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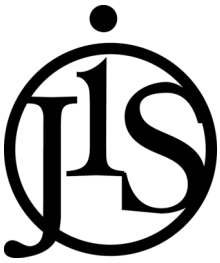

Parasitism of *Lygus* spp. nymphs by the parasitoid wasp, *Peristenus howardi,* in the alfalfa seed-growing region of the Pacific Northwest

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Abstract

The parasitoid, *Peristenus howardi* Shaw (Hymenoptera: Braconidae) has been found to parasitize a large proportion of *Lygus* species in some Washington and Idaho alfalfa seed fields. During 2002-2003 a survey was conducted to estimate the proportion of *Lygus* spp. (Hemiptera: Miridae) parasitized and the amount of that parasitism attributable to *P. howardi* in crop and non-crop plants attacked by *Lygus* in the alfalfa seed growing region of southwestern Idaho and eastern Oregon. Percentage parasitism was estimated from dissection of field-collected *Lygus* nymphs. Polymerase chain reaction (PCR) was used to amplify DNA extracted from parasitoid larvae followed by restriction endonuclease digestion of PCR products to distinguish *P. howardi* from other potentially co-occurring *Peristenus* species. Peak parasitism of *Lygus* nymphs occurred between the first and third weeks of July for both years for all host plants sampled. Of the parasitoid larvae recovered from *Lygus* nymphs in our study, 75% to 80% tested positive as *Peristenus* spp. and 76% of these matched the endonuclease digestion banding pattern for *P. howardi*. The identity of the remaining 20% to 25% of the parasitoids is not known.

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Introduction

Alfalfa (*Medicago sativa* L.) seed was produced on more than 30,000 ha (67,000 acres) in the US from 1991 to 2001 with annual production averaging about 36 million kg (80 million pounds) and an average value exceeding 100 million dollars annually during that period (NASS, 2003). Virtually all of this production originated from states in the western US. Idaho is the second largest alfalfa seed producing state behind California, accounting for 25% of the U.S. production, and is the leading producer of winter-hardy alfalfa seed. Although alfalfa grown for seed is considered a minor crop, it is the foundation of a forage alfalfa industry that ranks third nationally in planted acres behind corn and soybean (NASS, 2003).

Lygus spp., particularly *L. hesperus* Knight and *L. elisus* Van Duzee, (Heteroptera: Miridae) are the most serious pests of alfalfa grown for seed in the Pacific Northwest and California. In addition to direct yield reductions caused by feeding on alfalfa flowers and seeds (Sorenson, 1936, 1939), insecticides used to manage *Lygus* can indirectly cause further yield losses by reducing numbers and/or the activity of the alfalfa leafcutting bee, *Megachile rotundata* (F.), the principal pollinator of alfalfa seed in the Pacific Northwest (Peterson et al., 1992). Currently registered insecticides show little selectivity towards *Lygus*, increasing the cost of pest management through outbreaks of secondary pests due to the reduction of natural enemy populations (Godfrey, 2000). Moreover, insecticide-resistant *Lygus* populations have been reported in the Pacific Northwest (Xu and Brindley, 1994, Grafton-Cardwell et al., 2000). Improved knowledge of the role of parasitoids that attack Lygus may reduce dependency on insecticides harmful to pollinators and natural enemies found in alfalfa.

Native and introduced *Peristenus* spp. (Hymenoptera: Braconidae: Euphorinae) are known to parasitize *Lygus* in the northeastern US (Day, 1996) and the Canadian prairies (Braun et al., 2001). *Peristenus* are endoparasitoids that typically attack the nymphal stage of Heteroptera. Until recently, little information was available concerning parasitism of *Lygus* in the Pacific Northwest (Mayer et al., 1998; Waters et al., 2003). Earlier surveys reported that *Lygus* collected in Idaho and Utah were parasitized by *Peristenus pallipes* (Curtis) (Clancy and Pierce, 1966; Musebeck et al., 1951). More recently, a braconid wasp was found to parasitize a high percentage of *Lygus* nymphs collected from alfalfa seed in Idaho and Washington (Mayer et al., 1998). Although this parasitoid is morphologically similar to *P. pallipes* and *P. pseudopallipes* (Loan), it was determined to be a new species, *Peristenus howardi* Shaw, which is apparently native to the Pacific Northwest (Day et al., 1999).

