Host specificity of *Aphelinus* species considered for introduction to control *Diuraphis noxia*

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Abstract

Diuraphis noxia, the Russian wheat aphid, has become a major pest of wheat and barley since first being detected in the western USA in 1986. However, it is rarely a pest in Eurasia, its area of origin, and research has shown that natural enemies can limit its abundance there. Among the most important of natural enemies of *D. noxia* in Eurasia are parasitoids in the genus *Aphelinus*. Here we report results on host specificity of ten populations of seven species from two species complexes in the genus *Aphelinus*. Host specificity was not related to host plant species or the phylogenetic relatedness of the aphids or the parasitoids. While some species had very broad host ranges and others had intermediate host ranges, *Aphelinus hordei* had a very narrow host range, being restricted primarily to species in the genus *Diuraphis*, and especially *D. noxia*. We also report the results of experiments on the mechanisms of this host specificity. Most of the host specificity of *A. hordei* can be explained by differences in the behavior of females when they encountered different aphid species. Females of *A. hordei* rarely approach, sting, oviposit or host feed on aphids outside the genus *Diuraphis*, and they oviposit most frequently in *D. noxia*. From these results, we conclude that *A. hordei* is an excellent candidate for introduction into the USA to control *D. noxia*.

Keywords: biological control, Russian wheat aphid, *Aphelinus hordei*, *Aphelinus varipes* complex, *Aphelinus asychis* complex

1. Introduction

After *Diuraphis noxia* Kurdjumov (Hemiptera: Aphididae) was detected in the western USA in 1986 (Stoetzel, 1987), it rapidly became a major pest of wheat and barley (Brooks et al., 1994), causing over \$1 billion in damage and control costs in the first decade after it was found here (Morrison and Peairs, 1998). Wheat varieties resistant to *D. noxia* began to be used in 1996, and research has revealed multiple genes affecting resistance to *D. noxia* in wheat (Fazel-Najafabadi et al., 2015) and barley (Dahleen et al., 2015). However, aphid genotypes able to overcome one or more of these resistance genes began appearing by 2003 (Haley et al., 2004), and four virulent biotypes of *D. noxia* have been discovered, some of which have become widespread (Puterka et al., 2015; Randolph et al., 2009). Biological control, particularly in the form of introduced parasitoids that are specific to *D. noxia*, may not only provide direct control of this aphid, but also may slow the spread of virulent biotypes. Parasitoids could reduce the prevalence of virulent biotypes by causing density or frequency dependent mortality.

Diuraphis noxia is seldom a pest in Eurasia, its area of origin, although short-lived outbreaks have been reported (Fernandez et al., 1992; Grossheim, 1914; Tuatay and Remaudière, 1964). Field surveys and enclosure experiments have shown that natural enemies can limit *D. noxia* abundance in Eurasia (Basky and Hopper, 2000; Chen and Hopper, 1997; Hopper et al., 1995), but native natural enemies in the USA have had little impact (Feng et al., 1992; Lajeunesse and Johnson, 1991; Wraight et al., 1993). In Eurasia, parasitoids in the genus *Aphelinus* are among the most important of natural enemies of *D. noxia* (De Farias, 1995; Hopper et al., 1998). *Aphelinus atriplicis* Kurdjumov (Hymenoptera: Aphelinidae) was introduced and established against *D. noxia* by 1996 (Hopper et al., 1998) [although at the time, it was misidentified as *A. albipodus* (Heraty et al., 2007)]. This parasitoid has become the most frequently encountered natural enemy of *D. noxia* (Brewer et al., 2005), being found on 13-16% of sentinel pots of *D. noxia*-infested wheat in Colorado, Nebraska, and Wyoming (Noma et al., 2005). Nonetheless, *D. noxia* remains a pest, and a project was initiated to find additional candidates for introduction against it. To ensure introductions of exotic natural enemies would have minimum impact on non-target species, candidates were screened for host specificity.

The genus *Aphelinus* comprises >90 species (Hopper et al., 2012; Noyes, 2015; Shirley et al., 2016) that are all koinobiont endoparasitoids of aphids. They are small (~1 mm long) and weak fliers, searching for hosts and mates primarily while walking (Fauvergue and Hopper,

2009; Fauvergue et al., 1995). *Aphelinus* females prefer 2-4th instar aphids for oviposition, but will oviposit in all stages, including alate adults (Rohne, 2002). At 20°C, wasp progeny develop from an oviposited egg to pupation in about two weeks. At the third instar of the wasp, *Aphelinus* kill their hosts but leave the aphid exoskeleton intact, causing it to harden and turn black in a process called mummification (Christiansen-Weniger, 1994). Adults emerge about one week after pupation by chewing a hole through the aphid exoskeleton. Adult *Aphelinus* eat nectar and honeydew, but females also feed on aphids to get proteins and fatty acids for egg production and survival. Females emerge from their hosts with 10-15 mature eggs (Hopper and Diers, 2014). However, they continue to produce eggs throughout their lives and can parasitize an average 5-25 aphids per day, depending on age and species, and thus can parasitize ~100 aphids on average during their lifetimes (unpublished data).

The literature on the hosts of *Aphelinus* species suffers from confounding of cryptic parasitoid species (Heraty et al., 2007), which can increase estimates of host ranges when the parasitoid species have narrow and different host ranges. Host ranges may also be underestimated because of under-reporting of host aphids that are not pests (De Nardo and Hopper, 2004). Nonetheless, citations on host species in the Universal Chalcidoid Database (Noyes, 2015) suggest that host ranges of species within the genus *Aphelinus* vary from broad (e.g. 40 host species for *Aphelinus asychis*) to narrow (e.g. 2 host species for *A. hordei*). Here we report results on host specificity of 10 populations of seven species from two species complexes in the genus *Aphelinus* (Hymenoptera: Aphelinidae) originally collected from aphids that attack grain crops (Table 1). Host specificity did not depend on host plant species or the phylogenetic relatedness of the aphids or the parasitoids. While some species had very broad host ranges and others had intermediate host ranges, *Aphelinus hordei* had a very narrow host range, being restricted primarily to species in the genus *Diuraphis*, and especially *D. noxia*. We also report the results of experiments on the mechanisms of this host specificity.

