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Antixenosis, Antibiosis, and Potential Yield Compensatory Response in Barley Cultivars Exposed to Wheat Stem Sawfly (Hymenoptera: Cephidae) Under Field Conditions

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Subject Editor: Stefan Jaronski

Abstract

Wheat stem sawfly, *Cephus cinctus* Norton, is an economically serious pest of cereals grown in North America. Barley cultivars were previously planted as resistant crops in rotations to manage *C. cinctus*, but due to increasing levels of injury to this crop, this is no longer a valid management tactic in Montana. Therefore, we aimed to understand antixenosis (behavioral preference), antibiosis (mortality), and potential yield compensation (increased productivity in response to stem injuries) in barley exposed to *C. cinctus*. We examined these traits in eight barley cultivars. Antixenosis was assessed by counting number of eggs per stem and antibiosis was assessed by counting infested stems, dead larvae, and stems cut by mature larvae. Potential yield compensation was evaluated by comparing grain yield from three categories of stem infestation: 1) uninfested, 2) infested with dead larva, and 3) infested cut by mature larva at crop maturity. We found the greatest number of eggs per infested stem (1.80 ± 0.04), the highest proportion of infested stems (0.63 ± 0.01), and the highest proportion of cut stems (0.33 ± 0.01) in 'Hockett'. Seven out of eight cultivars had greater grain weight for infested stems than for uninfested stems. These cultivars may have compensatory responses to larval feeding injury. Overall, these barley cultivars contain varying levels of antixenosis, antibiosis, and differing levels of yield compensation. Our results provide foundational knowledge on barley traits that will provide a framework to further develop *C. cinctus* resistant or tolerant barley cultivars.

Key words: egg, larva, stem injuries, cut stem, grain weight

Complex and intertwined relationships exist between phytophagous insects and their host plants. Host plant direct and indirect defenses are often induced in response to insect injury (Dicke 2009; Piesik et al. 2011, 2013; Turlings and Erb 2018). Defense responses such as the synthesis of toxic secondary metabolites can negatively impact insect growth and development (Kos et al. 2012), but these processes can also have negative or positive effects on the host plant itself (Agrawal 1998). There is a well-known tradeoff between growth and plant defenses that typically reduce yield (Ballhorn et al. 2014). Feeding insects remove and ingest plant tissues or redirect tissue contents within the plant which usually creates stress responses in plants being fed on (Peterson and Higley 2000); however, sometimes stress outcomes are of little consequence to yield in the presence of insect injury. This type of resistance is called tolerance (Pedigo and Higley 1992, Pedigo 1995, Peterson et al. 2017). Overcompensation occurs when host plant yields can be positively correlated with plant

injury (Pedigo et al. 1986). Understanding the relationships between host plant responses and feeding injury is necessary to develop sustainable management strategies for most phytophagous insects. This study focuses on interactions between barley (*Hordeum vulgare* L.) and the wheat stem sawfly and reports on the relative roles of antixenosis, antibiosis, and possible yield compensation in innate plant defenses to this insect.

Wheat stem sawfly (*Cephus cinctus* Norton) is an economically devastating pest of wheat grown on the northern Great Plains of North America (Beres et al. 2011a). This native species (Lesieur et al. 2016) has a host range that includes both cultivated small grain cereals and native grasses over a large area of United States and Canadian wheat production (Criddle 1923, Cockrell et al. 2017). Female *C. cinctus* begin to deposit eggs in host stems early in the stem elongation process. At hatch, the larva starts to feed on the stem lining near the point of emergence from the egg. The resulting feeding

injuries to this parenchymous tissue and subsequently to vascular structures at the stem nodes may reduce the translocation of water and minerals from roots to shoots (Morrill et al. 1994, Delaney et al. 2010). As a result, the photosynthetic rate can be reduced in wheat (Macedo et al. 2005, 2006, 2007; Delaney et al. 2010).

As the host plant ripens, the mature larva moves to the base of the stem and makes a v-shaped groove around the interior of the stem. It does this by chewing a uniform gouge that encircles the stem interior and leads to lodging of the upper stems (Ainslie 1920, Weiss and Morrill 1992). The interior groove around the stem easily breaks due to wind and gravity. The resulting lodged stems, called cut stems, have lower grain weight at harvest (Holmes and Peterson 1965, Beres et al. 2007). As a result of physiological and physical impacts of herbivory estimated losses can range from approximately $50–80 million (USD) per year in Montana alone (Bekkerman 2014, Fulbright et al. 2017) and overall can cost $350 million per year in wheat (Beres et al. 2011a,b). The relative loss could be higher in barley due to strict grain quality requirements for higher value malt barley.

Grain yield in barley is composed of the number of seed heads per unit area, the number of seeds per head, and weight per seed. Additionally, grain weight is a function of the amount of photosynthetic assimilates translocated to the seeds at grain fill (Gallagher et al. 1976). Importantly, all tillers do not receive equal shares of assimilates especially if resources are limiting. As a result, many spikelets on more distal tillers are aborted and unable to bear grain (Simmons et al. 1982). This is also true in wheat injured by *C. cinctus* when feeding by larger larvae is not curtailed (Buteler et al. 2008, Bekkerman and Weaver 2018). Several studies have shown that *C. cinctus* injuries reduces grain yield from 2 to 30% in wheat heads (Holmes 1977, Morrill et al. 1992, Beres et al. 2007, Delaney et al. 2010). Barley may be able to better compensate for yield losses due to minor injury. Thus, relatively limited plant injuries, like those characteristic of *C. cinctus,* may not reduce grain yield in barley because of its ability to adapt to multiple stresses (Newton et al. 2011). Currently, there are no data reported on potential yield losses due to larval stem-mining injury in barley stems, nor is there any information on potential tolerance or induced yield compensation due to this type of injury.

Several management strategies can be implemented to limit losses due to *C. cinctus* injury but there has been limited success, for instance: insecticide applications (Knodel et al. 2009, Beres et al. 2011a); adjustment of planting dates to escape the peak of the *C. cinctus* oviposition period (Morrill and Kushnak 1999); deep plowing to bury stubble and shallow plowing to expose overwintered larvae to low temperatures and desiccation (Morrill et al. 1993); and temporal and spatial variation in parasitism rates (Morrill et al. 1998; Weaver et al. 2004, 2005). Therefore, host plant resistance is of critical importance. Although host plant resistance that is due to the presence of solid pith in the stem can be relatively successful for reducing stem cutting in wheat (Beres et al. 2013, Wu et al. 2013, Talbert et al. 2014, Carcamo et al. 2016, Varella et al. 2016, Adhikari et al. 2018), solid stems may be compromised by environmental effects on pith expression (Beres et al. 2009, 2017) and relative expression of the trait in different backgrounds (Cook et al. 2019, Varella et al. 2019, Bainsla et al. 2020). In addition to stem solidness, other defense-related metabolites, such as DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), tricetin, and lignin are downregulated in *C. cinctus*-infested wheat stems when compared with uninfested stems (Biyiklioglu et al. 2018). Among these identified compounds, DIMBOA is well investigated as a toxin from cereals for different classes of herbivores, notably aphids and

caterpillars (Martos et al. 1992, Erb et al. 2009, Niemeyer 2009). Tricetin activates lignin biosynthesis in secondary cell wall production (Moheb et al. 2013). However, *C. cinctus* feeding downregulated the synthesis of these metabolites, perhaps supporting the idea that these larvae disrupt signals and limit subsequent synthesis of these metabolites (Biyiklioglu et al. 2018). Cultivated barley has lost the biosynthetic capability to produce DIMBOA (Nomura et al. 2002, Grun et al. 2005), but there are other groups of compounds, such as flavonoids and alkaloids that may be synthesized to ward off insect pests (Balmer et al. 2013). The role of specific secondary metabolites leading to greater larval mortality in barley for *C. cinctus* larvae than in resistant wheat cultivars has yet to be explored (Varella et al. 2018).

While solid stem expression is the most common resistance deployed by plant breeders for wheat, cultivated barley has only hollow stems. However, the proportion of stems cut by *C. cinctus* larvae is lower in barley than in solid-stem wheat cultivars (Beres et al. 2013, Varella et al. 2018), primarily because in barley a greater proportion of larvae die before becoming mature enough to cut stems (Farstad and Platt 1946). Thus, the larval mortality in barley is based on factors other than the stem architecture. Historically, larval mortality rates up to 100% have been recorded in barley (Farstad and Platt 1946). A decade-long survey of barley in Montana reports increasing infestation rates and decreasing larval mortality in *C. cinctus* in Montana (Varella et al. 2018). The conflict between recent findings and early records could be due to diverse, overlapping *C. cinctus* populations (Lesieur et al. 2016) that facilitate better adaptation, first from grass to wheat (Ainslie 1920, Morrill and Kushnak 1996) and now to barley in Montana. Conversely, this change could be due to loss of significant sources of antixenosis and antibiosis in modern barley; like the reduced biosynthesis of benzoxazinoids (Grun et al. 2005). These two factors could also interact to cause the current trends. Increasing *C. cinctus* damage poses a serious threat to Montana barley production, which is third highest in the United States (U.S. Department of Agriculture 2020).