Parasitism of *Lygus* nymphs and adults has been described in several Pacific Northwest studies indicating that infestation by introduced *Peristenus* and perhaps other parasitic wasps can have an important impact on *Lygus* numbers in alfalfa seed, alfalfa forage and perhaps other crops (Braun et al., 2001). A great deal of information on the biology of *P. howardi* comes from collections made at a few locations in Washington and Idaho. Samples collected from various weed and crop hosts of *Lygus* in Washington from 1996-1997 showed similar and relatively high (30%-33%) mean levels of parasitism in feral alfalfa, hoary cress, *Cardaria draba* (L.) Desv., and annual pepper weed, *Lepidium latifolium* L., and lower (3%-23%) mean levels of parasitism in alfalfa seed, alfalfa hay, and red clover, *Trifolium pratense* L., (Mayer et al., 1998). A 2000 survey by Ball et al. (2001) found low parasitism of *Lygus* nymphs in southwestern Idaho and recovered no parasitoids from *Lygus* collected from alfalfa near Lovelock, Nevada. Since parasitism incidence in the above studies was determined by dissection, and since *Peristenus* spp. cannot be reliably separated as larvae from each other or from other euphorine parasites (Day and Saunders, 1990), the specific and generic identity of these parasites remains unknown. Mowry and Barbour (2004) used a molecular procedure restriction endonuclease digestion of polymerase chain reaction (PCR) products to distinguish *Peristenus howardi* from four other *Peristenus* species. The objective of this study was to use this molecular procedure to determine the proportion of parasitism of *Lygus* nymphs collected from selected crop and non-crop hosts attributable to *Peristenus howardi*.

Materials and Methods

Assessment of parasitism in field collected samples

Sweep net samples were collected from crop and non-crop *Lygus* hosts between 6 June and 12 August of 2002 and 2003 in alfalfa seed production areas of the Treasure Valley in Canyon County,

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Idaho and Malheur County, Oregon. The sampling period encompassed the seasonal peak of *Lygus* nymphal parasitism (Day et al., 1999) and the period of peak bloom in alfalfa seed when the crop is most susceptible to injury by *Lygus* (Sorenson, 1936). Sampling during this period would give the highest probability of detecting parasitism in *Lygus* collected from crop and non-crop hosts. Three crop plants, alfalfa seed, alfalfa forage, and red clover; and four non-crop plants, perennial pepperweed, hoary cress, kochia, and volunteer alfalfa were selected for sampling. The crop and non-crop plants were selected based on their acceptability as hosts for *Lygus*, their occurrence during the alfalfa seed-growing season, and for non-crop hosts, their occurrence in patches large enough to facilitate sampling. Non-crop host sites consisted of nearly pure patches of host plants that were 0.004-0.4 ha in size growing in uncultivated areas that received no agricultural inputs. Crop host sites consisted of 0.4-15 ha of cultivated land planted to the specific crop and were managed according to local grower practices. Four to six sites of each crop or non-crop host were sampled at approximately two-week intervals in each year of the study. An alfalfa seed field at the Parma Research and Extension Center was used as a reference site. It was also located in Treasure Valley and has received no insecticide applications since 1998, and has historically high levels of Lygus parasitism (Day et al., 1999). It was sampled during the same period to provide reference points from which to compare data collected from other crop and non-crop hosts. Each sample consisted of five, 180° sweeps through the upper one-third of the foliage using a 38 cm diameter sweep net. The first sweep was taken two steps from the edge of the site and subsequent sweeps were taken every other step towards the center of the site for a total of ten steps. Sweep net contents were transferred to 235 ml jars containing about 150 ml of 95% ethanol. In the laboratory *Lygus* nymphs were separated from other arthropods and debris. *Lygus* nymphs were dissected in 95% ethanol using a dissecting microscope to determine the number of nymphs containing parasitoid eggs or larvae. The percentage parasitism was estimated from the proportion of *Lygus* nymphs containing at least one parasitoid. Parasitoid eggs and larvae obtained from dissections were held at -20 °C in 95% ethanol for later identification.