2. Materials and methods

2.1. Parasitoid biology, collection, and rearing

Parasitoids were collected from 10 populations of seven *Aphelinus* species attacking *Diuraphis noxia*, *Rhopalosiphum padi*, or *Rhopalosiphum maidis* (Hemiptera: Aphididae) on wheat, barley, or maize in China, France, Israel, Republic of Georgia, and the United States

during 1999-2011 (Table 1). All these parasitoids are in the *asychis* or *varipes* species complexes of *Aphelinus*, and these complexes include several cryptic species that are reproductively incompatible and phylogenetically distinct (Heraty et al., 2007; Kazmer et al., 1996).

Parasitoids were shipped or hand-carried as mummified aphids to the containment facility at the USDA-ARS, Beneficial Insect Introductions Research Unit, Newark, Delaware, and maintained under the permits indicated in Table 1. Material was screened for hyperparasitoids and insect pathogens and then cultures were established using the founding numbers indicated in Table 1. To maintain genetic variation under laboratory rearing, each culture was split into 4-6 replicate subcultures after one generation, and each subculture was maintained with an adult population size >200 (Hopper et al., 1993; Roush and Hopper, 1995). Sex ratios of emerging adults were about 1:1 males:females. Parasitoids were reared in plant growth chambers on the host and host plant species on which they were collected at 20-25°C, 50-70% relative humidity, and 16:8 h (L:D) photoperiod. Vouchers for these populations are maintained at -20 °C in 100% molecular grade ethanol at the Beneficial Insect Introduction Research Unit, Newark, Delaware; voucher accession numbers are given in Table 1.

2.2 Aphid and host plants

In several experiments, we measured parasitism and behavior of the parasitoids when exposed to 16 aphid species in six genera and two tribes on six host plant species in four families (Table 2). These aphids and host plants were chosen to provide contrasts of aphid species in the same versus different genera and tribes on the same versus different host plant species. The goal was to explore the phylogenetic and host plant limits on parasitism, as has been recommended for host specificity testing of entomophagous insects (Kuhlmann et al., 2005).

Aphids for parasitoid rearing and host specificity experiments were from laboratory cultures at Newark started from field collections in the USA during 1996-2010. Aphids were reared on plants in plant growth chambers at 20°C, 50-70% relative humidity, and 16:8 h (L:D) photoperiod. Vouchers for these populations are maintained as described above for parasitoids.

2.3. Measurement of parasitism

We carried out several experiments to measure differences in parasitism among aphid and plant species for each parasitoid species and population. In the first set of experiments, we measured parasitism of seven aphid species (*D. noxia*, *M. persicae*, *R. maidis*, *R. padi*, *S.*

121 *graminum*, *A. glycines*, *A. gossypii*) on four host plant species by the 10 populations of *Aphelinus*
122 to test their specificity among aphid species across much of the phylogeny of aphids and on
123 several host plant species (Table 2). When these experiments were conducted, the parasitoids had
124 been in culture 12-52 generations.

125 Because the first set of experiments showed that *A. hordei* alone had a narrow host range
126 than included *D. noxia*, we measured parasitism by *A. hordei* in an experiment involving three
127 species of *Diuraphis* (*D. noxia*, *D. tritici*, *D. mexicana*), as well as well as 11 other aphid species
128 (*M. persicae*, *A. pisum*, *A. craccivora*, *A. helianthi*, *A. monardae*, *A. nerii*, *A. oestlundii*, *A.*
129 *rumicis*, *R. maidis*, *R. padi*, *S. graminum*) that included seven aphid and six plant species not
130 used in the first set of experiments. When this experiment was conducted, *A. hordei* had been in
131 culture 15-25 generations.

132 For these experiments, we used 1-5 day old females that had been with males and aphids
133 since emergence and thus had the opportunity to mate, host-feed, and oviposit. We isolated
134 females from males and aphids for 24 hr before using them in experiments to insure that the
135 females had a full egg load. In each experimental unit, we put a single female parasitoid in a cage
136 (10 cm diameter by 22 cm tall) enclosing the foliage of a potted plant of the appropriate species
137 with 100 aphids of mixed instars of a single species. Female parasitoids were removed after 24
138 hours and were used only once.

139 Because these parasitoids can parasitize a maximum of about 30 aphids in 24 hours, this
140 abundance of aphids and period of exposure allowed parasitoids to use their full egg load.
141 Furthermore, the density of aphids, amount of plant material, and cage size meant that
142 parasitoids were not limited by search rate. We waited until the larval parasitoids killed and
143 mummified their hosts before scoring parasitism. Therefore, we measured a combination of
144 acceptance of hosts for oviposition and suitability of hosts for parasitoid development. This type
145 of experiment is often called a no-choice experiment. However, female parasitoids had the
146 choice to oviposit or not when they sequentially encountered individuals of a particular host
147 species. This is frequently the choice aphid parasitoids have in the field because aphids are
148 distributed in colonies often of a single aphid species. The idea was to measure parasitism in an
149 environment that appears to harbor only one aphid species on only one plant species and where
150 female parasitoids re-encounter this combination repeatedly starting with a full egg load. Ten
151 days after exposure of aphids to parasitoids, we collected any mummified aphids and held them

for adult parasitoid emergence. After the adults emerged, we recorded the number mummified aphids and the number and sex of adult parasitoids.