We conducted a field study in Gallatin and Choteau counties of Montana to explore antibiosis, antixenosis, and yield compensation traits in barley cultivars in 2016 and 2017. The specific objectives of the study were 1) to determine the relative attractiveness of barley cultivars to *C. cinctus* females for oviposition (antixenosis); 2) to assess larval death rate in stems (antibiosis); and 3) to compare grain yield among uninfested and different categories of infestation (potential yield compensation).

This study will provide an understanding of these key types of host plant resistance in different barley classes, which should be the foundation of an integrated pest management (IPM) strategy for *C. cinctus* (Pedigo 1995, Beres et al. 2011b, Peterson et al. 2018). Our findings will bolster efforts to incorporate these traits into barley cultivars, which will ultimately lead to a significant reduction in future economic losses incurred due to *C. cinctus*.

Materials and Methods

Research Sites and Experimental Design
We assessed *C. cinctus* infested stems by counting the number of eggs and the proportion of dead larvae in cultivars that were seeded in the spring season at three sites in Montana: near Amsterdam in 2016 (45°45′27.3″ N, 111°24′00.9″ W) and 2017 (45°45′33.2″ N, 111°23′50.0″ W), and also near Big Sandy in 2017 (48°15′42.1″ N, 110°22′19.1″ W) (Fig. 1). Each planting of the experiment was conducted using a randomized complete
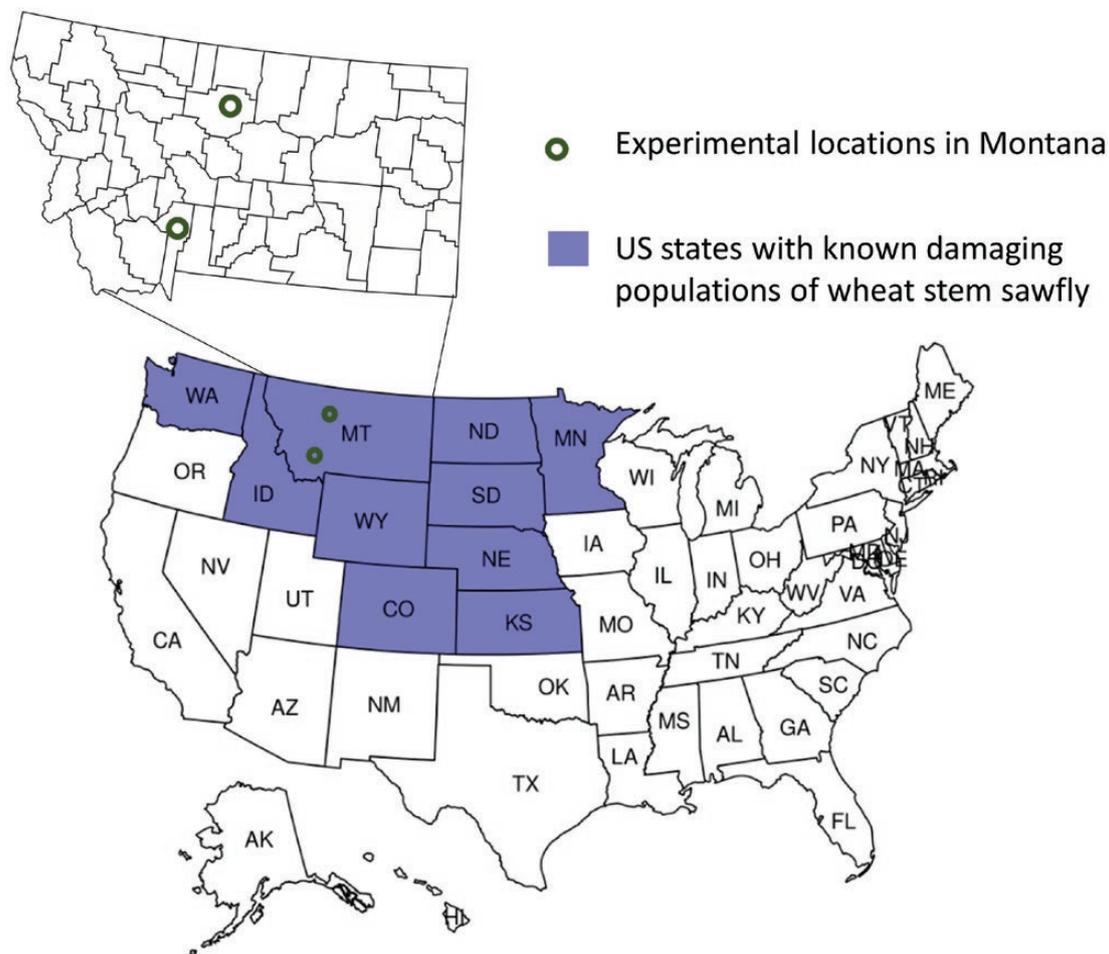
Fig. 1. Experimental locations 2016 and 2017 in Montana and U.S. states with known damaging populations of wheat stem sawfly.

block design. At a site, each block was equally divided into three subblocks based on field variability due to gentle slopes. Each subblock was divided into eight plots with a size of 1.8 × 3.6 m with 0.3-m spacing between each plot and block. The barley cultivars grown at all sites were: 'Celebration' (PI6B01-2218), a six-row malt barley; 'Champion' (YU501-385), a two-row feed barley; 'Craft' (PI646158), a two-row malt barley; 'Haxby' (MT950186), a two-row feed barley; 'Haybet' (CI16569), a two-row forage barley; 'Hockett' (PI657121), a two-row malt barley; 'Lavina' (MT981397), a two-row forage barley; and 'Tradition' (B2482), another six-row malt barley. We seeded each cultivar at a rate of 0.9 g/m² per plot. A self-propelled, seven-row seeder (Fabro Enterprises Limited, Swift Current, SK, Canada) was used for seeding near Amsterdam in 2016 and 2017, while a custom built, six-row self-propelled cone seeder with an Atom Jet paired row opener (BNM Customs, Havre, MT) was used near Big Sandy in 2017. Seeding was completed near Amsterdam in 2016 on April 4, near Amsterdam in 2017 on May 4, and near Big Sandy in 2017 on April 13. Local best management practices of participating wheat and barley growers were used to prepare and fertilize the soil before planting, but several manual weeding operations were subsequently performed to maintain better crop stands at all sites.

Sample Collection and Dissection

We began sample collections when plants were Zadoks stage 32–33 (Zadoks et al. 1974), which was from 43 to 59 d after seeding (DAS; at Big Sandy–43 DAS; at Amsterdam 2016–59 DAS; and 2017–51 DAS) because this is when *C. cinctus* infestation first occurs in elongating stems. All plants within a 0.3-m row length in each plot were uprooted and wrapped in labeled paper bags. This sampling procedure was repeated three times in each plot for each week of sampling. Samples were collected in nine consecutive weeks but were categorized into two groups from each site: green samples (samples from week 1 to week 8) and harvest samples (samples collected at crop maturity in week 9). From the green samples, we selected 35 stems that were suitable for oviposition or had elongated stems from each sample bag (105 stems per plot) and dissected all the selected stems lengthwise from base to head. Each dissected stem was visually examined to count the number of eggs and larvae as well as numbers of live and dead larvae for each life stage of the insect were recorded. It was not possible to dissect all the collected samples on the date of collection, so samples were stored at 4°C for dissection the following day. To prevent inflated counts of dead larvae due to prolonged storage, we selected only 35 primary stems from each sample bag (105 stems per plot). By doing this, we were able to dissect all the stems while they were in good condition (not withered or deteriorated) to get a precise determination of mortality for the *C. cinctus* eggs and larvae at the time of sample collection.