Identification of *Peristenus* spp. larvae

A sub-sample of parasitoid larvae recovered from dissection were identified using PCR to selectively amplify a 760 base pair region of the cytochrome oxidase I gene from *Peristenus* spp. followed by digestion of the PCR amplimer using the restriction endonuclease *Sfc* 1 to separate individual *Peristenus* (Mowry and Barbour, 2004; Tilmon et al., 2000). Genomic DNA was extracted from larvae using a modification of the protocol for the Qiagen DNeasy® Purification System kit (Qiagen Inc., www.qiagen.com). The extracted DNA was diluted 1:5 in nuclease-free water for use as a template in PCR reactions. The primers C1-J-2252 (Tilmon et al., 2000) and TL2-N-3014 (Simon et al., 1994) were synthesized by Integrated DNA Technologies, Inc. (www.idtdna.com), diluted to 6.4 µM in nuclease-free water, and stored in 50 µl aliquots at -25° C. Each 50 µl PCR reaction contained 5 µl of each 6.4 µM primer, 10 µl of 5 mM MgCl2, 25 µl of Promega PCR Master Mix (Promega Corporation, www.promega.com/), and 5 µl of diluted template DNA. Amplification was carried out in 35 cycles at 94° C for 60 seconds, 52° C for 60 seconds, and 72° C for 90 seconds using a PowerBlock® I thermocycler (Ericomp) equipped with a heated lid. PCR products were stained with ethidium bromide, electrophoresed in 1% gels (BioRad Laboratories Inc., www.bio-rad.com) in 1 x TBE buffer at 57 volts for 3 hours and visualized under UV (312 nm) light. DNA from larvae not producing an amplimer using the *Peristenus*-specific primers were subjected to PCR as described above but using 5.9 S-F and 28 S-R universal insect primers to ensure the presence of amplifiable DNA (Persad et al., 2004). PCR amplimers resulting from the use of *Peristenus*-specific primers were digested with the restriction endonuclease *Sfc* I following the protocol supplied with the enzyme (New England Biolabs, Inc., www.neb.com). Digestion reactions (20 µl) were carried out in 0.6 ml PCR tubes containing 9 µl of PCR product, 2 µl of NE buffer 4 (50 mM potassium acetate, 20 mM tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9), 8 µl of 0.25 mg/ml BSA, and 1 µl of *Sfc* I. Reactions were incubated for 1 hour at 37° C and the digestion products were electrophoresed in 3% Low Range Ultra Agarose (Bio-Rad Laboratories) gels in 1x TBE buffer at 57 volts for 4 hours. DNA banding patterns in the resulting gels were evaluated by comparison with similarly prepared gels containing digestion fragments from known specimens of *P. howardi, P. digoneutis*, *P. conradi*, P. *pallipes*, and *P. pseudopallipes*.

Statistical analyses

Percentage parasitism was measured by dividing the number of parasitized *Lygus* by the total

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number of *Lygus* collected. Percentage parasitism data were arcsine-square root transformed to equalize variances and analyzed as a split-plot in time ANOVA using the General Linear Model Procedure (SAS, 1999). Means among three or more treatments (hosts or host combinations) were separated using Tukey's HSD test at $P \leq 0.05$. Contrast statements were used to compare pooled parasitism rates for crop and non-crop hosts. Parasitism data from the Parma Research and Extension Center alfalfa seed field were not replicated and were not included in analyses, but are included in the results as a reference. Parasitism attributable to *Peristenus* spp., *P. howardi*, and to unknown *Peristenus* spp. parasitoids was determined for each *Lygus* host and all hosts in each year.