2.4. Mechanisms of host specificity in *Aphelinus hordei*

To determine the mechanisms affecting parasitism of aphid species by *A. hordei*, we directly observed behavior of female parasitoids exposed to aphids. We observed female parasitoids with each of nine aphid species (*D. noxia*, *D. mexicana*, *D. tritici*, *M. persicae*, *R. maidis*, *S. graminum*, *A. helianthi*, *A. monardae*, *A. rumicis*) that included species for which *A. hordei* did and did not produce parasitized aphids in the experiments described above. In each experimental unit, we exposed a day-old female parasitoid to ten second/third instar aphids, the preferred stages for parasitism, on an excised leaf of the appropriate plant species in small arenas (10 mm diameter by 4 mm height). The arenas were formed using two microscope slides with vinyl foam weather-stripping glued to them; on one slide, the weather-stripping had a 10 mm diameter hole bored in it, exposing the slide surface through which observations were made. A leaf freshly cut from a plant was clamped between the slides (weather-stripping against the leaf) and the leaf petiole placed in 1% agar in a small vial to maintain turgor. We placed aphids in the arenas about 30 minutes before beginning observations to allow them to settle and begin feeding on the leaves. We also exposed each parasitoid female to three *D. noxia* in an arena prior to each observation bout and counted the number fed upon. We used a randomized complete-block design with each block of nine aphid species done on a single day. When this experiment was conducted, the parasitoids had been in culture 27-78 generations.

We observed each female parasitoid and the aphids with which she was enclosed continuously for 25 minutes under a binocular stereoscope at 10-30X magnification. We recorded behavior of the parasitoids and aphids with a video camera attached to the microscope and connected to a digital videotape recorder (for date/time stamping) and a DVD burner (for archiving). Identities of aphids and sequences of behaviors were also noted by hand on maps of the arenas, and these locations and sequences were verified by reviewing the DVD recordings.

On encountering a potential host, *A. hordei* females stop at about half their body length away from the aphid. Without touching the aphid, females sway from side to side several times with antennae extended (which we call an approach). They then turn 180° to face away from the aphid, extend their ovipositor, and insert it into the aphid (which we call a sting). Females sometimes failed to contact the aphid with their ovipositor (which we call a miss) and made

several attempts at approaching and stinging the aphid. Besides ovipositing in an aphid, *Aphelinus* females may also paralyze them, make a wound with their ovipositor, and drink hemolymph from the wound (host-feeding). The aphid individuals paralyzed for feeding die, even if female parasitoids do not actually feed on them, and females do not oviposit in aphids paralyzed for feeding. When female parasitoids were exposed to aphid species that they did not approach or sting, they began to run and/or flit after a short period, suggesting that they would have left the aphid patch if they had not been constrained by the arena.

We recorded whether an aphid was approached, and if approached, the outcome of each encounter (stung, missed, host-fed). For the longest sting that did not lead to host feeding, we measured the duration of the encounter from approach until the parasitoid left the aphid (handling time), and for the longest sting that lead to host feeding, we measured the duration of the encounter from approach until the parasitoid left the aphid (host-feeding time). For aphids that were approached, we recorded the number of each type of defense behavior (kicking, bucking, rotating around while retaining mouth parts in the leaf, walking away, and cornicular secretion). We also measured patch-leaving time, which we defined as the time to flitting or running. From these data, we tabulated the number of aphids approached, the numbers of aphids stung, and the numbers of aphids fed upon. We calculated aphid defense as the number of aphids that defended themselves times the frequency of each defense behavior. We removed female parasitoids after the observation period, dissected their ovaries in Ringer's solution mixed with neutral red stain, and counted the number of mature eggs under 40-100X magnification (neutral red does not pass through the chorion of mature eggs and thus they remain unstained).

If a female parasitoid stung one or more aphids, we either dissected all the aphids from a given observation period to determine whether eggs were laid, or we transferred all the aphids from a given observation period to the appropriate host plant species and reared them to determine whether parasitoid progeny survived to aphid mummification and whether exposure to parasitoids otherwise affected aphid survival. We dissected aphids from half the replicates and reared aphids from the other half of the replicates, yielding five replicates of dissected aphids and five replicates of reared aphids per aphid species that female parasitoids stung. Aphids were dissected in batches of five on glass microscope slides, each batch placed in a small drop of Ringer's solution and covered with a separate cover slip. We counted parasitoid eggs from the

dissected aphids at 40-100X magnification. For replicates in which no aphids were stung, we neither dissected nor reared the aphids after observation.

2.5. Data analysis

In the 24-hour parasitism experiments, we tested the effects of aphid species on number of parasitized (mummified) aphids, the proportion of adult wasps that emerged from parasitized aphids, and the proportion of females among these adults. The experimental unit in these analyses was a mated, female parasitoid exposed to aphids of a single species on the appropriate host plant. Replicates in which females were not recovered or died before the end of the exposure period were not used in analyses. In the first set of experiments, missing or dead females constituted <8% (72/910) of those exposed with 0-4 missing for each combination of aphid species and parasitoid population. After these were removed, we had information for 6-29 females from each of the nine parasitoid populations on each of the seven aphid species for a total of 838 females. In the 24-hour experiment with *A. hordei*, missing or dead females constituted <8% (15/200) of those exposed with 0-2 missing for each aphid species. After these were removed, we had information for 9-28 females of *A. hordei* on 14 aphid species for a total of 185 females.

