PROGRESS AND OBSTACLES TO DEVELOPING LYGUS BUG PHEROMONES

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ABSTRACT
Various research groups have searched for Lygus bug pheromones for more almost 3 decades, but to date, the pheromones have defied all attempts to identify them. From trials using caged live females as lures, it is clear that females attract males with an airborne pheromone, but it has not even been possible to consistently produce female extracts that attract males. Furthermore, a robust bioassay still has not been developed, and attempts to develop a bioassay are hampered by the lack of an attractive stimulus as a positive control. Twenty or more compounds have been identified from Lygus bugs, but male and female extracts look remarkably similar, and no single component or reconstituted blend of components that have been tested to date have shown any indication of being attractive to male bugs. Efforts to identify Lygus bug pheromones will be reviewed, and possible reasons why the pheromone has proven so elusive will be discussed.

Keywords – *Lygus hesperus*, *Lygus lineolaris*, hexyl butyrate, E2-hexenyl butyrate, attractant, pheromone, electroantennogram

Lygus bug species are some of the most economically important true bug species in the world, causing damage to the entire spectrum of crops from forage and fiber through to perennial tree fruit and nut crops. Because of their importance, efforts to identify sex pheromones of Lygus bugs for monitoring and possibly control purposes began more than three decades ago, and have continued sporadically ever since. Some of the best chemical ecology research groups in the world have sought this elusive pheromone, so far to no avail. In this article, we will briefly review what is known about Lygus bug chemistry, and discuss possible reasons for the lack of progress in identifying a pheromone.

Evidence from numerous researchers has demonstrated that sexually mature female Lygus bugs almost certainly use a sex pheromone to attract males, with males being attracted to caged females used as trap baits. Because the females are in screen cages in the traps, visual cues can be ruled out, and anecdotal evidence also suggests that acoustic cues are not the prime cause of long-range attraction. For example, males have been observed to fly upwind towards caged females, using the characteristic zigzag flight patterns of an insect responding to a pheromone plume (J. McLaughlin, pers.comm.). In fact, the use of female-baited traps has been suggested as a method of monitoring *L. lineolaris* (Slaymaker and Tugwell, 1984). However, Strong et al. (1970) did note that female *L. hesperus* in the presence of quiescent males tapped on the substrate, which usually induced the males to begin courtship, so acoustic cues may play some role in the overall sequence of reproductive behaviors.

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From here, the picture becomes clouded, because no research group has reported the development of a reliable and reproducible laboratory bioassay. Even worse, none of the many researchers who have worked with Lygus bugs have ever been able to produce female extracts that are consistently attractive to males in either field or laboratory bioassays. The combination of these two facts presents a very serious obstacle to identification of the pheromone(s). For example, if a bioassay gives negative results, without some kind of positive control as a benchmark, one can never be sure whether the negative results were due to the wrong treatment being tested, or whether the insects were just not responsive on that particular day (e.g., wrong age, wrong time of day, wrong bioassay conditions, etc.). Without a positive control, and without a bioassay, researchers are essentially reduced to analyzing the chemicals that the bugs produce, and then trying to reconstitute an active blend by trial and error. Even this approach has problems, as will be described below.

Extracts of Lygus bugs have been made in a variety of ways, varying from simply soaking bugs in solvent, to the more sophisticated methods of dissection and extraction of secretory glands, or, possibly best of all, trapping of the chemicals released by live virgin bugs. These methods have shown that both males and females produce the same profile of chemicals, with the only apparent difference between the sexes being quantitative variations in some of the compounds. Twenty or more compounds have now been identified (Gueldner and Parrott 1978, Hedin et al. 1985, Aldrich et al. 1988, Ho 2000), of which the most abundant compounds in *L. lineolaris*, *L. hesperus*, and *L. elisus*, hexyl butyrate and (*E*)-2-hexenyl butyrate, are produced by the large metathoracic glands. Most of these compounds are probably used for defense, although some of them may also have a secondary role as pheromone components. Overall, we have no clear idea yet where the pheromone compounds might be produced, although there is limited evidence that suggests that one or more of the pheromone components may be produced somewhere in the abdomen (Graham 1988). In contrast, for the three cases in which pheromones of other mirid bugs have been identified (2 *Phytocoris* spp. and the mullein bug *Campyloma verbasci;* Millar et al. 1997, Millar and Rice 1998, Smith et al. 1991), it appeared likely that the pheromone was produced in the metathoracic area, so for Lygus bugs, we really cannot be sure.