Results

Assessment of parasitism in field collected samples

The mean number of *Lygus* nymphs collected per host over all weeks ranged from 11.5 to 81.6 per 5 sweeps but did not differ significantly among host plants in 2002 ($F = 2.77$, df = 6, $P = 0.0414$) or 2003 ($F = 1.65$, df = 5, $P = 0.1861$). Although ANOVA yielded a significant F value for 2002, means were not separable by Tukey's HSD at $P = 0.05$. The number of *Lygus* nymphs collected from crop and non-crop host plants did not differ in either year (2002, $F = 4.83$, df = 1, $P = 0.0702$; 2003, $F = 0.68$, df = 1, $P = 0.4349$; Fig. 1). Parasitized *Lygus* nymphs were found on all crop and non-crop host plants sampled with peak parasitism in each year occurring between the first and third week of July (Fig. 2). The total number of parasitoids recovered from dissection of *Lygus* nymphs on all host plants was 509 and 1087 for 2002 and 2003, respectively (Table 1). The total includes data from the reference alfalfa seed field at the Parma Research and Extension Center that contributed to more than half of the totals for each year. Except for *Lygus* collected from alfalfa seed in 2002 and hoary cress in 2003, percentage parasitism in bi-weekly samples from crop and non crop hosts never exceeded 20%. Peak parasitism for hosts sampled in 2002 was 26% in alfalfa seed, 12% in feral alfalfa, 10% in annual pepperweed, and 6% or less in clover seed, alfalfa forage and kochia. Peak parasitism in 2003 was 59% in hoary cress, 18% in annual pepperweed, 15% in alfalfa seed, 12% in clover seed, 11% in kochia, and 5% in alfalfa forage (Fig. 3). Parasitism rates in *Lygus* collected from the alfalfa seed field at the Parma Research and Extension Center were generally higher than those from other crop and non-crop hosts samples with peak parasitism rates of 66% and 81% in 2002 and 2003, respectively. The seasonal mean parasitism rates did not differ among host plants in 2002 ($F = 20.4$, df = 6, $P = 0.1158$) or 2003 ($F = 2.26$, df – 5, $P = 0.0808$), nor between crop and non-crop hosts in either year (2002, $P = 0.9491$); 2003, $P = 0.7295$) and was 10% or lower for all plant hosts (Fig. 3).

Identification of parasitoid larvae

Visual inspection of larval features during dissections indicated that all parasitoids removed from *Lygus* nymphs were species of Hymenoptera. The percentage of parasitoid larvae obtained from Lygus that were screened by PCR ranged from 41% to 100% in 2002 and 94% to 100% in 2003 for individual host plants except alfalfa seed at the

Figure 1. Seasonal mean (± SEM) number of *Lygus* spp. nymphs per 5 sweep sample collected from crop and non-crop plants during 2002 and 2003. The mean number of *Lygus* spp. nymphs collected did not differ significantly among host plants in 2002 ($F = 2.77$, df = 6, $P = 0.0414$) or 2003 ($F = 1.65$, df = 5, $P = 0.1861$) or between crop and non-crop host plants in either year (2002, $F = 4.83$, df = 1, $P = 0.0702$; 2003, $F = 0.68$, df = 1, $P = 0.4349$)