In the direct observation experiment, we tested the effects of aphid species on the number of aphids that *A. hordei* females approached and stung, the number on which they host fed or in which they oviposited, and the number that were mummified. We tested the effects of aphid species on leaving time and the survival of *A. hordei* progeny. Lastly, we tested the differences in the amount of defense behaviors among aphid species and the relationship between oviposition and aphid defense. The experimental unit in these analyses was an unmated, female parasitoid exposed to aphids of a single species on a leaf of the appropriate host plant. We observed 10 females of *A. hordei* for each of nine aphid species.

Because the dependent variables often had non-normal distributions with variances proportional to means, we used generalized linear models with appropriate distributions (e.g. normal, binomial, negative binomial) for the dependent variable to test for effects of model factors. We chose the distribution that gave highest model probability calculated from the residual deviance divided by residual degrees of freedom compared to the chi-square distribution (Littell et al., 1996). To test the effect of aphid species on leaving time, we used the Cox

proportional hazards model for right-censored data. Leave time data were right censored because parasitoids did not leave patches of aphid species that they parasitized.

Because counting eggs in aphids was destructive, we could not directly compare the number of aphids in which *A. hordei* oviposited with the number that mummified and produced adult wasps. Instead, we used Spearman's rank correlation coefficient (ρ) to test the relationship between the mean number of wasp progeny and the mean number of aphids in which *A. hordei* oviposited and the relationship between the amount of aphid behavior defenses and the numbers of aphids stung, oviposited in, and host-fed upon.

For these analyses, we used `cor.test`, `glm`, `glm.nb`, and `coxph` in R version 3.2.3 (R_Core_Team, 2014). Means are given with 95% asymptotic confidence intervals in brackets. Data are archived on the Ag Data Commons website (data.nal.usda.gov; DOI 10.15482/USDA.ADC/1337670)

3. Results

3.1. Patterns of parasitism among *Aphelinus* species

These *Aphelinus* species from grain aphids differed greatly in host range (Fig. 1). Four species (*A. albipodus*, *A. asychis*, *A. atriplicis*, and *A. certus*) parasitized all or all but one of the aphid species on all or nearly all the plant species tested. Two species (*A. kurdjumovi* and *A. varipes*) parasitized several, but not all, of the aphid species on several plant species. *Aphelinus hordei*, which parasitized one aphid species on one plant species, was the only species with a narrow host range. Within these categories, species showed different host specificities (Table 3). Parasitism by both populations of *A. asychis* did not differ among aphid species, nor was there an interaction between collection host and aphid species in their effects on parasitism. However, *A. asychis* originally collected from *R. padi* produced more parasitized aphids than those collected from *D. noxia*. Parasitism differed marginally among aphid species for *A. albipodus*, mostly because of low parasitism of *M. persicae*. Parasitism differed among aphid species for *A. atriplicis*, and collection country interacted with aphid species in their effects on parasitism. *Aphelinus atriplicis* from the Republic of Georgia parasitized few *M. persicae* and moderate numbers of *A. gossypii*, whereas *A. atriplicis* from the USA parasitized few *A. gossypii* and many *M. persicae*. *Aphelinus certus* readily parasitized all aphid species on all plant species, except for

D. noxia, which it rarely parasitized. *Aphelinus hordei* was the only parasitoid that specialized on *D. noxia*.

Rates of adult emergences from mummified aphids were high (means 0.7-1.0 adults per mummified aphid) for all aphids but *M. persicae*, for which parasitoid species that showed low parasitism tended to show low emergence (Fig. 2). This caused significant effects of aphid species on rates of adult emergences for six of the seven *Aphelinus* species studied (Table 4). For *A. hordei*, emergence rates were high, even for aphid species it rarely parasitized, except *M. persicae*, from which parasitoid adults rarely emerged.

Adult sex ratios were highly variable within aphid species (Fig. 3), and for five out of the seven *Aphelinus* species, sex ratio did not differ among aphid species they parasitized (Table 5). *Aphelinus kurdjumovi* and *A. varipes* had male-biased sex ratios when they parasitized *Aphis* species, suggesting that these aphids may be poor quality hosts for these parasitoid species. For the other *Aphelinus* species, sex ratio did not significantly differ from 1:1, although the sex ratios of *A. hordei* on *D. noxia* and *S. graminum* tended to be female-biased.

3.2. Host specificity of *Aphelinus hordei*

In the experiment on the host specificity of *A. hordei*, parasitism varied strongly among aphid species (model deviance = 98.0, df = 13; residual deviance = 111.7, df = 171; $P < 0.00001$). *Aphelinus hordei* parasitized primarily species in the genus *Diuraphis*, particularly *D. noxia*, and little or no parasitism outside this genus (Fig. 4). Rates of adult emergences from mummified aphids were high for *D. noxia* (mean = 0.89 [0.77-1.00]), *D. mexicana* (0.84 [0.69-1.00]), and *S. graminum* (0.95 [0.75-1.00]), but lower for *D. tritici* (0.66 [0.46-0.85]) and *A. monardae* (0.56 [0.26-0.85]). However, the low number of replicates with parasitized aphids for species other than *D. noxia* meant that adult emergence rates were not quite significantly different among aphid species (model deviance = 0.6, df = 4; residual deviance = 3.0, df = 43; $P = 0.07$). Adult sex ratios did not differ from 1:1 and were not affected by aphid species (model deviance = 0.4, df = 3; residual deviance = 5.7, df = 39; $P = 0.48$).

3.3. Mechanisms of host specificity in *Aphelinus hordei*

Most of the host specificity of *A. hordei* can be explained by differences in the behavior of females when they encountered different aphid species (Fig. 5; Table 6). When exposed to species in the genus *Diuraphis*, *A. hordei* females approached 5-8 aphids on average during the

25-minute observation periods. *Rhopalosiphum maidis* and *S. graminum* were the only non-*Diuraphis* species approached with even moderate frequency.

