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Common facultative endosymbionts do not influence sensitivity of cereal aphids to pyrethroids

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Abstract

1. Cereal aphids, including the bird cherry-oat aphid, *Rhopalosiphum padi*, and the grain aphid, *Sitobion avenae*, can transmit viruses that significantly reduce crop yields. To mitigate against yield losses, insecticides are routinely used to manage aphid populations.
2. Aphids can form relationships with endosymbionts that confer fitness benefits or consequences to the aphid. Recent artificial inoculation experiments indicate that endosymbionts could increase aphid susceptibility to insecticides, but this has not been explored using aphid populations naturally infected with endosymbionts.
3. Here, we sampled aphids from an important cereal production region in Lower Saxony, Germany. We characterized the endosymbiont profile of these aphid populations and conducted pyrethroid dose–response assays to test the hypothesis that facultative endosymbionts increase aphid susceptibility to insecticides.
4. We find that the level of insecticide susceptibility is highly variable in *S. avenae* and we identify populations that are sensitive and tolerant to pyrethroids, including populations collected from the same field. For *R. padi*, we find evidence for decreased sensitivity to pyrethroids, representing the first report of reduced sensitivity to pyrethroids in *R. padi* sampled from Central Europe.
5. We detected high endosymbiont infection frequencies in the aphid populations. 84% of aphids carry one facultative endosymbiont and 9% of aphids carry two facultative endosymbionts. We detected associations with *Regiella insecticola*, *Fukatsia symbiotica*, and *Hamiltonella defensa*. However, we do not identify a link between endosymbiont infection and insecticide susceptibility, indicating that other factors may govern the development of insecticide resistance and the need for alternative management strategies.

KEYWORDS
Dose response, Endosymbionts, Insecticide tolerance, Pest management, Pyrethroid

INTRODUCTION

Cereal aphids, including the bird cherry-oat aphid, *Rhopalosiphum padi*, and the grain aphid, *Sitobion avenae*, are important herbivorous insects. Cereal aphids are classed as agricultural pest species on many grasses and cereals, including wheat and barley (Van Emden & Harrington, 2017). Cereal aphids are widely distributed across Central Europe and can cause significant damage to cereal crops. Aphid

damage can be caused through direct feeding (Dedryver et al., 2010) and via the transmission of plant viruses, including barley yellow dwarf virus (BYDV) (Perry et al., 2000). High levels of BYDV infection in cereal crops can result in yield losses of *c*. 80% (Nancarrow et al., 2021).

Insecticides remain the method that is most commonly used to manage aphid populations, with pyrethroids widely used for the management of cereal aphids on spring and winter cereal crops across Europe (Dewar & Foster, 2017). The high reliance on pyrethroid insecticides increases the evolutionary pressure on aphid populations, increasing the risk that insecticide resistant aphid populations will emerge (Dewar & Foster, 2017). Insecticide resistant populations can have devastating consequences on effective aphid management and increase potential aphid-derived yield loss (Dewar & Foster, 2017), making these an urgent priority for the development of alternative management strategies. Resistance to insecticides evolves over time, and monitoring surveys of herbivorous insect populations can detect the emergence of insecticide resistance by identifying populations that are less sensitive to insecticides (Umina et al., 2020; Walsh, Ferrari, et al., 2020). Resistance against pyrethroids has been described in both *S. avenae* and *R. padi* populations (Foster et al., 2014; Wang et al., 2020). Pyrethroid resistance is associated with mutations in voltage-gated ion channels (Foster et al., 2014; Wang et al., 2020) and this resistance mechanism is referred to as *knock down resistance* (*kdr*). Two mutations conferring *kdr* resistance have been described, namely *kdr* (Foster et al., 2014) and *super-kdr* (Wang et al., 2020).

Pyrethroid resistance has been described in *S. avenae* populations, with heterozygous *knockdown resistance* (*kdr*-SR) resistant populations detected in China, Ireland and the UK (Foster et al., 2014; Gong et al., 2021; Walsh, Schmidt, et al., 2020). However, the composition of resistant populations is variable and appears to differ between survey years and across regions. Field surveys of *S. avenae* in Ireland indicate that the composition of individuals containing the *kdr*-SR heterozygous mutation can range from 25–54% (Walsh, Schmidt, et al., 2020). Resistance against pyrethroids was recently reported in an *R. padi* population collected from Jingyang, Shaaxi Province, China (Wang et al., 2020) and subsequent field surveys have detected additional pyrethroid-resistant populations in multiple locations across China (Gong et al., 2021). According to the Arthropod Pesticide Resistance Database, a global databank of insecticide resistance cases, no other occurrences of pyrethroid resistance in *R. padi* have been reported. This indicates that full pyrethroid resistance is yet to evolve, or be detected, in *R. padi* populations outside of China.

