extract with high performance liquid chromatography, and at the present time we possess neither the equipment nor the experience necessary to perform such an analysis. We hope in future to be able to establish a cooperative effort with scientists capable of assisting us with such work.

Stimulation of Egg Deposition:

We conducted experiments to explore some of the cues stimulating egg deposition once a female had drilled into a substrate with her ovipositor. Our observations of females drilling through filter paper treated with the probing/drilling stimulant suggested that some cue critical for stimulating egg deposition was absent. The most obvious candidate for this cue is the body of the host itself. According to an unpublished description of its oviposition behavior (Bushing, 1967), *R. xylophagorum* will typically locate a host's body with its ovipositor, sting the host with a venom that renders it immobile, and then deposit an egg on or near the host. On two occasions we succeeded in obtaining egg deposition by placing a host larval surrogate (consisting of homogenized insect larvae sandwiched between layers of Parafilm) beneath filter paper treated with the probing/drilling stimulant. However, we were unable to obtain this egg deposition consistently or from more than a tiny minority of parasitoids.

We wished to see if ovipositor contact with a live host was necessary for stimulating egg deposition, and, if not, whether a host surrogate suitable for a mass rearing scheme might be substituted for a live host. Bark sandwiches (21 cm^2 pieces of fresh, excised loblolly pine bark pressed to microscope slides) were infested with third instar *I. grandicollis* larvae that were allowed to feed for two days. The sandwiches were then subjected to a variety of treatments to alter host presence and condition. On some sandwiches, hosts were carefully dissected out of the bark by peeling back the phloem tissue concealing the insects' mines. These hosts were either replaced live into their mines (treatment b), replaced freeze-killed into their mines (treatment d), or the mines were left empty (treatment c). In all cases the phloem tissue was carefully replaced over

the mines and replaced hosts. Some sandwiches were not dissected but were tested either without further manipulation (treatment a) or having been placed in a deep freezer for ~3 h and then allowed to warm to room temperature (treatment e). Immediately following preparation, sandwiches were enclosed in glass petri plates containing three female parasitoids for a 3 h period. Afterward, the sandwiches were dissected and parasitoid eggs were counted.

We found that, while oviposition rates were somewhat reduced, *R. xylophagorum* females would nonetheless readily oviposit into host larval galleries or pupal chambers if the host was either removed or freshly dead (Table 1). On two bark sandwiches in which *I. grandicollis* larvae were replaced with the larvae of the cowpea weevil, we found that *R. xylophagorum* could successfully parasitise this surrogate host species (however, only one of four eggs laid completed development). Our data demonstrate that the presence of a live host is not necessary to stimulate egg deposition in *R. xylophagorum*, although the presence of a host or a surrogate may increase the numbers of eggs laid. This finding provides further evidence that a host-free mass rearing system for *R. xylophagorum* is possible.

The Role of Host-Associated Microorganisms

Several researchers of bark beetle ecology have proposed beetle-associated microorganisms as a probable source of cues involved in host seeking and/or oviposition behavior of parasitoids. Parasitoids of bark beetles, including *R. xylophagorum*, can be attracted to tree bolts inoculated with bark beetle associated microbes in the absence of the beetles themselves, and other evidence has pointed to a role for microorganisms in stimulating oviposition as well. This hypothesis raises the possibility that it might be possible to stimulate oviposition artificially with the microbial associates (or their metabolic products) alone, and this in turn might permit a "beetle-free" method of obtaining parasitoid eggs. It also implies that oviposition stimulation in *R. xylophagorum* might be impossible if the host's microbial associates are not incorporated into the process.

We tested this idea by performing an experiment in which female *R. xylophagorum* were presented with bark sandwiches (21 cm^2) infested with 6-10 third instar *I. grandicollis* larvae either reared from surface sterilized eggs (and therefore lacking associated microorganisms) or removed from naturally-infested pine bolts (and presumably carrying a "normal" complement of microbial associates). The larvae of both treatments had been allowed three days to feed prior to the sandwiches being presented to the parasitoids. The oviposition-related behaviors of the parasitoids were observed and, after the removal of the parasitoids, the sandwiches were dissected to count the number of hosts parasitised and eggs laid (table 2)

The absence of its host's microbial associates made no significant difference in the oviposition behavior of *R. xylophagorum* with regard to either time spent searching, numbers of distinct acts of ovipositor probing and drilling, hosts parasitised, or eggs laid. Our data indicate that the presence of the host's microbial associates is neither requisite nor beneficial for successful host finding and oviposition by *R. xylophagorum*.

(2) *Studies conducted to fully evaluate the fitness of* R. xylophagorum *reared on artificial diet.*

We encountered and potentially solved several problems in developing an artificial diet for *R. xylophagorum*, but final success has yet to be achieved. In this part of the study, *R. xylophagorum* eggs were implanted in cells partially filled with artificial diet, and the subsequent behavior of the hatched larvae observed. Difficulties were first encountered in retaining the highly active larvae within the cells and with bacterial and fungal contamination of the diet during implantation. These problems were overcome by better sealing of the cells and improved sterile technique. We then encountered a problem with excess moisture in the cells, which was overcome by inserting a drying period during dispensing of the diet. It was then discovered that the pH of the diet was too acidic, causing high mortality of the larvae. This problem can be easily fixed by adjusting the pH with a KOH solution. The diet with KOH added does appear to be palatable to *R. xylophagorum*, because in all cases the larvae were observed feeding on the diet. We plan additional work to correct these problems, beyond the end of the STDP.