Within the genus *Diuraphis*, *A. hordei* females stung most of the aphids they approached, although there was a tendency to sting *D. noxia* more per approach than *D. mexicana* and *D. tritici* (90% and 84% of approaches, respectively, led to stings versus 96% for *D. noxia*). Outside the genus *Diuraphis*, *A. hordei* females stung both *R. maidis* and *S. graminum* 45% of the time they were approached, but the other non-*Diuraphis* species were never stung. Although *A. hordei* females approached and stung *D. mexicana* and *D. tritici* as often or more often than *D. noxia*, they oviposited more often in *D. noxia* (3 [2-6] aphids with eggs) than in the other *Diuraphis* species (2 [1-4] aphids with eggs). This meant that the proportion of aphids in which parasitoids oviposited per aphid stung was higher for *D. noxia* (0.64) than for *D. mexicana* (0.42) or *D. tritici* (0.28). *Aphelinus hordei* females rarely oviposited in *S. graminum*, and never oviposited in other non-*Diuraphis* species. Handling time of aphids that were not fed upon varied among aphid species (Table 6), from a mean of 69 [48-100] seconds for *D. noxia* to a mean of 24 [16-36] seconds for *R. maidis* (Fig. 6). This variation may have arisen from longer times for stings involving oviposition than for stinging without oviposition, together with fewer ovipositions in species other than *D. noxia* and particularly in non-*Diuraphis* species. Mean duration of stings of females correlated with the number of eggs they laid in aphids (Spearman's $\rho = 0.78$, $P < 0.00001$), suggesting that stings with oviposition took longer than stings without oviposition. The distribution of sting durations was bimodal, and the mean sting duration for females of *A. hordei* that laid one or more eggs in aphids was 3.5-fold longer than that for females that laid no eggs (14 [11-16] seconds versus 48 [44-52] seconds, respectively), which also supports the idea that stings for oviposition took longer.

Survival of *A. hordei* progeny in *D. noxia* was higher (75%) than in *D. mexicana* (36%) or *D. tritici* (22%), which may explain part of the differences in realized parasitism among these aphid species in the experiments above. The mean number of aphids that were mummified was strongly correlated with the mean number of aphids in which females oviposited (Spearman's $\rho = 0.98$, $P < 0.00001$).

The numbers of aphids that were host fed varied with aphid species, with female *A. hordei* feeding only on *Diuraphis* species (Table 6). Female *A. hordei* consumed 0.7 [0.3-1.5] *D. noxia*, 0.5 [0.2-1.2] *D. tritici*, and 0.4 [0.2-1.1] *D. mexicana*, during the 25-minute observation

period, but these differences were not significant (model deviance = 0.9, df = 2; residual deviance = 24.8, df = 27; $P = 0.65$). Handling times for aphids that were fed upon were 9-17 fold longer than for aphids that were not fed upon, being 687 [494-956] seconds for *D. mexicana*, 631 [512-778] seconds for *D. noxia*, and 537 [435-662] seconds for *D. tritici*, but the differences were not significant (Table 6). The main difference was in the duration of stings that were 6-8 times longer than for aphids that were not host-fed and the duration of host-feeding that were as long or longer than the duration of stings.

Among aphids approached by *A. hordei*, defense behavior varied among aphid species (Table 6), with *D. noxia* defending itself least and *S. graminum* most (Fig. 7). Neither the number of aphids in which females oviposited nor the number of aphids on which they host-fed correlated with the amount of defense behavior by aphids (Spearman's $\rho = 0.11$, $P = 0.15$; $\rho = 0.09$, $P = 0.27$, respectively), and the number of aphids stung correlated positively, rather than negatively correlated, with the amount of defense behavior (Spearman's $\rho = 0.59$, $P < 0.00001$).

The time to patch leaving by *A. hordei* females were much longer for *Diuraphis* species than for other aphid species (Fig. 8; Cox proportional-hazards likelihood ratio = 39.5, df = 8, $P < 0.00001$).

At the start of the observation periods, female *A. hordei* with four ovarioles carried 13 [12-14] eggs and females with six ovarioles carried 17 [14-20] eggs (range 2-33). Female *A. hordei* oviposited 8% of their eggs on average during the 25-minute observation periods. Although two females carried only two eggs and one carried three eggs, the rest of the females carried at least six eggs, and a maximum of four eggs were laid in aphids, so these females did not appear to be egg-limited. Furthermore, there was no correlation between the numbers of aphids in which female *A. hordei* laid eggs and their egg loads (Spearman's $\rho = -0.09$, $P = 0.70$).

4. Discussion

Among the seven *Aphelinus* species we tested, which included all species found on grain crops during exploration for parasitoids of *D. noxia* in Eurasia (Heraty et al., 2007; Hopper et al., 1998), only *A. hordei* specialized on *Diuraphis* species, and in particular, *D. noxia*. The specialization on *Diuraphis* species resulted from oviposition restricted almost exclusively to aphids in this genus. Female *A. hordei* very rarely approached aphids on host plants other than barley, and even with aphids on barley, walked over non-host aphid species, completely ignoring them. Furthermore, *A. hordei* rarely stung non-*Diuraphis* species but did sting exuviae of

Diuraphis species. Female *A. hordei* rarely touched host aphids or exuviae with their antennae but did orient head-first towards them at a distance before stinging them. Within *Diuraphis*, female *A. hordei* oviposited more and their progeny survived better in *D. noxia* than in other species. Although *A. hordei* females approached and stung as many or more *D. mexicana* and *D. tritici* as *D. noxia*, the proportion of aphids in which parasitoids oviposited per aphid stung for *D. noxia* was 51% higher than for *D. mexicana* and 127% higher than for *D. tritici*, suggesting that parasitoid evaluation of host quality may be mediated in part by sensilla on their ovipositors. Together, these results suggest two steps may be involved in *A. hordei* parasitism of hosts: recognition of a potential host, perhaps by detection of cuticular hydrocarbons in the aphid cuticle with sensilla on the antennae (or elsewhere), and then acceptance of the aphid for oviposition mediated by sensilla on the ovipositor after potential hosts are stung. Females of *A. hordei* also specialized on *Diuraphis* species for host feeding, and they host fed more on *D. noxia* than on the other *Diuraphis* species, which suggests that the same cues may be used for recognition and acceptance of aphid species for both feeding and oviposition.