All stems were processed from the harvest samples. From every stem in each harvest sample, we removed the seed head and saved it in a uniquely labeled envelope. From the first internode, stem outside diameter was measured to the nearest 0.01 mm using a micrometer (Mitutoyo Outside Micrometer, Billings, MT) as per Buteler and Weaver (2012). Individual grain numbers from each seed head

were recorded using a grain counter (Masch. Nr. 77314, Pfeuffer GmbH, Kitzingen, Germany) with the following settings: container 2, speed-80, sizes-24, and present-3000. Total grain weight for each head was recorded using a digital balance (model SC 2020, Merck KGaA, Damstadt, Germany). For the variables grain weight, grain number, and stem diameter, the stems were separated into one of three groups based on their infestation categories: 1) infested cut by a mature larva at crop maturity (stem girdled at the base that resulted in a mature larva in a hibernaculum); 2) infested with a dead larva (the stem contained larvae that died before maturity); and 3) uninfested stems (stem with no evidence of a larva or larval feeding). To compare grain weight and grain number per head based on infestation categories, we removed occasional outliers with the smallest (<1 mm) and the largest (>5 mm) stem diameters (Supp Fig. 1 [online only]). Additionally, we excluded stems with less than seven grains per head which indicated a rare loss of grains during handling. Next, we compared grain weight and grain number based on stem infestation categories within a cultivar and used stem diameters as a covariate. We did not compare grain yield among the cultivars, irrespective of *C. cinctus* stem injuries because we included different classes of barley cultivars, such as two-row and six-row malt barley, as well as cultivars from the feed and forage classes. With different end uses intended, these barley classes have different grain yield potential, irrespective of *C. cinctus* stem injury.

We calculated the proportion of infested stems by recording the number of infested and uninfested stems. A stem was categorized as infested if it contained a live or dead egg, larval frass, or either live or dead larvae. Otherwise, the stem was categorized as uninfested. Similarly, the proportion of dead larvae was calculated by first assessing all larvae in each cultivar. Thereafter, we categorized the dead larvae into three categories: 1) dead due to plant factors if the larva died within one internode early in development; 2) dead due to parasitism, if a stem contained parasitoid larvae or cocoons or a parasitoid emergence hole; and 3) dead due to other factors, a later instar larva died from other than the above mentioned reasons.

Statistical Analyses

We used a generalized linear model (GLM; Marschner 2011) with a quasi-Poisson distribution for number of eggs per stem, number of eggs per infested stem (after correcting for stems without eggs), and GLM with a binomial distribution for proportion of infested stems, proportion of dead larvae, and proportion of cut stems. Additionally, a linear regression model was used to compare grain weight within a cultivar among the stem infestation categories. Regression coefficients of grain weight and stem diameter with different infestation categories were compared using the function lstrends in lsmeans package (Lenth 2016). Post-hoc tests were conducted by using Tukey's HSD test in multcomp (Hothorn et al. 2008). Tukey's HSD was used to reduce error due to unequal sample sizes across cultivars. We prepared maps for experimental locations and U.S. states with known damaging populations of wheat stem sawfly using the package usmap (Di Lorenzo 2019). All the statistical analysis and data visualizations were performed using ggpubr (Kassambara 2018) and ggplot2 (Wickham 2016) in R version 3.6.2 (2019).

Results

Mean Number of Eggs

In total, 26,632 stems were split to assess the number of eggs per stem among eight cultivars over three sites × years. Most of the cultivars had eggs during the first four weeks of sampling (from first to fourth week), except at Amsterdam 2017 where eggs were lacking in the later weeks (Fig. 2). The mean number of eggs per sampled stem differed by cultivars and by sites (GLM; $F = 3.52$; df = 14, 164; $P < 0.001$). A similar pattern was found for the mean number of eggs per infested stems, where there was an interaction between cultivars and sites (GLM; $F = 3.65$; df = 14, 164; $P < 0.001$). Overall, 'Hockett' had the greatest number of eggs per stem and per infested stem, relative to the other cultivars (Table 1 and 2).

Proportion of Dead Larvae per Stem

We observed the first dead larvae in all cultivars and sites during the second sampling week, except for in 'Celebration' and 'Champion' in Amsterdam 2016 (data not shown). We found an interaction between cultivar and site for proportion of dead larvae (GLM; $F = 3.02$; df = 14, 164; $P < 0.001$). The proportion of dead larvae was greatest in 'Craft' and least in 'Hockett' at all sites; while the rest of the cultivars were intermediate (Fig. 3).

Categories of Larval Mortality

There was no interaction between cultivar and site for percentage of larvae dying within a single internode (GLM; $F = 1.67$; df = 14, 102; $P = 0.07$); similarly, we did not find an interaction between cultivar and site for percentage mortality due to parasitism (GLM; $F = 0.74$; df = 14, 102; $P = 0.73$) or percentage mortality due to other factors (GLM; $F = 0.83$; df = 14, 102; $P = 0.64$). However, the greatest percentage of larvae dying within a single internode occurred in 'Craft' stems (79.4 ± 2.4) and the lowest percentage in 'Hockett' stems (37.2 ± 4.4) (Table 3). There was no difference among cultivars for percentage mortality due to parasitism. However, 'Hockett' had the highest percentage of larval mortality due to other factors (58.1 ± 3.8), whereas the lowest was in 'Craft' (18.8 ± 3.1) (Table 3).

Proportion of Infested Stems

To assess the proportion of infested stems, a total of 25,108 and 15,843 stems were dissected for Amsterdam 2016 and 2017, respectively, whereas 23,125 stems were dissected from Big Sandy in 2017. The proportion of infested stems differed by cultivars and by sites (GLM; $F = 6.17$; df = 14, 164; $P < 0.001$ (Fig. 4). Overall, a lower proportion of infested stems (< 50% except for Haxby and Hockett) was found near Big Sandy in 2017.

Proportion of Cut Stems

To assess the proportion of stems cut by mature larvae, a total of 3,960 and 2,514 stems were collected at harvest for subsequent dissection from near Amsterdam in 2016 and 2017, respectively, whereas 4,255 stems from near Big Sandy were similarly collected and dissected in 2017 (Table 4). There was an interaction between cultivar and site for the proportion of cut stems (GLM; $F = 2.96$; df = 14, 164; $P < 0.001$). The proportion of cut stems was lower in all cultivars near Big Sandy in 2017 than it was near Amsterdam in 2016 and 2017. 'Hockett' had the greatest proportion of cut stems compared with other cultivars at all site × years (Fig. 5).

Grain Yield per Stem

Across cultivars, there was a mixed trend for grain weight per head in stems based on infestation categories. However, whenever there was a difference in grain yield per stem by stem infestation categories, a greater grain weight per head occurred in stems containing dead larvae and also in stems cut by mature larvae than for uninfested stems across site × years except in Lavina (Fig. 6). The six-row malt
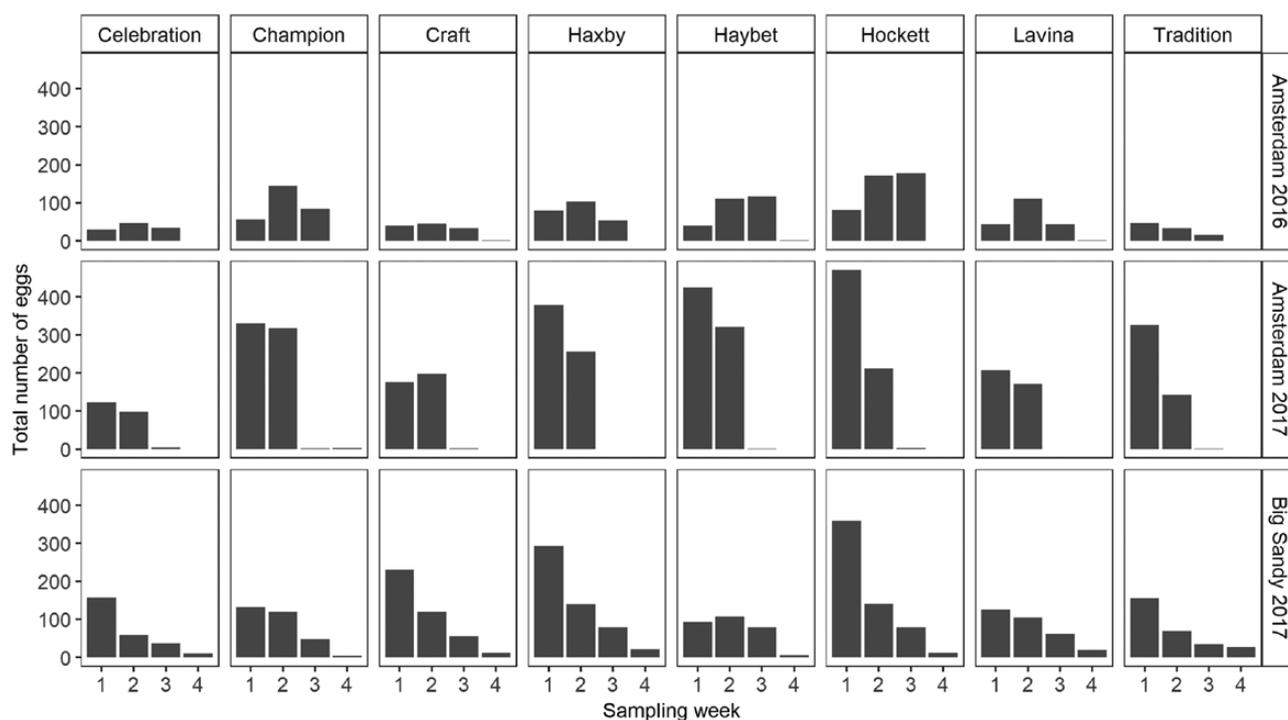
Fig. 2. Number of eggs by cultivar and site across sampling week over three sites × years. Sampling week equivalents for Amsterdam 2016 [1 = 59 d after seeding (DAS), 2 = 67 DAS, 3 = 73 DAS, 4 = 81DAS], Amsterdam 2017 [1 = 51 DAS, 2 = 58 DAS, 3 = 65 DAS, and 4 = 72 DAS], and Big Sandy 2017 [1 = 43 DAS, 2 = 50 DAS, 3 = 57 DAS, and 4 = 64 DAS].