The development of insecticide resistance can be monitored through dose–response assays to detect the emergence of populations showing reduced sensitivity to insecticides. *R. padi* populations with reduced sensitivity to pyrethroids have been recently detected in Ireland (Walsh, Ferrari, et al., 2020) and Australia (Umina et al., 2020), suggesting that resistance could be evolving. However, on average, field concentrations of pyrethroids are still effective at controlling over 90% of the aphid population (Umina et al., 2020; Walsh, Ferrari, et al., 2020; Zuo et al., 2016).

The lack of high prevalence of resistant populations across regions and years (Gong et al., 2021; Walsh, Schmidt, et al., 2020) suggests that fitness consequences could be associated with insecticide resistance traits. Recent research has provided some evidence to support this: it was recently reported that *S. avenae* populations with heterozygous *kdr*-SR resistance to pyrethroids exhibit increased vulnerability to the parasitoid *Aphidius ervi* (Jackson et al., 2020). Studies have also identified additional fitness trade-offs resulting from *kdr*-SR heterozygous resistance to pyrethroids, including lower aphid abundance and reduced growth in *S. avenae* populations (Jackson et al., 2020) and reduced fecundity in *R. padi* populations carrying *super-kdr* resistance (Wang et al., 2021).

A key driver of phenotypic diversity in aphid populations is the presence of facultative endosymbionts (Zytynska et al., 2021; Zytynska & Weisser, 2016). The majority of aphid species form an essential relationship with the endosymbiont *Buchnera aphidicola*, with *B. aphidicola* providing nutritional supplementation to the aphid diet (Douglas & Prosser, 1992; Sasaki et al., 1991). Aphids can also form non-essential, or facultative, relationships with a range of additional endosymbionts (Guo et al., 2017; Zytynska & Weisser, 2016). The most common facultative endosymbionts detected in aphid populations are *Spiroplasma spp*., *Regiella insecticola*, *Hamiltonella defensa*, *Rickettsiella sp*., *Fukatsia symbiotica* (previously pea aphid x-type symbiont, *PAXS*), *Serratia symbiotica*, *Rickettsia spp*., and *Arsenophonus spp*. (Beekman et al., 2022; Guo et al., 2017; Zytynska & Weisser, 2016). These facultative endosymbiotic relationships occur naturally in aphid populations (Beekman et al., 2022; Guo et al., 2019; Henry et al., 2015; Leybourne, Bos, et al., 2020). The phenotypic consequence of endosymbiont infection is not always clear, and the phenotypic traits conferred by a specific endosymbiont species are not consistently observed between aphid species, aphid genotypes within the same species, or even between different endosymbiont strains (Cayetano et al., 2015; McLean & Godfray, 2015; Oliver & Higashi, 2019; Vorburger et al., 2010). One common beneficial trait that is often conferred through facultative endosymbiont infection across a range of aphid-endosymbiont combinations is resistance against parasitoid wasps (Asplen et al., 2014; Leybourne, Bos, et al., 2020; Oliver et al., 2003, 2009). A diverse range of other phenotypic traits that can be conferred by endosymbiont infection have also been described, including lower fecundity (Zytynska et al., 2021).

In cereal aphids, endosymbiont-conferred phenotypes include protection against parasitoid wasps (Leybourne, Bos, et al., 2020), altered feeding behaviour (Leybourne, Valentine, et al., 2020), adjusted life-history parameters, including reduced growth and development (Leybourne, Bos, et al., 2020; Liu et al., 2019; Luo et al., 2020), and moderate increase in susceptibility to bacterial pathogens (Álvarez-Lagazzi et al., 2021). Recently, studies have indicated that endosymbiont infection can also influence the susceptibility of the aphid host to insecticides. A study examining aphid susceptibility to a range of insecticides found that wheat aphids, *S. miscanthi*, infected with *H. defensa* were more susceptible to low concentrations of insecticide when compared with uninfected aphids (Li et al., 2021). This indicates that there could be a phenotypic link between facultative endosymbiont communities and aphid susceptibility, or tolerance, to insecticides. An association between endosymbiont infection and insecticide resistance could provide an explanation for high variation