Numerous trials extending over more than 2 decades have failed to show any indication of attraction of bugs of either sex to any partial or total reconstituted blend of the compounds identified from *L. lineolaris* (Gueldner and Parrott 1978, Hedin et al. 1985). In our field trials with *L. hesperus* in 1997 and 1998, we field tested what we thought were likely combinations of some of the compounds, focusing on those compounds for which there were quantitative differences between males and females. We then tested all possible 2-component blends of the 15 most abundant compounds (a total of 120 different blends) found in volatiles collected from virgin female *L. hesperus*, at 2 different field sites. There was no indication that any of the blends were attractive. We and others have also tried using electroantennogram (EAG) assays to locate likely pheromone components in female *L. hesperus* (Ho 2000) and *L. lineolaris* (Chinta et al. 1994, Dickens et al. 1995) extracts, with ambiguous results. With these assays, a live insect antennae is stimulated with a test chemical, and the antennal response is amplified and quantified. The antennae of both male and female insects responded to a number of compounds in the extracts from both sexes, and there were some quantitative differences in the responses to some compounds, but no compounds were found that stimulated primarily or exclusively the male antennae, as might be expected for a sex pheromone.

We have also tried approaching the problem from the other end, by screening compounds that are similar to the pheromone components known from other mirid bugs, and similar to the chemicals that we have collected from Lygus bugs. This approach has also proven fruitless to date, with screening trials extending over 3 field seasons yielding no clear leads.

So why have Lygus pheromones proven to be so difficult to identify? There are a number of possible reasons, which concern both the reproductive biology of the insect, and the methods that have been used to try and collect and analyze the pheromone. First, some possible biological reasons:

1. From studies with many insects, we know that the physiological state of insects is critically important to pheromone production and response. With Lygus bug, we know that it takes several days for adult bugs to become sexually mature and start producing and responding to pheromone. We also have found that rearing and holding conditions are critically important; for example, only 6% of female *L. hesperus* reared in groups mated within 30 min of being put with a male, whereas 33% of females reared individually mated within 30 min (Ho 2000). It also seems logical that bugs will produce pheromone under lab rearing conditions, because we are able to maintain lab colonies for multiple generations. However, there is the possibility that bugs, in the crowded conditions customary of lab rearing, produce little pheromone, and that males encounter females frequently enough that some mating occurs even without pheromone.

2. Whereas there have been numerous demonstrations of the attraction of male Lygus bugs to live females, we have no real idea how powerful the attraction might be, and over what range a pheromone might act. Anecdotal evidence from observers suggests that the pheromone must have a range of at least several meters (J. McLaughlin, pers. comm.), but trap catches tend to be relatively low. For example, traps baited with virgin females usually catch less than 3 males per female/trap/day (Strong et al. 1970; Graham 1987; Ho, unpub. data). To place this in context, a single female moth can attract tens or even a hundred or more moths per night. Thus, we may be looking for a relatively weak response. Having said that, though, a good bioassay should be able to detect even a relatively weak response.

3. It is possible, and even likely, that at least some of our extracts do indeed contain the pheromone, but that its effects are being masked or inhibited by the other compounds in the extracts. For example, whole body extracts will inevitably contain large amounts of the defensive compounds from the metathoracic glands, and these compounds are known to produce an alarm response (and repellent effect) in bugs of both sexes (Ho, 2000). Consequently, extracts contaminated with these compounds will probably not be attractive.

   Extracts prepared by collection of the volatiles produced by undisturbed bugs should in theory be less contaminated with the defensive compounds. However, in order to collect enough material to analyze, we usually need to collect for periods of at least an hour or two, so that the extracts are time averages of volatiles production over extended periods of time, rather than snapshots of the chemical profile produced at any given instant. Thus, if female bugs are able to regulate the blend of chemicals that they produce, and if they only produce an attractive pheromone blend in short bursts interspersed with their more general and constant background odor, then this collection method may still have severe limitations.