Table 1. Mean (±SE) number of eggs per sampled stem by cultivar over three sites × years in Montana

Cultivar	Amsterdam 2016	Amsterdam 2017	Big Sandy 2017	Mean
Celebration	0.01 ± 0.012^a	0.30 ± 0.025^a	0.25 ± 0.022^a	0.2 ± 0.011
Champion	0.21 ± 0.015^{bc}	0.78 ± 0.042^{ac}	0.27 ± 0.022^{ab}	0.38 ± 0.015
Craft	0.09 ± 0.008^a	0.46 ± 0.032^{ab}	0.38 ± 0.026^{ac}	0.28 ± 0.013
Haxby	0.17 ± 0.012^{ac}	0.76 ± 0.043^{bc}	0.47 ± 0.029^{bc}	0.42 ± 0.016
Haybet	0.17 ± 0.012^{ac}	0.90 ± 0.052^c	0.24 ± 0.017^a	0.36 ± 0.015
Hockett	0.30 ± 0.017^c	0.82 ± 0.048^c	0.50 ± 0.031^c	0.49 ± 0.018
Lavina	0.13 ± 0.010^{ab}	0.45 ± 0.031^{ab}	0.25 ± 0.016^a	0.25 ± 0.01
Tradition	0.08 ± 0.008^a	0.58 ± 0.040^{bc}	0.28 ± 0.024^{ac}	0.27 ± 0.014
F-value	6.76	8.66	5.35	
df	7, 60	7, 37	7, 60	
P-value	<0.001	<0.001	<0.001	

Mean values with different letters are different ($P < 0.05$) within a site according to Tukey's HSD.

barley 'Celebration' (lm; $F = 1.31$; df = 4, 788; $P = 0.44$) and two-row forage barley 'Haybet' (lm; $F = 1.42$; df = 4, 1101; $P = 0.22$) had a similar grain weight per head across infestation categories and site × year. The remaining cultivars displayed interactions between infestation categories and site (Champion: lm; $F = 13.41$; df = 4, 1213; $P < 0.001$. Craft: lm; $F = 5.10$; df = 4, 1270; $P = 0.01$. Haxby: lm; $F = 2.73$; df = 4, 1433; $P < 0.002$. Hockett: lm; $F = 2.28$; df = 4, 12197; $P = 0.05$. Lavina: lm; $F = 12.01$; df = 4, 1519; $P < 0.001$. Tradition: lm; $F = 7.24$; df = 4, 802; $P < 0.001$; Fig. 6).

Correlation Between Grain Weight and Stem Diameter

All cultivars displayed a strong positive correlation [correlation coefficient (R) from 0.59 to 0.80] between per stem diameter and per stem grain weight that was independent of stem infestation

categories and cultivar (Fig. 7). Similarly, a strong correlation (correlation coefficient from 0.73 to 0.97) (Supp Fig. 2 [online only]) between grain weight and grain number was also found irrespective of stem infestation categories. The pairwise comparisons of regression coefficients for stem diameter that were made for uninfested stems versus infested stems with dead larvae; uninfested stems versus infested stems cut by mature larvae; and infested stems with dead larvae versus infested stems cut by mature larvae, were similar in most of the cultivars(Supp Table 2 [online only]). But the regression coefficients for 'Celebration' (near Amsterdam 2016 and 2017), 'Hockett' and 'Tradition' (near Amsterdam 2017), and 'Champion', 'Haxby', and 'Hockett' (near Big Sandy 2017) were not similar within the cultivar (Supp Table 2 [online only]). Notably 'Hockett' and 'Champion' grain weight was somewhat reduced in infested stems cut by mature larvae.

Table 2. Mean (±SE) number of eggs per infested stem by cultivar over three sites × years in Montana

Cultivar	Amsterdam 2016	Amsterdam 2017	Big Sandy 2017	Mean
Celebration	1.202 ± 0.085[ab]	1.523 ± 0.064[a]	1.469 ± 0.075[ab]	1.429 ± 0.043
Champion	1.274 ± 0.038[ab]	2.038 ± 0.064[bc]	1.520 ± 0.070[ac]	1.669 ± 0.037
Craft	1.026 ± 0.015[a]	1.728 ± 0.065[ab]	1.672 ± 0.068[bc]	1.564 ± 0.039
Haxby	1.150 ± 0.028[ab]	2.089 ± 0.070[bc]	1.636 ± 0.064[bc]	1.681 ± 0.038
Haybet	1.201 ± 0.035[ab]	2.349 ± 0.088[c]	1.335 ± 0.048[ab]	1.721 ± 0.045
Hockett	1.345 ± 0.038[b]	2.261 ± 0.081[bc]	1.810 ± 0.074[c]	1.798 ± 0.040
Lavina	1.157 ± 0.031[ab]	1.712 ± 0.066[ab]	1.230 ± 0.037[a]	1.376 ± 0.029
Tradition	1.010 ± 0.010[a]	2.048 ± 0.084[ac]	1.487 ± 0.083[ab]	1.647 ± 0.051
F-value	2.94	4.69	5.63	
df	7, 60	7, 37	7, 60	
P-value	0.01	<0.001	<0.001	

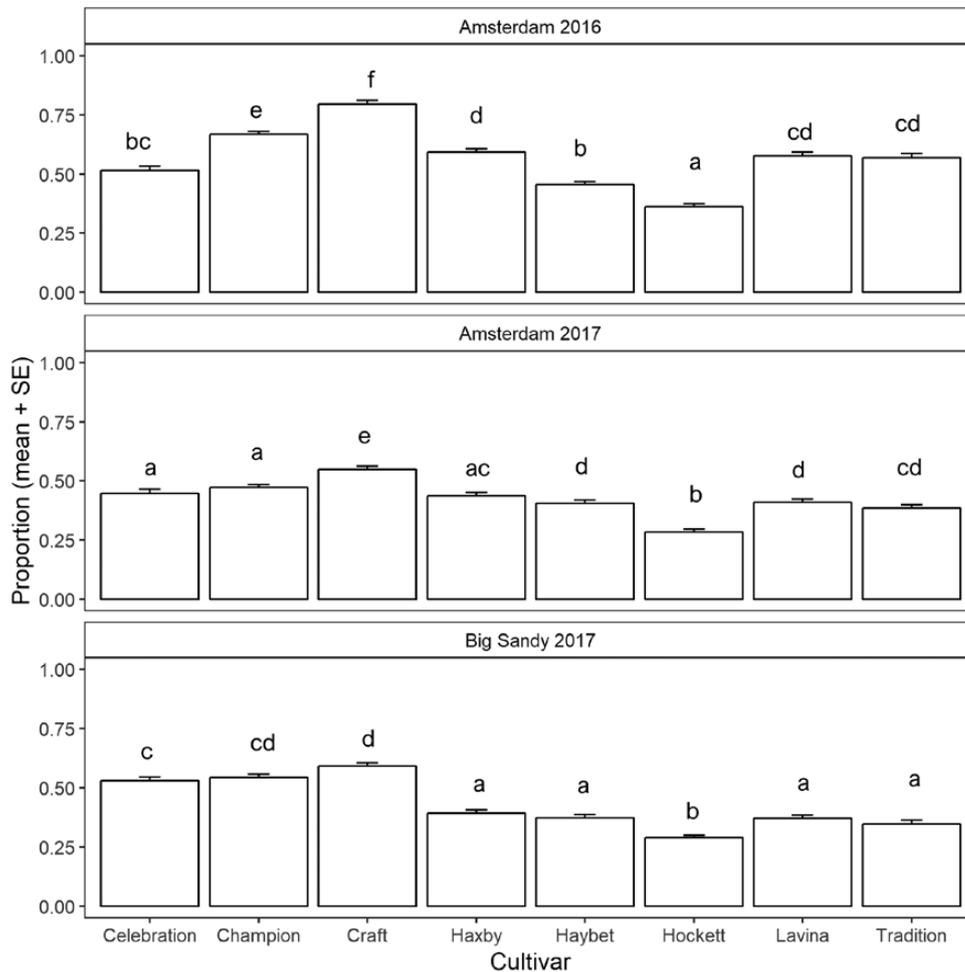Mean values with different letters are different ($P < 0.05$) within a site according to Tukey's HSD.