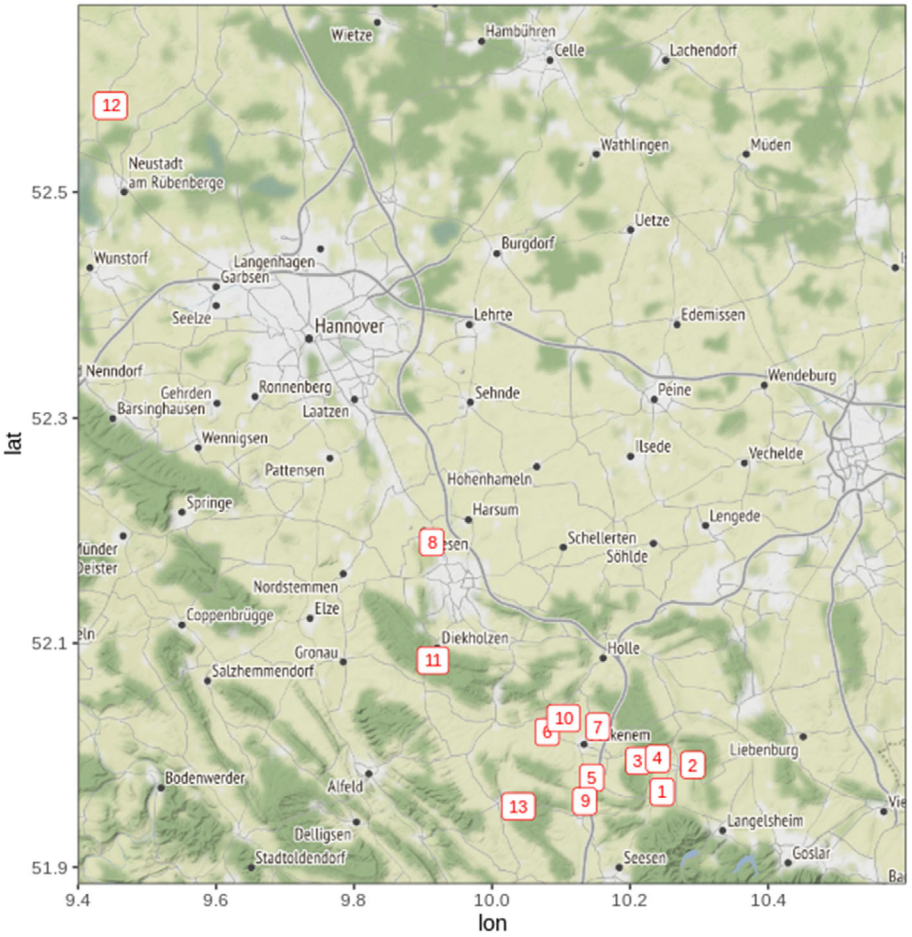

FIGURE 1 Location of the 13 sample sites. Field 12 was a winter rapeseed field, all other fields were winter wheat. The coordinates (longitude and latitude) for each field are displayed in Table S1

in endosymbiont prevalence and *kdr*-SR prevalence in aphid populations (Guo et al., 2019; Walsh, Schmidt, et al., 2020).

Here, we report the results of pyrethroid dose–response bioassays for cereal aphid populations sampled from a key cereal production region in Northern Germany. We sampled 25 *S. avenae* and seven *R. padi* populations from 13 field sites. We find that, for *S. avenae*, the level of insecticide susceptibility is highly variable, with populations sensitive and tolerant to pyrethroid exposure, including populations collected from the same field. In *R. padi* populations, we find evidence for decreased sensitivity, indicating that resistance to pyrethroids is starting to evolve in German *R. padi* populations. Furthermore, we explore the hypothesis that endosymbiont infection increases aphid susceptibility to insecticides by characterizing the endosymbiont communities of these populations.

METHODS

Aphid sampling and establishment of lab populations

Cereal aphid populations were sampled in summer and autumn 2021 from 13 agricultural fields in Lower Saxony, Germany (Figure 1; Table S1). Sample sites comprised 12 winter cereal fields (collected in summer) and one winter rapeseed field (collected in autumn). Single adult aphids (apterous or alate) were collected and used to establish laboratory populations; from the 13 fields 25 *Sitobion avenae* and seven *Rhopalosiphum padi* populations were established. Populations were maintained on one-week old wheat plants (approximately GS 11–13) in ventilated plastic cups under glasshouse conditions. Where multiple samples were collected from one field the samples were either collected on different dates or with a 20 m minimum distance between sampling points.

Insecticide sensitivity testing

Aphid populations were screened for susceptibility and sensitivity to the synthetic pyrethroid Decis Forte® (Bayer CropScience, Germany), a Class 3A synthetic pyrethroid (active ingredient deltamethrin at 100 g L^{-1} formulation). This insecticide was selected as it is approved for use on arable and field crops in Germany. A stock solution was prepared in water at a concentration comparable to the recommended field rate, equating to a concentration of 357 mg a.i. L^{-1}. Serial dilutions were prepared from the stock solution. Five insecticide dilutions were used in the assay: stock, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, with distilled water included as a negative control.