The short times to patch-leaving behavior by *A. hordei* on non-*Diuraphis* species also suggest that it would not parasitize these species in the field. The occasional parasitism of non-*Diuraphis* species in both the behavioral experiments and the 24-hour experiments may have resulted from parasitoids revisiting patches of aphids that they would not have revisited if they had been allowed to disperse.

Such taxonomic specialization might suggest that host specificity in *Aphelinus* species is related to phylogenetic relatedness of the aphid species or to the chemistry of their host plants. However, *A. kurdjumovi* and *A. varipes* both parasitized *R. padi* and *S. graminum* equally well, but rarely parasitized *R. maidis*, a congener of *R. padi*. The four other parasitoid species attacked most or all of the aphid species to which they were exposed, so phylogenetic relatedness of the aphids could not have an effect on them. Host specificity was not determined by host plant species. For example, *A. hordei* parasitized *Diuraphis* species on barley but ignored the other species on this host plant, while *A. kurdjumovi* parasitized only two of the four species on barley, but *A. varipes* parasitized aphids on barley, cotton and soybean. Lastly, the four generalist parasitoids parasitized aphids on most or all the host plant species, some of which had very different chemistries.

Phylogenetic relatedness of parasitoids also failed to explain patterns in host specificity. *Aphelinus hordei* and *A. kurdjumovi* are closely related (Heraty et al., 2007), but their host specificities are non-overlapping. *Aphelinus atriplicis* and *A. varipes* are also closely related and show low levels of reproductive compatibility, with 8% of crosses between *A. atriplicis* females and *A. varipes* males yielding female progeny (Heraty et al., 2007). Although they both parasitized all the aphid species tested, *A. atriplicis* did equally well on most species, while *A. varipes* had high fitness on only two species.

The shorter handling times of *A. hordei* for species other than *D. noxia* may reflect female parasitoid rejection of poor host quality during stings, which would lead to less frequent oviposition and thus shorter handling times, which is supported by significantly shorter handling times for parasitoids that did not lay eggs in aphids than for those that did. Like other *Aphelinus* species, *A. hordei* has relatively large, anhydropic eggs that appear to take a long time to pass through their long, thin ovipositors into host aphids. In support of this idea, the handling times for *A. hordei* are much longer than those reported for the other major group of aphid parasitoids, the aphidiine braconids (Wu et al., 2011) that carry much smaller eggs and have much shorter ovipositors with a large egg canal (Le Ralec et al., 1986). These long handling times have implications for the impacts of intraguild predation on parasitoid foraging behavior (Martinou et al., 2010), and probably mean that *A. hordei* is unlikely to attack ant-defended aphids (Wyckhuys et al., 2009).

Our results on the lack of correlation between defense behavior by aphids and oviposition or host feeding by *A. hordei* females, together with the positive correlation between defense behavior and stinging of aphids by *A. hordei* females, suggest that more parasitoid attacks elicited more, but ultimately ineffective, defensive behaviors by the aphids.

Comparing our results with *Aphelinus* host specificity from the literature is difficult because there are no previous publications that evaluate *Aphelinus* specificity experimentally. All the published evidence on *Aphelinus* specificity is from field collections of parasitized aphids. Host specificity obtained by rearing parasitoids from field-collected hosts suffers from two major problems. First, publications about host species from field collections almost always state that the parasitoid species was reared from a host species but do not estimate parasitism levels. In our experiments, we found parasitism of some aphid species at very low levels for some parasitoid species, and field records might include these aphids in the host range of the parasitoids even

though they are rarely attacked. Second, closely related *Aphelinus* species can be very difficult to distinguish so that host records from the literature may confound cryptic species of parasitoids. In particular, Yasnosh (1978) reported that *D. noxia* was a host of *A. varipes*, but later reported that this was a misidentification of what was actually *A. hordei* (Yasnosh, 2002). Ferriere (1965) synonymized *A. hordei* with *A. varipes* and the synonymy was supported by Graham (1976), but this synonymy is incorrect because the two species are reproductively incompatible and phylogenetically distinct (Heraty et al., 2007), despite being difficult to distinguish morphologically (Prinsloo et al., 2002). The results reported here show that *A. varipes* does not parasitize *D. noxia*, whereas *A. hordei* is a specialist on *Diuraphis* species, and especially *D. noxia*.

Narrow host ranges are rare among aphidiine braconids, but some species in this group do show relatively narrow host ranges (Desneux et al., 2009). Nonetheless, the limited experimental results on aphidiine braconids compared with our results on *Aphelinus* species suggest that the latter have a greater diversity of host specificities, including extremes within the same species complex, like *A. hordei* with a very narrow host range versus *A. atriplicis* with a very broad host range.