Fig. 3. Proportion (mean ± SE) of dead larva by cultivar and site for three site × years. Amsterdam 2016 ($F = 19.44$; df = 7, 60; $P < 0.001$), Amsterdam 2017 ($F = 16.35$; df = 7, 37; $P < 0.001$), and Big Sandy 2017 ($F = 20.94$; df = 7, 60; $P < 0.001$). Bars with different letters are different ($P < 0.05$) within a site × year using a Tukey HSD.

Discussion

We aimed to explore antixenosis, which determines *C. cinctus* host selection behavior and oviposition, as well as antibiosis, which reflects larval mortality and impacts the number of nodes injured by feeding. We also measured potential yield compensation that could indicate a variation in responses to larval feeding injuries. We provide a foundational characterization of traits to target for development of resistant barley cultivars to reduce economic losses incurred by *C. cinctus* in barley.

Barley Antixenosis

Number of eggs per stem varied depending on cultivar, indicating oviposition preference for certain cultivars. We found that 'Hockett' had the greatest number of eggs per stem and the greatest number

of eggs per infested stem relative to all other cultivars across site × years (Table 2). This indicates that 'Hockett' barley has traits, similar to those reported for wheat, that are more attractive to

Table 3. Mean (±SE) percentage larval mortality by types of mortality by cultivar over three sites × years in Montana

Cultivar	Type of mortality		
	Dead in one internode	Parasitism	Other factors
Celebration	68.3 ± 3.6[b]	3.3 ± 1.0[a]	28.4 ± 3.1[b]
Champion	69.3 ± 3.8[a]	3.0 ± 1.4[a]	27.8 ± 3.4[c]
Craft	79.4 ± 2.4[d]	1.9 ± 1.3[a]	18.8 ± 3.1[a]
Haxby	60.4 ± 4.2[a]	3.0 ± 1.1[a]	36.5 ± 4.2[c]
Haybet	46.3 ± 5.5[b]	4.7 ± 2.3[a]	49 ± 4.8[b]
Hockett	37.2 ± 4.4[c]	4.7 ± 2.3[a]	58.1 ± 3.8[d]
Lavina	54.9 ± 4.7[b]	5.6 ± 2.6[a]	39.5 ± 4.3[b]
Tradition	56.3 ± 4.8[ab]	3.7 ± 1.5[a]	40 ± 4.5[bc]
F-value	20.06	2.0	16.87
df	7, 102	7, 102	7, 102
P-value	<0.001	0.06	<0.001

Column with different letters are different ($P < 0.05$) within type of mortality, using a Tukey's HSD.

foraging *C. cinctus* females (Weaver et al. 2009, Buteler et al. 2010, Buteler and Weaver 2012) and lead to oviposition in the stem lumen after exploration (Buteler et al. 2009, Varella et al. 2017). Although the cause of preference in barley is unknown, previous studies in wheat have shown that gravid females select stems using specific cues. For instance, females are attracted to wheat plants that release large amounts of several compounds, including (*Z*)-3-hexenyl acetate (Piesik et al. 2008, Weaver et al. 2009, Buteler et al. 2010) and (*E*)- and (*Z*)-β-ocimene (Buteler and Weaver 2012), and also prefer hollow stems that are taller (Buteler et al. 2009) and thicker (Morrill et al. 2000, Carcamo et al. 2005) as well as those with less stem solidness (Varella et al. 2017, 2018).

Overall, we found a higher proportion of infested stems and more eggs per stem near Amsterdam in 2017 compared with near Amsterdam in 2016 and near Big Sandy in 2017 (Table 1 and 2 and Fig. 4). This could be due to a shorter crop duration (110 d in 2017 vs 140 d in 2016), because re-seeding was necessary near Amsterdam in 2017. A potential reason could be that the late planting provided an ample number of succulent, newly elongating stems that are preferred for oviposition (Seamans 1928, Holmes and Peterson 1960, Weaver et al. 2009, Buteler et al. 2010). This is because plants experienced warmer temperatures and required amounts of rainfall that accelerated growth and development in the later 2017 planting but resulted in oviposition occurring in only two sampled weeks
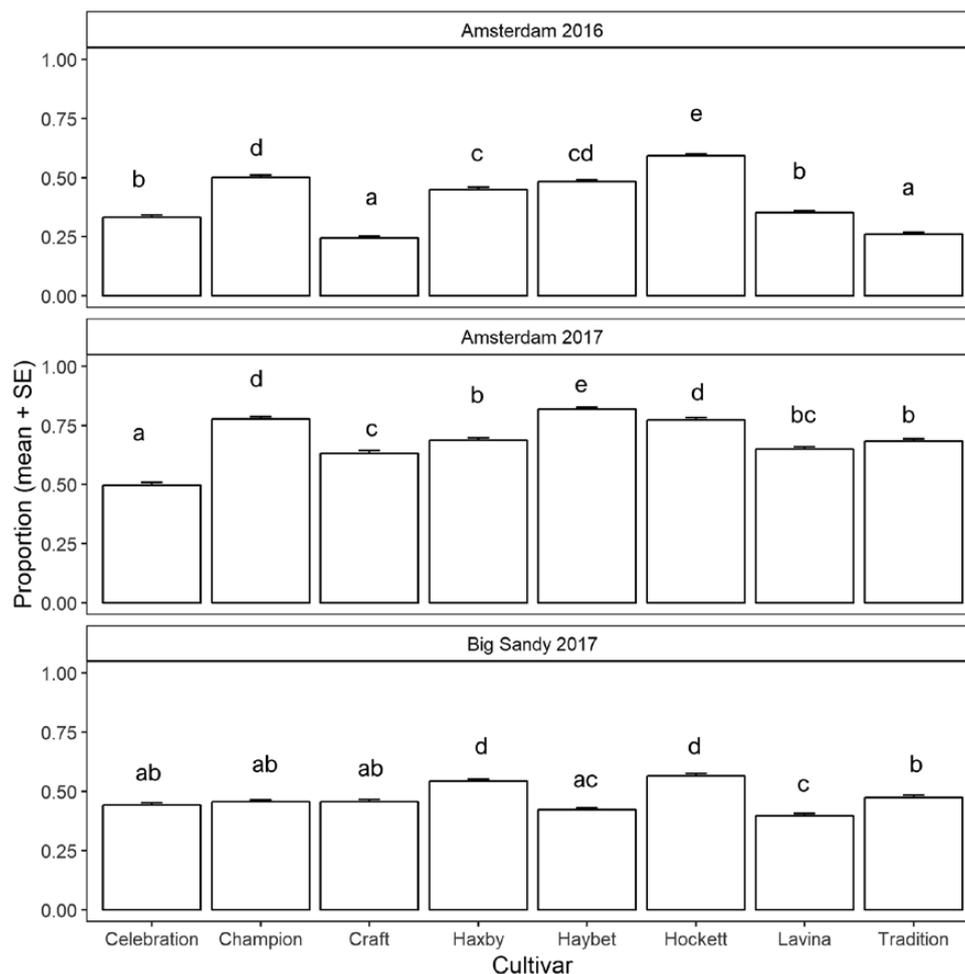

Fig. 4. Proportion of infested stems (mean ± SE) by cultivar and site across three sites × years in Montana. Amsterdam 2016 (*F* = 16.40; df = 7, 60; *P* < 0.001), Amsterdam 2017 (*F* = 8.24; df = 7, 37; *P* < 0.001), and Big Sandy 2017 (*F* = 8.81; df = 7, 60; *P* < 0.001). Bars with different letters are different (*P* < 0.05) within a site × year, according to Tukey's HSD.