TABLE 1 Insecticide bio-assay results

Clone name	Species	Field	Week sampled	Number tested (number of biological replicates)	Slope coefficient (±SE)	EC_{50} (mg a.i. L^{-1})	95% confidence interval	Resistance category
SA-1 ^{AB}	*S. avenae*	6	21.06.2021	153 (4)	0.50 (0.08)	0.62	0.09–3.00	Susceptible
SA-2 ^{BCDEF}	*S. avenae*	5	21.06.2021	173 (4)	0.70 (0.18)	9.59	2.99–36.71	Susceptible
SA-3 ^{AB}	*S. avenae*	1	21.06.2021	126 (3)	0.89 (0.11)	1.81	0.49–6.11	Susceptible
SA-4 ^{ABCD}	*S. avenae*	7	21.06.2021	123 (3)	0.67 (0.11)	1.91	0.37–8.57	Susceptible
SA-5 ^{A}	*S. avenae*	10	21.06.2021	168 (4)	0.79 (0.09)	0.38	0.10–1.13	Susceptible
SA-6 ^{AB}	*S. avenae*	2	21.06.2021	174 (3)	0.66 (0.18)	1.09	0.28–3.64	Susceptible
SA-8 ^{CDEF}	*S. avenae*	5	21.06.2021	124 (3)	0.69 (0.15)	15.67	3.84–96.17	Moderately susceptible
SA-9 ^{CDEF}	*S. avenae*	1	21.06.2021	126 (3)	0.57 (0.38)	24.62	5.03–295.95	Moderately tolerant
SA-10 ^{CDEF}	*S. avenae*	1	21.06.2021	176 (4)	0.60 (0.33)	20.39	5.64–114.40	Moderately tolerant
SA-11 ^{BCDE}	*S. avenae*	7	21.06.2021	192 (4)	1.05 (0.18)	4.14	1.17–10.04	Susceptible
SA-12 ^{BCDEF}	*S. avenae*	3	21.06.2021	192 (4)	0.53 (0.58)	33.14	8.60–258.43	Moderately susceptible
SA-13 ^{EF}	*S. avenae*	6	21.06.2021	174 (4)	0.38 (0.09)	68.56	10.19–3319.38	Tolerant
SA-14 ^{CDEF}	*S. avenae*	6	21.06.2021	192 (4)	0.48 (0.12)	24.52	5.75–215.58	Moderately tolerant
SA-15 ^{F}	*S. avenae*	7	21.06.2021	144 (3)	0.38 (0.10)	190.08	19.80–11356.90	Tolerant
SA-16 ^{EF}	*S. avenae*	7	21.06.2021	156 (3)	0.45 (0.11)	80.53	13.31–3572.30	Tolerant
SA-17 ^{CDEF}	*S. avenae*	9	21.06.2021	126 (3)	0.75 (0.16)	20.29	5.39–114.46	Susceptible
SA-18 ^{ABCDE}	*S. avenae*	3	21.06.2021	156 (4)	0.69 (0.08)	2.86	0.77–10.34	Moderately susceptible
SA-19 ^{BCDEF}	*S. avenae*	7	21.06.2021	174 (4)	0.63 (0.30)	9.08	2.60–39.21	Moderately susceptible
SA-20 ^{CDEF}	*S. avenae*	3	21.06.2021	174 (4)	0.53 (0.17)	23.01	5.57–184.19	Moderately tolerant
SA-21 ^{DEF}	*S. avenae*	3	05.07.2021	126 (3)	0.59 (0.13)	39.30	8.27–573.09	Moderately tolerant
SA-22 ^{CDEF}	*S. avenae*	9	21.06.2021	174 (4)	0.52 (0.17)	16.87	4.07–120.94	Moderately tolerant
SA-23 ^{AB}	*S. avenae*	13	21.06.2021	149 (4)	0.62 (0.18)	0.85	0.16–3.33	Susceptible
SA-24 ^{DEF}	*S. avenae*	6	05.07.2021	173 (4)	0.63 (0.19)	22.71	6.49–123.98	Moderately susceptible
SA-25 ^{EF}	*S. avenae*	9	21.06.2021	174 (4)	0.63 (0.64)	33.94	9.74–209.92	Moderately tolerant
SA-26 ^{BCDEF}	*S. avenae*	12	04.10.2021	170 (4)	0.63 (0.13)	9.15	2.57–41.39	Susceptible
RP-1 ^{ZY}	*R. padi*	2	21.06.2021	189 (4)	0.81 (0.07)	1.13	0.37–3.11	Susceptible
RP-2 ^{ZY}	*R. padi*	5	05.07.2021	192 (4)	0.73 (0.10)	1.55	0.49–4.54	Susceptible
RP-3 ^{Z}	*R. padi*	5	21.06.2021	189 (4)	0.66 (0.15)	0.44	0.11–1.40	Susceptible
RP-4 ^{ZY}	*R. padi*	11	21.06.2021	192 (4)	0.63 (0.18)	1.41	0.39–4.63	Susceptible
RP-5 ^{Y}	*R. padi*	13	21.06.2021	192 (4)	1.50 (0.11)	5.32	2.51–11.29	Susceptible
RP-6 ^{ZY}	*R. padi*	4	21.06.2021	187 (4)	0.82 (0.09)	1.63	0.55–4.55	Susceptible
RP-7 ^{ZY}	*R. padi*	4	05.07.2021	192 (4)	0.78 (0.07)	2.03	0.69–5.69	Susceptible