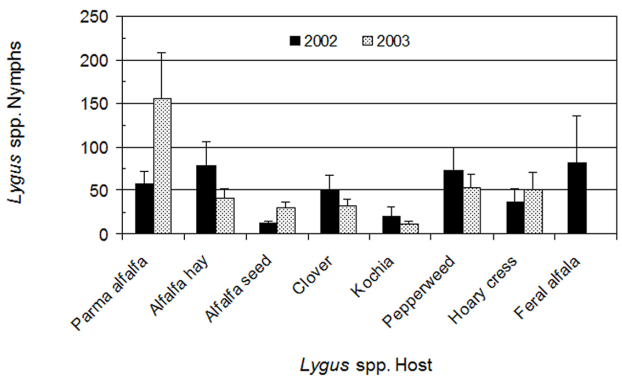

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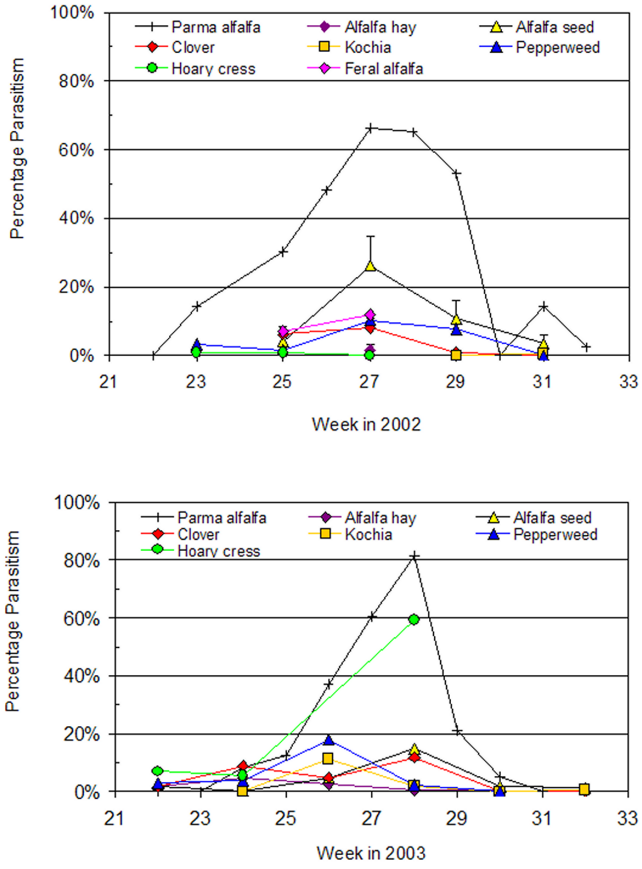
Figure 2. Mean (± SEM) percentage parasitism of *Lygus* spp. nymphs collected from crop and non-crop hosts for each sample date in A) 2002 and B) 2003. Percentage parasitism was determined by dissection of the nymphs. Percentage parasitism for PREC alfalfa represents non-replicated data included for reference.



Parma Research and Extension Center reference field. The percentage of larvae obtained from Lygus obtained from the reference field screened by PCR was 7% and 9% in 2002 and 2003, respectively. The percentage of parasitoid larvae from all host plants sampled that tested positive for the 760 base pair *Peristenus* spp. DNA amplimer was 77% and 81% in 2002 and 2003, respectively (Table 1). Endonuclease restriction products of amplimers with *Sfc1* indicated that 0% to 100% and 67% to 100% of parasitoids from the different host plants tested positive as *P. howardi* in 2002 and 2003, respectively. The percentage parasitism, as determined by dissection, was higher for *Lygus* collected from alfalfa seed at the reference field than for hosts at other sites; however, the percentage of parasitoids testing positive as *Peristenus* spp. and as *P. howardi* was similar in the reference field and all the other crop and non crop host plants sampled (Table 1) in both years of the study. A total of 26 parasitoid larvae (8 in 2002 and 18 in 2003) testing positive as *Peristenus* spp., gave *Sfc* 1 banding patterns not matching those of reference *Peristenus* used in this study and were classified as unknown *Peristenus*. One parasitoid larva in 2002 and two in 2003 gave *Sfc* 1 banding patterns matching that of *P. pallipes* or *P. pseudopallipes*. Thirty seven, or 6%, of the 586 parasitoid larvae failed to produce an amplimer when PCR was conducted using either the *Peristenus*-specific primers or the universal primers.