Aphidiine braconids antennate and bend their abdomens towards aphid exuviae (essentially empty cuticles) and chemicals extracted from these exuviae, which can include hydrocarbons, wax esters, alcohols, and aldehydes (Muratori et al., 2006). *Aphelinus* species will also sting aphid exuviae, suggesting that exuviae contain chemicals involved in *Aphelinus* host recognition. Although *A. hordei* females stung aphid species other than *D. noxia*, including species in genera other than *Diuraphis*, *A. hordei* females almost never laid eggs in species in other genera. Furthermore, the ratio of ovipositions to stings was higher for *D. noxia* than for other *Diuraphis* species. These results suggest that *A. hordei* females use cues on aphid cuticles to decide whether to sting and cues inside aphids to decide whether to oviposit. We are now studying the parasitoid genes that could be involved in these decisions.

Our results show that *A. hordei* is specialist on *Diuraphis* species, and particularly *D. noxia*. This specialization results from almost exclusive acceptance of *Diuraphis* species for oviposition and from higher oviposition and survival of progeny in *D. noxia* than in the other *Diuraphis* species. The narrow specificity of *A. hordei* makes it an excellent candidate for introduction into the USA to control *D. noxia*.

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Figure captions

Fig. 1. Parasitism of seven species of aphids exposed to species in the genus *Aphelinus* collected from wheat, barley, and maize. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles. Host plants on which aphids were exposed are indicated by the fill colors of the boxes: green = barley; brown = radish; yellow = soybean; white = cotton.

Fig. 2. Adult emergences from seven species of aphids parasitized by species in the genus *Aphelinus* collected from wheat, barley, and maize. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles. Host plants on which aphids were exposed are indicated by the fill colors of the boxes: green = barley; brown = radish; yellow = soybean; white = cotton.

Fig. 3. Sex ratios of adults emerging from seven species of aphids parasitized by species in the genus *Aphelinus* collected from wheat, barley, and maize. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles. Host plants on which aphids were exposed are indicated by the fill colors of the boxes: green = barley; brown = radish; yellow = soybean; white = cotton.

Fig. 4. Parasitism of 14 species of aphids exposed to *Aphelinus hordei*. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles.

Fig. 5. Behavior of *Aphelinus hordei* when exposed to nine species of aphids. Symbols represent means and vertical lines are asymptotic 95% confidence intervals of the means.

Fig. 6. Handling times of aphids not host-fed for *Aphelinus hordei* on species of aphids in which female parasitoids oviposited. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles.

Fig. 7. Amount of defense behavior by aphid species in which female *Aphelinus hordei* would oviposit. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles.

Fig. 8. Leaving times *Aphelinus hordei* when exposed to nine species of aphids. Diamonds are means and vertical lines are asymptotic 95% confidence intervals of the means. Upper and lower sides of the boxes indicate lower and upper quartiles and the horizontal line indicates the median. Grey dots are outliers beyond the quartiles.

A. asychis from *D. noxia* *A. atriplicis* from Republic of Georgia *A. hordei* from 2000 *A. asychis* from *R. padi* *A. atriplicis* from USA *A. kurdjumovi* *A. albipodus* *A. certus* *A. varipes*

rates of adult emergences

aphid species

sex ratios (proportion males)

aphid species

number of parasitized aphids

number of aphids

behavior

- approached
- stung
- oviposited
- mummified

aphid defense

D. noxia

D. mexicana

D. tritici

R. maidis

S. graminum

aphid species

leave time (minutes)

Table 1. Origins of *Aphelinus* species collected from grain aphids and tested for host specificity on *Diuraphis noxia*. All

<i>Aphelinus</i>		Aphid							
complex	species	host	Country	Region	Locations	Number	Year	Collectors	Permit and Voucher
<i>asychis</i>	<i>asychis</i> Walker	<i>D. noxia</i>	France	Herauld	Prades-le-Lez	>200	2000	N. Ramualde, D. Coutinot, J. Lopez	P526P-15-04274, AFr00_Dn
	<i>asychis</i> Walker	<i>R. padi</i>	France	Herauld	Prades-le-Lez	>200	2000	N. Ramualde, D. Coutinot, J. Lopez	P526P-13-02503, AFr00_Rp
	<i>albipodus</i> Hayat & Fatima	<i>R. padi</i>	Israel	Judea	Gedera	81	1999	K. Hopper	P526P-13-02503, VIsr99
<i>varipes</i>	<i>atriplicis</i> Kurdjumov	<i>D. noxia</i>	Republic of Georgia	Tbilisi	Satchergesi	101	2000	D. Coutinot	P526P-15-04274, VGg00_Dn
	<i>atriplicis</i> Kurdjumov	<i>D. noxia</i>	USA	Colorado, Nebraska, Wyoming	Sterling, Scottsbluff, Cheyenne	584	2006	K. Hopper	P526P-15-04274, VUS06_Co12
	<i>certus</i> Yasnosh	<i>R. maidis</i>	China	Hebei	Langfang	88	2005	K. Hopper	P526P-13-02503, VCh05_Lfm
	<i>hordei</i> Kurdjumov	<i>D. noxia</i>	France	Herauld	Prades-le-Lez	40	2000	N. Ramualde, D. Coutinot, J. Lopez	P526P-15-04274, VFr00_Dn
	<i>hordei</i> Kurdjumov	<i>D. noxia</i>	France	Herauld	Montpellier	69	2011	G. Mercadier, M. Roche	P526P-15-04274, VFr11_Dn
	<i>kurdjumovi</i> (Kurdjumov)	<i>R. padi</i>	Republic of Georgia	Tbilisi	Satchergesi	80	2000	D. Coutinot	P526P-13-02503, VGg00_Rp
	<i>varipes</i> (Förster)	<i>R. padi</i>	France	Herauld	Prades-le-Lez	107	2000	N. Ramualde, D. Coutinot, J. Lopez	P526P-13-02503, VFr00_Rp

Table 2. Aphids and host plants on which parasitism by *Aphelinus* species was measured.