Note: The EC_{50} values for populations followed by the same letter do not differ significantly, based on observation of overlapping confidence intervals (Foster et al., 2011). Populations have been allocated a resistance category based on the observed mortality (% aphids affected) at the field concentration (375 mg a.i. / L) treatment: Susceptible (95–100% mortality), moderately susceptible (90–94% mortality), moderately tolerant (71–89% mortality), tolerant (≤70% mortality).

The insecticide sensitivity assays broadly followed the IRAC leaf-dip method (IRAC, 2016) and the method deployed by (Umina et al., 2020). Briefly, *c.* 25 mm sections of wheat leaves were submerged for *c.* 10 s in one of the test solutions. Control leaves were dipped first, then the leaves were dipped sequentially from the lowest concentration (10^{-4}) to the field rate stock solution. Once dipped,

leaves were left to dry on paper towels for approximately 1 h before they were placed abaxial side up on agar (1 g L^{-1}) in a plastic Petri Dish; a droplet of water was added to the surface of the agar to aid leaf adhesion. Aphids were transferred to each Petri Dish using a fine-haired paintbrush, Petri Dishes were moved to a controlled environment room (20°C ± 2°C, L16:D8), and Petri Dishes were inverted to simulate aphid feeding from the underside of the leaf. Between 4–8 aphids were transferred to each Petri Dish (i.e., 4–8 'replicate aphids' were included per bio-assay); each population was tested in at least three experimental repeats ('biological replicate') and the total number of repeats is indicated in Table 1. After 48 h, aphids were scored as either alive, moribund or dead. Aphids were classed as alive if they were able to return to an upright position when placed on their back (i.e., they were capable of coordinated movement). Moribund and dead aphids were grouped together as "affected", in-line with previous dose–response assays (Foster et al., 2012; Umina et al., 2020).

DNA extraction and diagnostic PCR for endosymbiont characterization

A sample of five aphids (mixture of apterous adults and nymphs) were collected from each population and DNA was extracted using the Norgen® Plant and Fungi DNA extraction kit (Norgen Biotek, Germany) following manufacturer's instructions. An extraction blank was included with each batch of extractions.

Successful DNA extraction was confirmed using a PCR marker for the primary aphid symbiont *B. aphidocola*. The presence of facultative endosymbionts was determined using a three-step multiplex diagnostic PCR assay (Beekman et al., 2022). Multiplex assays were used to detect the presence of the main aphid secondary endosymbionts: *Spiroplasma spp*., *Regiella insecticola*, *Hamiltonella defensa*, *Rickettsiella sp*., *Fukatsuia symbiotica*, *Serratia symbiotica*, *Rickettsia spp*., and *Arsenophonus spp*. All PCR primer details are described in Table S2. PCR assays were conducted in a final reaction volume of 12 μl consisting of: 2 μl DNA, 6 μl 2X Kappa2G Fast PCR Ready Mix (Merck, Germany). Primer concentrations and volumes differed between the multiplex assays and are detailed in Table S2. The final reaction mixture was made to 12 μl using nuclease-free DEPC-treated water (CarlRoth, Germany). PCR conditions followed Beekman et al., 2022, i.e.: denaturation at 94°C for 3 min followed by 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 60 s with a final extension step at 72°C for 10 min. Positive DNA (mixed DNA containing positive DNA extracts for all target endosymbionts) was included as a positive control, an extraction blank was used as an extraction negative control, and DNA-free PCR mastermix was included as PCR negative control. Endosymbiont presence was detected by separation of PCR products on a 1% agarose gel stained with GelRed® (Biotium, Germany), and reactions were visualized under UV light; a 100 bp DNA ladder (ThermoFischer, Germany) was used to estimate band size. Positive identification of the presence of endosymbionts in the multiplex assay were confirmed in additional singleplex assays. All PCR assays were conducted in a Biometra TRIO 48, Thermocycler (Analytik Jena, Germany).