Discussion

Results from our survey are consistent with those of previous surveys that found similar rates of parasitism of *Lygus* nymphs among crop and non-crop host plants in the Pacific Northwest (Clancy and Pierce, 1966; Mayer et al., 1998; Waters et al., 2003). These parasitism rates were much lower than those observed in the reference alfalfa seed field at the Parma Research and Extension Center since 1996 (Day et al., 1999). Parasitism of *Lygus* by hymenopteran parasitoids

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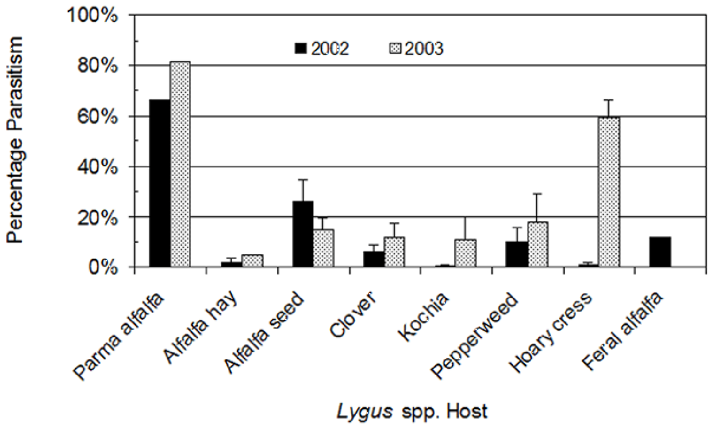
Table 1.

Year	Host	Dissection Results		Polymerase Chain Reaction				Endonuclease Digestion		
		Nymphs[1]	Parasitism[2]	Larvae[3]	*Peristenus*[4]	Other[5]	Unamplifiable[6]	*P. howardi*[7]	*P.pallipes*[8]	Unknown[9]
2002	Parma alfalfa[10]	575	267	20	20	0	0	18	0	2
	Alfalfa hay	390	7	3	3	0	0	3	0	0
	Alfalfa seed	462	56	23	18	4	1	18	0	0
	Clover seed	590	34	28	24	3	1	21	1	2
	Kochia	160	2	1	0	1	0	0	0	0
	Pepperweed	1379	85	57	34	10	13	31	0	3
	Hoary cress	257	3	3	2	0	1	1	0	1
	Feral alfala	327	55	35	30	3	2	30	0	0
	Total	**4140**	**509**	**170**	**131**	**21**	**18**	**122**	**1**	**8**
2003	Parma alfalfa[10]	2018	725	62	46	14	2	42	0	4
	Alfalfa hay	979	12	12	11	1	0	8	0	3
	Alfalfa seed	1799	83	78	65	8	5	62	2	1
	Clover	893	28	28	25	2	1	25	0	0
	Kochia	219	8	8	7	1	0	7	0	0
	Pepperweed	1396	172	170	146	20	4	138	0	8
	Hoary cress	775	59	57	45	9	3	43	0	2
	Total	**8079**	**1087**	**415**	**345**	**55**	**15**	**325**	**2**	**18**
Both years		**12219**	**1596**	**585**	**476**	**76**	**33**	**447**	**3**	**26**