In fact, there is a clear precedent for this type of scenario. Volatile chemicals from males and females of the rice bug *Leptocorisa chinensis* showed no qualitative sex-specific differences, but eight compounds elicited strong EAG responses from antennae of both sexes (Leal *et al*., 1996). Four of the EAG-active chemicals were not tested as attractants on the basis that they were probably defensive compounds, but the mixture of the remaining four compounds, or any mixture of 3 of the 4 compounds, was not attractive. Only a specific blend of two components attracted adult males. Thus, the attractive pheromone consisted of a specific subset of compounds from the indistinguishable and unattractive total extracts from males and females. Other components of the time-averaged trapped volatiles must inhibit response to the pheromone, and so the bugs must be able to regulate the blend of volatiles they produce.

4. Along the same lines, it is possible that females produce pheromone as they need it, and do not store it in any specialized glandular tissues, as some other insects do. Thus, the amount of pheromone present at any given time on a female, or in her tissues, may be minute. If this were the case, whole body extracts would be virtually useless, because the tiny traces of pheromone would be buried under the much larger amounts of defensive compounds and other extractable lipids.

5. We know that Lygus bug extracts contain at least 20 or so volatile compounds that could potentially be pheromone components. We also know, or at least strongly suspect, that at least some of the components are inhibitory or repellent, because the crude extracts are not attractive to bugs of either sex. Thus, we cannot simply reconstruct the entire mixture, and then remove components one by one to determine which ones result in a loss of activity when removed. Instead, we can only try and guesstimate which combinations might be important, and screen those combinations. As mentioned above, there were 120 possible combinations of the 15 most abundant compounds, and this group of 120 combinations included only a single ratio of each pair of components. It becomes logistically impossible to begin screening more complex blends consisting of three or more components, as does screening more than one ratio of each of the two-component blends.

   The dose of pheromone to test may provide a further twist, because we have no firm idea what dose range may be appropriate. With many insects, dose is critically important to obtaining the correct behavioral responses, and doses that are too large usually result in decrease or even total inhibition of attraction. All we have to go on is the dose range that typically results in responses from other insects (~0.1 to 5 mg, depending on the insect species), and these are the doses that we and other researchers typically have tested, with no responses.

The points mentioned above cover a few of the biological reasons that may have resulted in the search for Lygus pheromones being unsuccessful so far. In addition to these, there are limitations to the available methodology for analyzing pheromones that also may have contributed to our lack of success. For example:

1. Most of our methodology is optimized for the collection and extraction of nonpolar (i.e., oil-soluble) chemicals, because these are typically the types of chemicals that insects use as pheromones. However, it is possible that one or more of the pheromone components are small water-soluble chemicals that might not be extracted by the solvents that are typically used to make extracts. Small water-soluble chemicals might also be missed by the analytical methods that we normally use in pheromone identifications, particularly in trace amounts. This possibility has not been investigated in detail, in part because it is extremely difficult to fractionate and work with microscale amounts of volatile, water-soluble chemicals without losing them altogether.

2. It is possible that the pheromone is very unstable, and is destroyed during extraction or analysis. For example, we have shown that when Lygus bugs are ground up for whole body extractions, the grinding releases enzymes that rapidly degrade the defensive compounds (Ho, 2000), and the same thing may happen to the pheromone components. Alternatively, the pheromone could be destroyed by heat or sunlight, and there are a number of precedents of unstable insect pheromones of this type.

Overall, any one, or any combination of the above factors could be contributing to the lack of progress in identifying Lygus bug pheromones. Alternatively, some quirk in the biology of the insects may have been missed. Whatever the case, one of the keys to identifying Lygus bug pheromones will probably be the development of a fast, reliable, and reproducible bioassay that can be used with small amounts of material, so that fractions of extracts can be screened rapidly. It has also become clear that small-scale and sporadic efforts to identify the pheromone will probably not be successful. The bad news is that what may be required is a concentrated effort by a team of chemists and biologists dedicated specifically to this project, so that all aspects of the biology and chemistry can be examined in minute detail. The good news is that the identification of the pheromone of one species will almost certainly open the door to the identification of pheromones for other species in the genus, particularly as there is some evidence for cross-attraction between species (Graham 1987).

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