Statistical analysis

All statistical analysis was carried out using R (v.4.1.2) and R Studio (v.1.3.1093). The following R packages were used for data visualization: ggplot2 (Wickham, 2016) and ggmap (Kahle & Wickham, 2013).The estimated concentration of active ingredient required to achieve 50% mortality (EC_{50} value), EC_{50} 95% confidence intervals, slope, intercept and associated standard errors for the dose–response curve were calculated for each aphid population using probit estimation regression (Finney, 1952). To achieve this, the "ProbitEPA" function in the R package ecotoxicology (v.1.0.1) was used. Differences in the dose response between populations was detected using an ANOVA, as done in similar studies (Umina et al., 2020); to achieve this the intercepts of the dose–response curves were estimated using linear models with aphid population, facultative endosymbiont infection status, and facultative endosymbiont diversity (Simpson's diversity) included as explanatory variables in individual models. Linear models were tested for significance using Type-II ANOVA. Simpson's diversity was calculated using the vegan package (v.2.5–7). Where significant differences in model intercepts were detected, the differing aphid populations were identified by observing the overlapping confidence intervals. This method has been used previously to identify differences in insecticide susceptibility between aphid populations from estimated concentration of active ingredient required to achieve 50% population mortality (EC_{50}) values (Foster et al., 2012).

Use of Abbot's formula to adjust for mortality in the control treatment

For toxicology studies, such as insecticide resistance monitoring, it is sometimes recommended that values are adjusted to account for observed mortality within the control treatment (Finney, 1949). The World Health Organization advises that these adjustments are only applied if the observed mortality in the control treatment exceeds 5%, and that results are discarded if mortality in the control treatment exceeds 20% (WHO, 2016). During our tests, mortality within the control treatment was rarely observed and only one aphid population (SA-12) had mortality above 5% (9.37%). Therefore, we only applied an Abbot's adjustment (Finney, 1949) to the data for *S. avenae* population SA-12.

RESULTS

Pyrethroid sensitivity is variable in Sitobion avenae populations

Based on mortality at field rate concentration (i.e., mortality in the stock treatment, 357 mg a.i. L^{-1} deltamethrin), *S. avenae* populations were grouped into four broad categories (Table 1): Susceptible (mortality at field concentration 95–100%), moderately susceptible (mortality at field concentration 90–94%), moderately tolerant (mortality at field concentration 71–89%) and tolerant (mortality at field concentration ≤70%). Field rate concentration only achieved complete aphid

control in five of the 25 *S. avenae* populations, namely populations SA3, SA-5, SA-6, SA-11 and SA-23 (Figure 2). Three *S. avenae* populations (SA-13, SA-15, SA-16) showed tolerance to pyrethroid exposure, with ≤70% population mortality following exposure to field rate concentrations of deltamethrin (Figure 2; Table 1).

The estimated effective dose required for 50% population control (EC_{50}) ranged from 0.38 mg a.i. L^{-1} to 190.08 mg a.i. L^{-1} deltamethrin (Table 1). Comparison of the dose response model intercepts highlighted differences in EC_{50} amongst the *S. avenae* populations examined ($F_{24,92} = 4.44$; $p = < 0.001$). Observation of the overlapping 95% confidence intervals (Figure 2; Table 1) indicates that differences in EC_{50} are between the three tolerant populations (SA-13, SA-15, SA-16) and seven of the 10 susceptible populations (SA-1, SA-3, SA-4, SA-5, SA-6, SA-11, SA-23).

Decreased pyrethroid sensitivity in a Rhopalosiphum padi population collected from Germany

Based on mortality at field rate concentration, all *R. padi* populations were categorized as susceptible to deltamethrin (mortality >95%; Table 1; Figure 3); however, two populations (RP-5 and RP-6) had a reduced mortality of 96% (Figure 3).

The estimated effective dose required for 50% population control (EC_{50}) ranged from 0.44 mg a.i. L^{-1} to 5.32 mg a.i. L^{-1} deltamethrin (Table 1). Comparison of model intercepts highlighted differences in EC_{50} amongst the *R. padi* populations examined ($F_{6,27} = 7.43$; $p = <0.001$). Observation of the overlapping 95% confidence intervals (Figure 3; Table 1) indicates that differences in EC_{50} are between one of the populations with reduced mortality, RP-5, and the susceptible population RP-3 (Figure 3; Table 1).