Table 4. Number of stems cut by mature larvae and the number of stems collected at harvest by cultivar over three sites × years

Cultivar	Amsterdam 2016	Big Sandy 2017	Amsterdam 2017	Total sampled stems
Celebration	83 (380)[1]	15 (286)	19 (188)	854
Champion	96 (490)	69 (583)	51 (264)	1337
Craft	40 (533)	32 (479)	52 (356)	1368
Haxby	142 (616)	72 (631)	71 (354)	1601
Haybet	194 (400)	72 (643)	98 (342)	1385
Hockett	272 (542)	98 (561)	137 (423)	1526
Lavina	144 (625)	82 (741)	70 (389)	1755
Tradition	26 (374)	39 (331)	65 (198)	903
Total stems	3960	4255	2514	

[1]Number in parentheses is the sum of uninfested stems and infested stems with dead larvae.
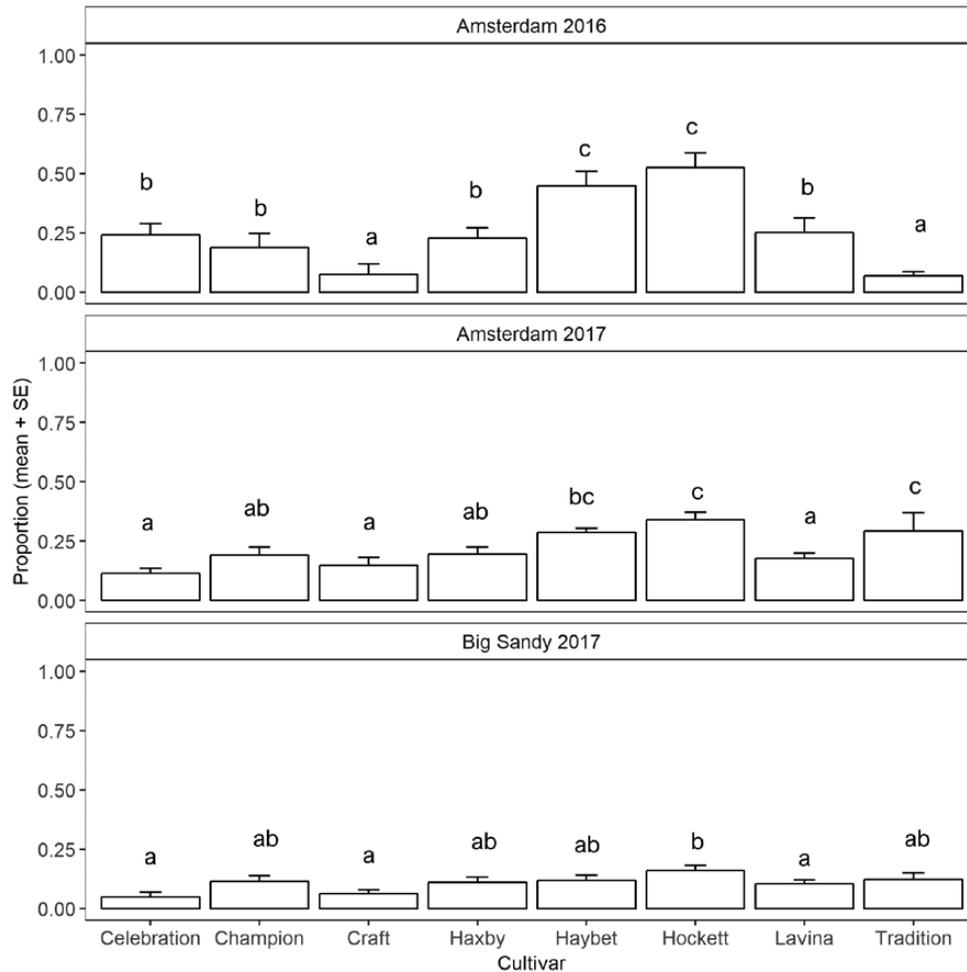

Fig. 5. Proportion of cut stems (mean ± SE) by cultivar and site across three sites × years in Montana. Amsterdam 2016 ($F = 8.88$; df = 7, 60; $P < 0.001$), Amsterdam 2017 ($F = 4.61$; df = 7, 37; $P < 0.001$), and Big Sandy 2017 ($F = 2.69$; df = 7, 60; $P = 0.01$). Bars with different letters are different ($P < 0.05$) within a site × year, according to Tukey's HSD.

rather than in all four (Fig. 2). Additionally, abundance of elongated stems, preferable oviposition sites, was synchronized with the peak flight period of WSS. In Montana, WSS flight period starts in mid- to late-May and ends early in July (Morrill and Kushnak 1999, Nansen et al. 2005). In contrast, the 2016 plantings yielded earlier plants that were exposed to less optimal temperatures from evening to early morning in the early stages of development (this is normal

for spring planted barley). As a result, those plants produced an adequate, but lower number of tillers that were suitable for oviposition. Further study is needed to assess the production of volatile attractants in field grown barley, which will complement results from laboratory efforts (Piesik et al. 2011, 2013). Similarly, detailed characterizations of stem exploration leading to oviposition in barley by gravid *C. cinctus* are warranted.
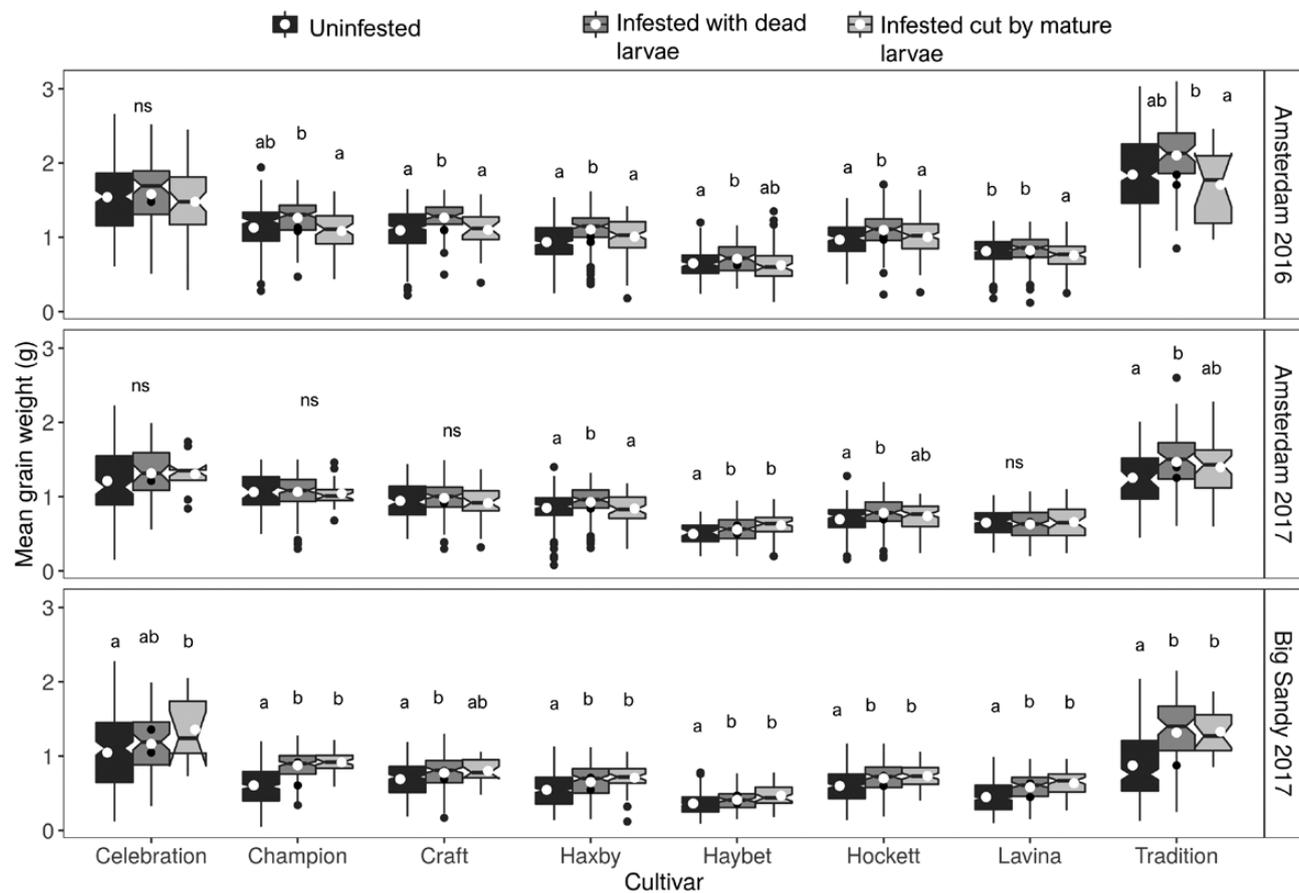
Fig. 6: Box plots illustrating the distribution of grain weight per head in gram by stem infestation categories (uninfested, infested with dead larvae, and infested cut by mature larvae) by cultivar and site across three sites × years in Montana. The median is marked with a black line; mean is marked by white circle; the first and third quartiles are represented as the lower and upper edges of the box, respectively. Amsterdam 2016 (Celebration: lm, $F = 0.80$, df = 2, 345, $P = 0.44$; Champion: lm, $F = 21.50$, df = 2, 437, $P < 0.001$; Craft: lm, $F = 20.24$, df = 2, 497, $P < 0.001$; Haxby: lm, $F = 39.79$, df = 2, 569, $P < 0.001$; Haybet: lm, $F = 5.72$, df = 2, 279, $P = 0.006$; Hockett: lm, $F = 12.17$, df = 2, 387, $P < 0.001$; Lavina: lm, $F = 3.14$, df = 2, 548, $P = 0.04$; Tradition: lm, $F = 13.12$, df = 2, 353, $P < 0.001$), Amsterdam 2017 (Celebration: lm, $F = 1.13$, df = 1, 171, $P = 0.27$; Champion: lm, $F = 0.06$, df = 2, 230, $P = 0.99$; Craft: lm, $F = 1.98$, df = 2, 324, $P = 0.13$; Haxby: lm, $F = 4.84$, df = 2, 315, $P = 0.008$; Haybet: lm, $F = 7.17$, df = 2, 290, $P < 0.001$; Hockett: lm, $F = 6.94$, df = 2, 363, $P = 0.001$; Lavina: lm, $F = 1.5$, df = 2, 341, $P = 0.22$; Tradition: lm, $F = 6.49$, df = 2, 155, $P = 0.001$), Big Sandy 2017 (Celebration: lm, $F = 3.6$, df = 2, 267, $P = 0.02$; Champion: lm, $F = 121.7$, df = 2, 541, $P < 0.001$; Craft: lm, $F = 13.10$, df = 2, 444, $P < 0.001$; Haxby: lm, $F = 24.48$, df = 2, 544, $P < 0.001$; Haybet: lm, $F = 12.92$, df = 2, 527, $P < 0.001$; Hockett: lm, $F = 19.23$, df = 2, 464, $P < 0.001$; Lavina: lm, $F = 56.97$, df = 2, 635, $P < 0.001$; Tradition: lm, $F = 33.45$, df = 2, 289, $P < 0.001$). Bars with different letters are different ($P < 0.05$) within cultivar and within a site × year, according to Tukey's HSD.