DOCUMENTATION:

Barras, S.J. 1976. Role of symbiotic microorganisms in the development of the southern pine beetle. Study Final Report. USDA Forest Service, Southern Forest Experiment Station, Forest Insect Research, Pineville, LA.

Bushing, R.W.. 1967. Parasites of the western pine beetle, *Dendroctonus brevicomis* Leconte (Coleoptera: Scolytidae). Ph.D. Thesis, University of California, Berkeley.

Sullivan B.T., E.M. Pettersson, K.C. Seltmann, and C.W. Berisford. 2000. Attraction of the bark beetle parasitoid *Roptrocerus xylophagorum* (Hymenoptera: Pteromalidae) to host-associated olfactory cues. *Environ. Entomol.* 29: 1138-1151.

Sullivan. B.T., K.C. Seltmann and Berisford, C.W. 1999. A simple continuous rearing technique for the bark beetle parasitoid, *Roptrocerus xylophagorum* (Ratzeburg). *J. Entomol. Sci*. 34:260-264.

FIRST YEAR FUNDED: FY 1999
YEAR SCHEDULED TO END: FY 2000
ACTUAL YEAR TO END: FY 2000

PRODUCTS AND DUE DATES IDENTIFIED IN THE ORIGINAL PROPOSAL:
A cost-efficient process for mass rearing the parasitoid *R. xylophagorum* for use in inundative releases against bark beetle pests. We expect this process to be developed by the year 2000.

STATUS OF PRODUCTS: A probing/drilling stimulant has been identified, and work continues to identify an oviposition stimulant. A palatable diet has been developed, and it is being refined to increase larval survival. The mass rearing system is nearing completion and work will continue beyond the end of the STDP.

FUNDS OBLIGATED FROM BEGINNING OF PROJECT THROUGH END: $53,477 ($19,390 STDP funds).

POST PROJECT TECHNOLOGY SUPPORT: The transfer of this technology will involve all cooperators on this proposal (University of Georgia, Southern Research Station, and FHP personnel). Once a mass rearing procedure for *R. xylophagorum* has been developed, we plan to conduct field trials of augmentation in *D. frontalis* or *Ips* infestations, and funding for this work is needed. If efficacy were demonstrated at this level, the technology would be made available to various private companies that rear beneficial insects, who would provide the parasitoids to federal, state, and private landowners. We envision that the companies that are interested in this technique would provide the support to produce and market it. Landowners would be trained on the use of this method by FHP personnel, with the help and support of the company.

LOOK TO THE FUTURE: We expect augmentation of *R. xylophagorum* to become one part of an IPM program for SPB. To accomplish this integration, other questions should be investigated.

1. Is this technique applicable to other SPB parasitoids?
2. How does augmentation meld with other new methods of SPB suppression such as supplemental parasitoid feeding and the use of semiochemicals?
3. Recommendations as to when and how to augment parasitoid populations must be developed. For example, does augmentation work best at the onset of SPB epidemics, or toward the end when natural enemy populations are increasing? How many parasitoids should be released based on infestation size, host type, mean tree DBH, etc.?
4. Can augmentation, alone or in concert with other techniques, suppress individual spots, and/or are the benefits evident on a larger scale, providing a reduction in the duration and severity of outbreaks?

Table 1. The importance of host presence and host viability to oviposition by female *R. xylophagorum.* Bark sandwiches (21 cm^2), infested with 8 third instar *I. grandicollis* larvae two days previously, were confined in a petri dish with three *R. xylophagorum* females for 3 h. Afterwards, the sandwiches were dissected to determine the number of hosts ostensibly parasitised and eggs laid.

Bark sandwich treatment	Parasitism rate[1]	Eggs per host[2]
A. Unaltered sandwich - control	82%	2.5
B. Hosts removed then replaced live	53%	1.5
C. Hosts removed and not replaced	27%	1.7
D. Hosts removed then replaced freeze-killed	20%	1.5
E. Entire sandwich frozen then thawed	36%	2.0

[1]Percentage of distinct pupal/larval chambers with eggs present at dissection
[2]Number of eggs per pupal/larval chamber

Table 2. Host-seeking and oviposition behavior of female *R. xylophagorum* presented with bark sandwiches infested with 8-10 host larvae that were either (a) axenic (reared from surface-sterilized eggs to eliminate all microbial associates, or (b) "wild" (removed from a naturally-infested bolt and possessing a presumably "normal" microbial complement).

	During 15 min observation period of single parasitoids (n=15)		After 90 min exposure to 3 parasitoids	
	Time spent searching (min)	Oviposition attempts	% Parasitism	Eggs laid per host
a) Axenic Hosts	7.66 ± 1.90	2.9 ± 0.7	54 ± 10	1.3 ± 0.1
b) "Wild" Hosts	7.04 ± 1.68	2.5 ± 0.8	50 ± 7	1.6 ± <0.1