Facultative endosymbionts occur at high frequencies in aphid populations but they do not influence pyrethroid sensitivity

Of the 32 aphid populations 27 were infected with at least one facultative endosymbiont (Table 2). The endosymbiont community differed between the two cereal aphid species: endosymbionts were detected in 92% of *S. avenae* populations and 57% of *R. padi* populations (Table 2). In *S. avenae* endosymbiont communities were dominated by *R. insecticola*, with *R. insecticola* present in 72% of *S. avenae* populations. Low levels of infection with *F. symbiotica* (8%) and co-infection of *R. insecticola* and *F. symbiotica* (12%) were detected in the *S. avenae* populations. For *R. padi*, the defensive endosymbiont *H. defensa* was detected in 28% of the populations and *F. symbiotica* was detected in 28% of the populations. For both aphid species, neither endosymbiont infection status nor endosymbiont diversity influenced aphid sensitivity to deltamethrin: *S. avenae* facultative endosymbiont infection status ($F_{1,23} = 0.37$; $p = 0.778$; Figure 2) and diversity ($F_{1,23} = 0.06$; $p = 0.816$), *R. padi* facultative endosymbiont infection status ($F_{2,4} = 0.65$; $p = 0.569$; Figure 3) and diversity ($F_{2,4} = 1.11$; $p = 0.341$).

DISCUSSION

Our results provide insecticide dose–response data for 32 aphid populations against a synthetic pyrethroid approved for aphid management in arable crops in Germany. We observe wide variation in dose–response amongst the 25 *S. avenae* populations and we detect reduced sensitivity to deltamethrin in one of the *R. padi* populations tested. We detect natural infection with the facultative endosymbionts *R. insecticola* in *S. avenae*, *H. defensa* in *R. padi* and *F. symbiotica* in both species, including co-infection of *R. insecticola* and *F. symbiotica* in a subset of *S. avenae* populations.

For the *S. avenae* populations we detected variable response to pyrethroid exposure, with EC_{50} values ranging from 0.38 mg a.i. L^{-1} to 190.08 mg a.i. L^{-1}. This wide variation in dose–response in *S. avenae* indicates that our *S. avenae* populations comprise individuals that are highly sensitive to pyrethroids, tolerant to pyrethroids and populations with intermediate susceptibilities. Indeed, the low EC_{50} value of 0.38 mg a.i. L^{-1} detected in SA-5 is comparable with EC_{50} values in *S. avenae* populations that are sensitive to pyrethroid exposure (0.50 mg Bifenthrin L^{-1}; 2.40 mg Beta-cypermethrin L^{-1}; Gong et al., 2021) and the high EC_{50} values estimated for the three tolerant populations, SA-15 (190.08 mg a.i. L^{-1}), SA-16 (80.53 mg a.i. L^{-1}), SA-13 (68.56 mg a.i. L^{-1}), are similar to the EC_{50} values reported for *S. avenae* populations tolerant to pyrethroids, including those that harbour heterozygous *kdr*-SR resistance (Foster et al., 2014; Gong et al., 2021; Walsh, Ferrari, et al., 2020). Our field survey also indicates that pyrethroid susceptibility is highly variable between populations collected from the same field. We sampled five *S. avenae* populations from field 7 and the estimated EC_{50} for these populations ranged from 1.91 mg a.i. L^{-1} 190.08 mg a.i. L^{-1}. Based on the observed population control at field rate concentrations, this field contained populations grouped into the susceptible, moderately tolerant and tolerant categories. Similar observations were made for other locations where more than one population was sampled: Field 3 contained four *S. avenae* populations comprising those categorized as moderately tolerant and moderately susceptible; field 9 had three populations comprising those categorized as susceptible and moderately tolerant; four individuals were collected from field 6 comprising those categorized as susceptible, moderately tolerant and tolerant; and three populations were sampled from field 1 comprising populations categorized into susceptible and moderately tolerant. The diverse range of susceptibility to and tolerance of pyrethroids, including wide variation within the same sampling locations, that we report is in-line with recent surveys conducted in Ireland (Walsh, Schmidt, et al., 2020) and China (Gong et al., 2021) However, these studies did not relate insecticide susceptibility or tolerance to the presence or absence of facultative endosymbionts within the local aphid populations.