Aphid		Host plant			
Tribe	Species	Family	Species	Variety	Common name
Macrosiphini	<i>Diuraphis noxia</i> (Kurdjumov)	Poaceae	<i>Hordeum vulgare</i> L.	Lacey	barley
	<i>Diuraphis tritici</i> (Gillette)				
	<i>Diuraphis mexicana</i> (Baker)		<i>Bromus marginatus</i> Nees ex Steudel	Garnet	mountain brome
	<i>Myzus persicae</i> (Sulzer)	Brassicaceae	<i>Raphanus sativus</i> L.	Cherry Belle	radish
	<i>Acyrtosiphon pisum</i> Harris	Fabaceae	<i>Vicia faba</i> L.	Windsor	fava bean
	<i>Aphis craccivora</i> Koch				
Aphidini	<i>Aphis glycines</i> Matsumura		<i>Glycine max</i> (L.)	Pioneer 91Y70	soybean
	<i>Aphis gossypii</i> (Glover)	Malvaceae	<i>Gossypium hirsutum</i> L.	SG105	cotton
	<i>Aphis helianthi</i> Monell	Asteracea	<i>Helianthus annuus</i> L.	-	common sunflower
	<i>Aphis monardae</i> Oestlund	Laminacea	<i>Monarda fistulosa</i> L.	-	wild bergamot
	<i>Aphis nerii</i> Boyer de Fonscolombe	Apocynaceae	<i>Asclepias syriaca</i> L.	-	common milkweed
	<i>Aphis oestlundii</i> Gillette	Onagraceae	<i>Oenothera biennis</i> L.	-	common evening-primrose
	<i>Aphis rumicis</i> L.	Polygonaceae	<i>Rumex altissimus</i> Wood	-	pale dock
	<i>Rhopalosiphum maidis</i> (Fitch)	Poaceae	<i>Hordeum vulgare</i> L.	Lacey	barley
	<i>Rhopalosiphum padi</i> (L.)				
	<i>Schizaphis graminum</i> (Rondani)				

Table 3. Analyses of deviance for effect of aphid species on parasitism (mummies formed after 24 h exposure) by parasitoids in the genus *Aphelinus* collected from aphids on wheat, barley, and maize.

parasitoid species	factor	model		residual		<i>P</i>
		df	deviance	df	deviance	
<i>asychis</i>	collection host	1	6.3	134	179.1	0.01
	aphid species	6	7.4	128	171.7	0.28
	interaction	6	2.7	122	169.0	0.85
<i>albipodus</i>	aphid species	6	12.3	44	62.5	0.06
<i>atriplicis</i>	collection country	1	8.9	189	262.9	0.003
	aphid species	6	17.3	183	245.6	0.008
	interaction	6	15.7	177	229.9	0.02
<i>certus</i>	aphid species	6	48.0	65	82.6	<0.00001
<i>hordei</i>	aphid species	6	19.9	214	262.6	0.003
<i>kurdjumovi</i>	aphid species	6	161.3	110	96.1	<0.00001
<i>varipes</i>	aphid species	6	84.5	116	139.1	<0.00001

Table 4. Analyses of deviance for effect of aphid species on adult emergence rate of parasitoids in the genus *Aphelinus* collected from aphids on wheat, barley, and maize.

parasitoid species	factor	model		residual		<i>P</i>
		df	deviance	df	deviance	
<i>asychis</i>	collection host	1	0.004	114	2.8	0.65
	aphid species	6	0.7	108	2.2	<0.00001
	interaction	6	0.07	102	2.1	0.72
<i>albipodus</i>	aphid species	6	0.5	36	1.3	0.02
<i>atriplicis</i>	collection country	1	0.2	172	7.2	0.02
	aphid species	6	0.7	166	6.6	0.002
	interaction	6	1.4	160	5.1	<0.00001
<i>certus</i>	aphid species	6	0.1	53	1.0	0.56
<i>hordei</i>	aphid species	3	0.8	31	0.7	<0.00001
<i>kurdjumovi</i>	aphid species	5	0.4	49	1.8	0.05
<i>varipes</i>	aphid species	6	1.4	77	4.0	0.0002

Table 5. Analyses of deviance for effect of aphid species on sex ratio of parasitoids in the genus *Aphelinus* collected from aphids on wheat, barley, and maize.

parasitoid species	factor	model		residual		<i>P</i>
		df	deviance	df	deviance	
<i>asychis</i>	collection host	1	0.1	113	9.0	0.32
	aphid species	6	0.2	107	8.8	0.83
	interaction	6	0.6	101	8.2	0.26
<i>albipodus</i>	aphid species	5	0.5	31	2.7	0.36
<i>atriplicis</i>	collection country	1	0.6	159	15.6	0.02
	aphid species	6	0.5	153	15.2	0.60
	interaction	6	0.4	147	14.8	0.74
<i>certus</i>	aphid species	5	0.4	51	2.9	0.18
<i>hordei</i>	aphid species	1	0.1	31	3.3	0.31
<i>kurdjumovi</i>	aphid species	4	1.6	43	3.9	0.001
<i>varipes</i>	aphid species	5	2.1	68	8.0	0.004

Table 6. Analysis of deviance for effect of aphid species on behavior of *Aphelinus hordei* and the aphid species it attacked.

		model		residual		<i>P</i>
		df	deviance	df	deviance	
number of aphids	approached	8	171.2	81	82.0	<0.00001
	stung	8	204.8	81	63.3	<0.00001
	oviposited	8	32.4	15	18.0	0.00008
	host-fed	8	36.0	81	24.8	0.00002
handling time	not host-fed	4	14.5	33	39.4	0.006
	host-fed	2	1.9	9	12.1	0.38
aphid defense		4	10.9	46	57.8	0.03