Barley Antibiosis

The primary source of nutrition for developing larvae includes parenchymous tissue throughout stem internode and vascular bundles at the stem node (Macedo et al. 2005, 2007). The greatest proportion of larval mortality occurred within a single internode near where the neonate emerges from the egg, and relatively soon after larval feeding begins (Table 4). If the larva survives initial feeding, it eventually proceeds into obligate diapause after a mature larva makes a girdling cut around the interior base of the stem. The lowest proportion of cut stems, 0.06 ± 0.02 (mean ± SE), occurred in 'Craft' near Big Sandy in 2017, and the highest proportion of cut stems, 0.50 ± 0.02 occurred in 'Hockett' near Amsterdam 2016 (Fig. 5). Additionally, the proportion of infested stems was 0.25 ± 0.01 in 'Craft' near Amsterdam 2016 and was 0.76 ± 0.02 in 'Hockett' near Amsterdam in 2017 (Fig. 4). We can see a clear difference between the patterns for the proportion of infested stems and the proportion of cut stems in these two cultivars at corresponding sites, indicating that more larvae died before they were fully matured in 'Craft' compared with 'Hockett'. Similar results were recorded in a field survey of barley in Montana, with larval mortality ranging from 51 to 100% (Varella et al. 2018). One major factor causing larval mortality is host plant antibiosis in barley and certain wheat cultivars (Wallace and McNeal 1966, Buteler et al. 2015, Varella et al. 2018). Some defense related secondary metabolites, such as DIMBOA (2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one), tricetin, and lignin are downregulated in *C. cinctus*-infested wheat stems (Biyiklioglu et al. 2018), indicating that these compounds are synthesized in response to and have a negative effect on *C. cinctus*. Although cultivated barley has lost the ability to biosynthesize DIMBOA (Nomura et al. 2002, Grun et al. 2005), other groups of defensive compounds, such as benzoxazinoids, flavonoids, and alkaloids can be synthesized to ward off insect pests (Balmer et al. 2013). For instance, two alkaloids: hordenine (N, N-dimethyltriamine) and gramine (N, N-dimethylindolemethyl-amine) are feeding deterrents to grasshoppers (Bernays and Chapman 1977, Westcott et al. 1992), *Heliothis* (Bernays et al. 2000), and aphids (Zuniga et al. 1988). We did not analyze the amounts of these secondary metabolite(s) that were potentially associated with larval mortality; however, our results showed compelling evidence that the antibiotic trait plays a key role in overall larval mortality in barley.
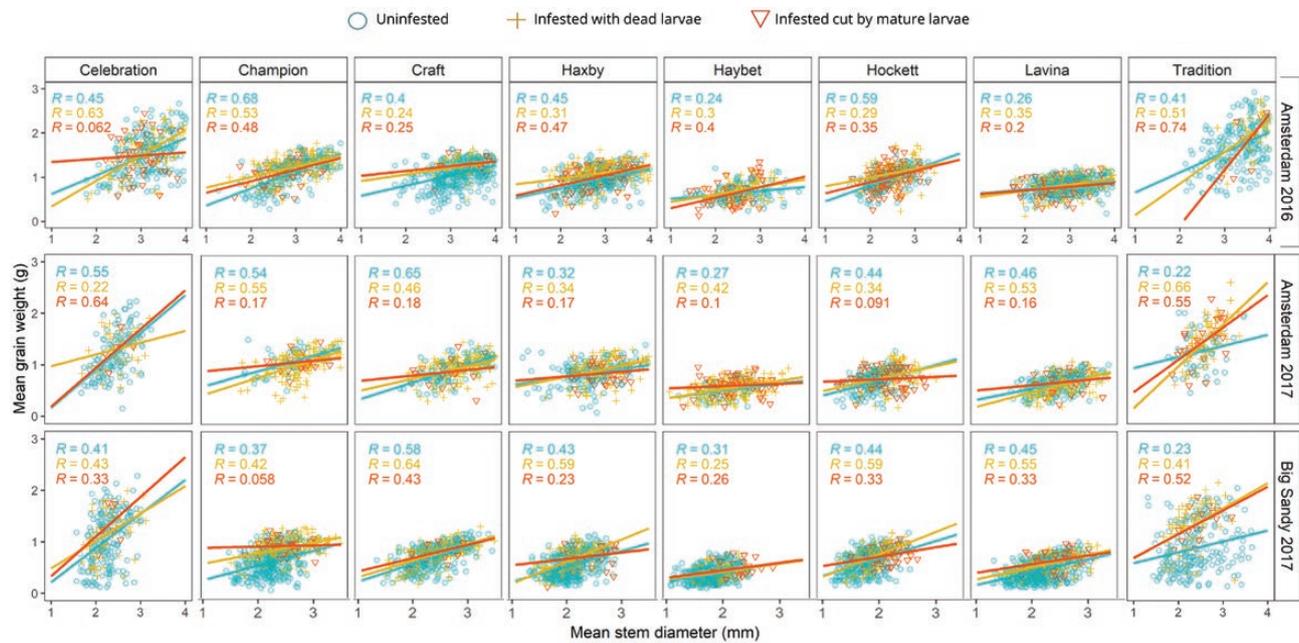
Fig. 7. Correlation of stem diameter and grain yield by stem infestation categories (uninfested, infested with dead larvae, and infested cut by mature larvae) by cultivar and site across three sites × years in Montana. *R* values indicate the correlation coefficient in respective cultivars and are arranged by from top to bottom; top—uninfested stems, middle—infested stems with dead larvae, and bottom—infested stems cut by mature larvae.