[1] Total number of *Lygus* spp. nymphs collected from the respective plant hosts.
[2] Number of *Lygus* spp. nymphs with at least one parasitoid at dissection.
[3] Number of parasitoid larvae subjected to polymerase chain reaction.
[4] Number of parasitoid larvae that produced an amplimer using *Peristenus*-specific primers C1-J-2252 and TL2-N-3014.
[5] Number of parasitoid larvae that produced an amplimer using universal insect primers 5.9S-F and 28S-R, but not with Peristenus-specific primers; interpreted as non-*Peristenus* parasitoid species.
[6] Number of parasitoid larvae that produced no amplimer with either primer set; interpreted as unamplifiable DNA.
[7] Number of *Peristenus*-specific amplimers that produced an *SfcI* digestion banding pattern matching that of the known *P. howardi* reference.
[8] Number of *Peristenus*-specific amplimers that produced an *SfcI* digestion banding pattern matching that of the known *P. pallipes* or *P. pseudopallipes* references, which are indistinguishable using this endonuclease.
[9] Number of *Peristenus*-specific amplimers that produced an *SfcI* digestion banding pattern not matching any of the *Peristenus* spp. references.
[10] Unreplicated data from Parma Research and Extension Center alfalfa seed field provided for comparison.

Figure 3. Seasonal mean (± SEM) percentage parasitism of *Lygus* spp. nymphs collected from crop and non-crop hosts for each sample date in 2002 and 2003. Percentage parasitism was determined by dissection of the nymphs. Mean parasitism rate did not differ among host plants in 2002 (F = 20.4, df = 6, P = 0.1158) or 2003 (F = 2.26, df – 5, P = 0.0808), nor between crop and non-crop hosts in either year (2002, P = 0.0414); 2003, P = 0.1861. Percentage parasitism for PREC alfalfa represents non-replicated data included for reference.



in the Pacific Northwest has often been assumed to due to *P. howardi*. Our survey confirms that assumption, but also indicates that other parasitoid species are responsible for a portion of the observed *Lygus* parasitism. Approximately 81% of the PCR-tested parasitoid larvae recovered from *Lygus*

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nymphs in our study tested positive as *Peristenus* spp. The identity of the remaining 19% is not known. All or part of the unknown parasitoids may be hyperparasitoids of *Peristenus*. *Mesochorus curvulus* Thompson (Hymenoptera: Ichneumonidae) is a known endoparasitoid of braconids parasitizing mirids, including *Peristenus* (Day, 2002). This hyperparasitoid has been recovered from *Lygus* nymphs assumed to be parasitized by *P. howardi* (Day, 2002) and from emergence traps placed to collect overwintering *Peristenus* adults (Barbour, unpublished data). It is also possible that mirid parasitoids in other genera, *e.g.*, *Leiophron* spp., are responsible for a portion of the parasitism not attributable to *Peristenus* spp. observed in this study.

Approximately 6 % (29 out of 476) of the parasitoid larvae identified as *Peristenus* spp. by PCR tested negative as *P. howardi* following digestion of PCR amplimers by *Sfc1*. The banding patterns of 26 individuals produced by digestion of amplimers from these larvae did not conform to the patterns produced by *P. digonuetus*, *P. conradi*, *P. pallipes*, or *P. pseudopallipes*. The specific identity of these parasitoids is, therefore, unknown. Differences in voltinism and sex ratios between *P. howardi* collected in southern and northern Idaho and changes over time in the sex ratio of *P. howardi* collected in southern Idaho suggest that *P. howardi* may be a two-species complex; *P. howardi* and *P.* near *howardi* (W. H. Day unpublished data). In northern Idaho, *P. howardi* appears to be largely univoltine with an approximately 50:50 male-to-female sex ratio. In southern Idaho, two overlapping *P. howardi* generations seem to occur that consist of nearly 100% female wasps. The molecular procedure used in this study does not distinguish between wasps, authoritatively determined by W. H. Day, to be specimens of these proposed species (Barbour, unpublished data).

Correctly assigning the cause of mortality is critical to establishing host-parasitoid interactions and to establishing biological control programs based on these interactions (Zhu et al., 2004). Additional studies using molecular methods in combination with rearing and dissection studies are needed to clarify *Lygus* spp.-parasitoid interactions in the Pacific Northwest.

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