Although the cause of early death is unknown, greater proportions of dead larvae within one internode could be due to traits that limit further development of neonates via toxins or a critical limit of essential nutrients (Holmes 1982, Perez-Mendoza et al. 2006). For instance, a wounded plant produces protease inhibitors (PIs) that limit the protein digestion in herbivorous arthropods and subsequently reduces the protein uptake from the plant diets (Green and Ryan 1972). Barley plants synthesize PIs in the form of the trypsin inhibitor CMe which reduces the survival rate of *Sitophilus oryzae* L. (Alfonso-Rubi et al. 2003) in seeds, but impact of these PIs to *C. cinctus* larval survival in stems is yet to be studied.

Potential Yield Compensation

The trends in grain weight per stem were dissimilar across cultivars when considering the stem infestation categories (Figs. 6 and 7). When there was a difference due to stem infestation categories within a cultivar, we found a greater grain weight per stem for both infested stems containing dead larvae and infested stems cut by mature larvae, relative to uninfested stems (Fig. 6). The observation that despite larval feeding injuries in the stem interior, infested stems attained a greater grain weight than uninfested stems can be explained in two ways. First, *C. cinctus* females are strongly biased to select the best and most productive main stems for oviposition (Buteler et al. 2009, 2010), which could result in greater weight per head. Second, larval feeding injuries in vascular tissues might stimulate compensatory growth via the infested stems and attain greater yield, because more stem reserves are built and mobilized during grain filling (Schnyder 1993).

First, our results show a strong positive correlation between grain weight and stem diameter (Fig. 7) as well as grain weight and grain number (Supp Fig. 2 [online only]) in all the cultivars, as expected. In barley, the primary spike is the main factor in increasing grain yield (Alqudah and Schnurbusch 2014), while the other tillers are only supportive components in gaining greater overall yield (Sakamoto and Matsuoka 2004, Sreenivasulu and Schnurbusch 2012). Further,

not all tillers are able to produce grains because the more distal florets of the tillers are not directly connected to the primary phloem (Hanif and Langer 1972). Subsequently, carbohydrate partitioning during grain filling is greater to the main stem than to tillers (Hay 1995, Peltonen-Sainio et al. 2008). As a result, the tertiary tillers are aborted due to an insufficient amount of carbohydrates at anthesis (Sakamoto and Matsuoka 2004, Gonzalez et al. 2011, Mäkelä and Muurinen 2011), which leads to reduced grain yield in tillers, but could contribute to increased weight in the primary stem.

Second, overcompensating and reallocating resources in response to *C. cinctus* larval injuries could play a role in an increased grain yield in the infested stems compared with the uninfested stems. Despite larval injury in the vascular tissues, the infested plants might accumulate excess amounts of photosynthetic assimilates during the vegetative stage to recover the loss due to injured areas within the stem. Later, the stem remobilizes assimilates, a common trait in cereals (Rae et al. 2005, Saint Pierre et al. 2010, Bainsla et al. 2020). The stored assimilates contribute heavily to grain fill, which improves yield stability in these crops (Slewinski 2012). A supporting study showed that the barley plants had higher photosynthetic assimilation in plants with partial spikelet removal than in plants with intact spikelets (Serrago et al. 2013). Additionally, yellow stem borer, *Scirpophaga incertulas* Walker infested rice, *Oryza sativa* L., plants compensate the potential reduced grain yield by producing a greater number of tillers than uninfested rice (Rubia et al. 1996). Thus, in our results, some of the cultivars may have the capacity to deliver larger amounts of photosynthetic assimilates in response to larval stem injuries, which could lead to greater grain yield in these cultivars.

From our results, it is unclear if the increase in grain weight we observed in association with infestation and cutting is due to female bias for robust stems or due to yield compensation or both. Most cultivars with a significant difference in head weight between infested and uninfested had a weaker correlation between stem diameter and grain weight in uninfested stems than infested, for

example: 'Tradition' (2017 Amsterdam and Big Sandy), 'Lavina' (all three sites × years), 'Champion' (Big Sandy 2017), 'Craft' (Big Sandy 2017), 'Haybet' (Amsterdam 2016 and 2017), 'Hockett' (Big Sandy 2017), and 'Haxby' (Amsterdam 2016 and 2017) (Fig. 7). In addition, we found a greater number of seeds per stems in the infested stems than in the uninfested stems for most of the cultivars (Supp Fig. 3 [online only]). The results indicate that most of the robust primary stems were oviposited by females, whereas only a few primary stems and many tertiary stems remain uninfested. However, in some cultivars during certain location years, infested stems in both categories have a weaker correlation between stem diameter and grain weight, indicating a lack of female bias contributing to greater head weight in 'Craft' (Amsterdam 2016), 'Haxby' (Amsterdam 2016), and 'Hockett' (Amsterdam 2016 and 2017) (Fig. 7). Thereby, the greater head weight could be due to a compensatory mechanism. Even more surprising is in some cases infested cut stems had significantly higher grain weight than uninfested stem (Fig. 6). This is surprising because larvae with the opportunity to cut have a more prolonged feeding interval to injure stems. We found that in stems cut by mature larvae, stem mining injured 3.3 ± 0.04 (mean ± SE) nodes per stem; while the number of injured nodes in infested stems with dead larvae was 2.5 ± 0.03 (mean ± SE) (Supp Table 1 [online only]). In the case of 'Tradition', greater weight per head for cut stems is likely due to female bias for larger stems since the correlation between grain weight and stem diameter is higher in cut stems versus uninfested stems. However, in several cases the correlation between weight per head and stem diameter is lower in cut versus uninfested stems, for instance, 'Champion', 'Haxby', and 'Hockett' (Big Sandy 2017) (Fig. 7), indicating potential yield compensation.

We found higher proportion of infested stems in fields near Amsterdam compared to the field near Big Sandy. It could be because the *C. cinctus* population in the Amsterdam area has been exposed continuously to barley for more than two decades (personal communication—M. Flikkema), whereas *C. cinctus* population exposure to the barely crop near the Big Sandy area is relatively naïve, with no barley grown in nearly four decades (personal communication—L. Edwards). Overall, the levels of *C. cinctus* injury were lower across cultivars near Big Sandy. Yet, even for pest populations that had never historically encountered this host, 'Hockett' experienced the greatest injury. This pattern remains true at sites near Amsterdam with continuous exposure of the pest to barley, but the magnitude of injury is greater. Since the increase in damaging populations is relatively recent (Varella et al. 2018), the greater vulnerability of 'Hockett' is worrisome given the popularity of this cultivar with barley growers. By area of production, 'Hockett' was the third most commonly grown malt barley cultivar in Montana in 2019 (AMBA 2020).

Although there were some discrepancies within cultivars against *C. cinctus*, we can conclude that 'Hockett' had the most eggs per infested stem compared to all other cultivars across sites (Table 2). Further study may provide insights into which cultivar traits—visual, chemical, and tactile cues or weaker antixenosis traits relative to other cultivars—attract a higher number of gravid females compared with other cultivars. Additionally, the greatest proportion of dead larvae (Fig. 3) and highest percentage of dead larvae occurring within a single internode was in 'Craft' relative to all other cultivars across site × years (Table 3). This could be a result of more antibiotic compounds or a greater deficiency of essential nutrients, which are explicit mechanisms of antibiosis. Heritability of antibiosis and antixenosis in the biotic interaction between barley and *C. cinctus* is a promising area for future study. In terms of grain yield, understanding underlying compensatory mechanisms in response to larval feeding injury could provide opportunities to develop *C. cinctus* tolerant barley cultivars in the future. Further studies exploring remobilization of stored photosynthetic assimilates to grain fill in response to stem injury in barley cultivars would also be an asset towards developing more stable barley production.

Finally, these findings in barley cultivars show varying levels of antixenosis, antibiosis, and potential yield compensation (tolerance) traits that provide a foundation to understand the specificity of host plant interactions. Knowledge of these interactions can be advanced by conducting transcriptomic, proteomic, and metabolomic studies that can help to decipher key resistance pathways in these cultivars. This knowledge will empower selection of traits for developing novel barley cultivars, and possibly other cereal grain cultivars, with greater resistance to *C. cinctus*.

Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

Acknowledgments

Author contributions

Conceptualization: D.K.W., B.B.A. Data collection and formal analysis: B.B.A. Resources: D.K.W. Writing-original draft: B.B.A., and D.K.W. Writing-review and editing: D.K.W., B.B.A., J.D.S., G.V.P.R., R.K.D.P.

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