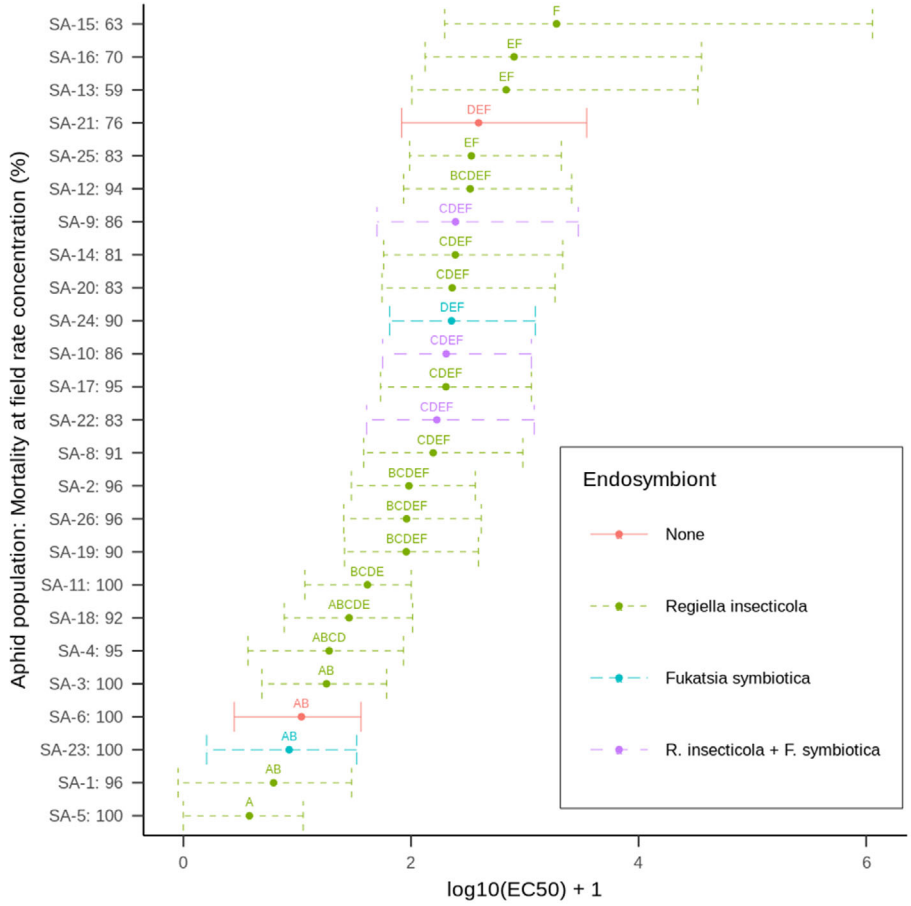

FIGURE 2 Estimated EC_{50} values and 95% confidence intervals for *Sitobion avenae* populations. EC_{50} values are shown on the $\log_{10}+1$ scale to aid interpretation. Aphid population on the y-axis is followed by the percentage population control at field rate concentration. Letters indicate differences between the aphid populations based on overlapping confidence intervals

In our *R. padi* populations we detected reduced sensitivity to the pyrethroid deltamethrin in one population. This reduction in pyrethroid sensitivity was detected in population RP-5, where we estimated an increased EC_{50} of 5.32 mg a.i. L^{-1}. We also detected a reduction in the level of population control in RP-5, with only 96% of the aphid population effectively managed at the field rate concentration of 357 mg a.i. L^{-1}, compared with 100% population control in RP-3. Our increased EC_{50} for RP-5 represents the first observation of reduced sensitivity to pyrethroids in *R. padi* populations collected in Germany. Reduced sensitivity against pyrethroids has recently been reported in three *R. padi* populations in Australia, these three populations were estimated to have EC_{50} values of 15.34, 16.22 and 24.57 mg a.i. L^{-1} (Umina et al., 2020). These values are at least three-fold higher than the EC_{50} value estimated for RP-5 and are associated with a further reduction in effective population control under field rate concentrations, down to 91% effective control (Umina et al., 2020), compared with 96% control in RP-5. Recent surveys in Ireland have also detected reduced pyrethroid sensitivity in one *R. padi* population, where EC_{50} levels were compared with a susceptible *kdr*-SS *S. avenae* population (Walsh, Ferrari, et al., 2020). Although we detect reduced sensitivity in *R. padi* in Germany, effective population control remains relatively high at 96%, thus this should not adversely affect aphid management strategies or impact crop yields in the immediate term. However, this finding indicates that resistance to pyrethroids is starting to evolve and, upon the evolution of *kdr*-SR resistance, the impact on crop yields could become more apparent as conventional control strategies fail. Surveys should be continued in order to monitor the development of the situation over the coming years while developing and prioritizing the use of alternative strategies for aphid management that do not rely on resistance-inducing insecticides. In China, decreased sensitivity to pyrethroids was first detected in populations sampled in 2013 from multiple locations across China, including Xianyang, Shaaxi Province (Zuo et al., 2016). *Super-kdr*-SR heterozygous resistance against pyrethroids (shown to be effective against two active ingredients: alpha-cypermethrin and deltamethrin) was detected 6 years later from populations collected in 2019 from the same Province (Wang et al., 2020).

Aphids are often considered as a single homogeneous population, however, it is clear that aphids can comprise populations with contrasting intra-species diversity. Intra-species diversity in cereal aphids is associated with genetic diversity and the composition of

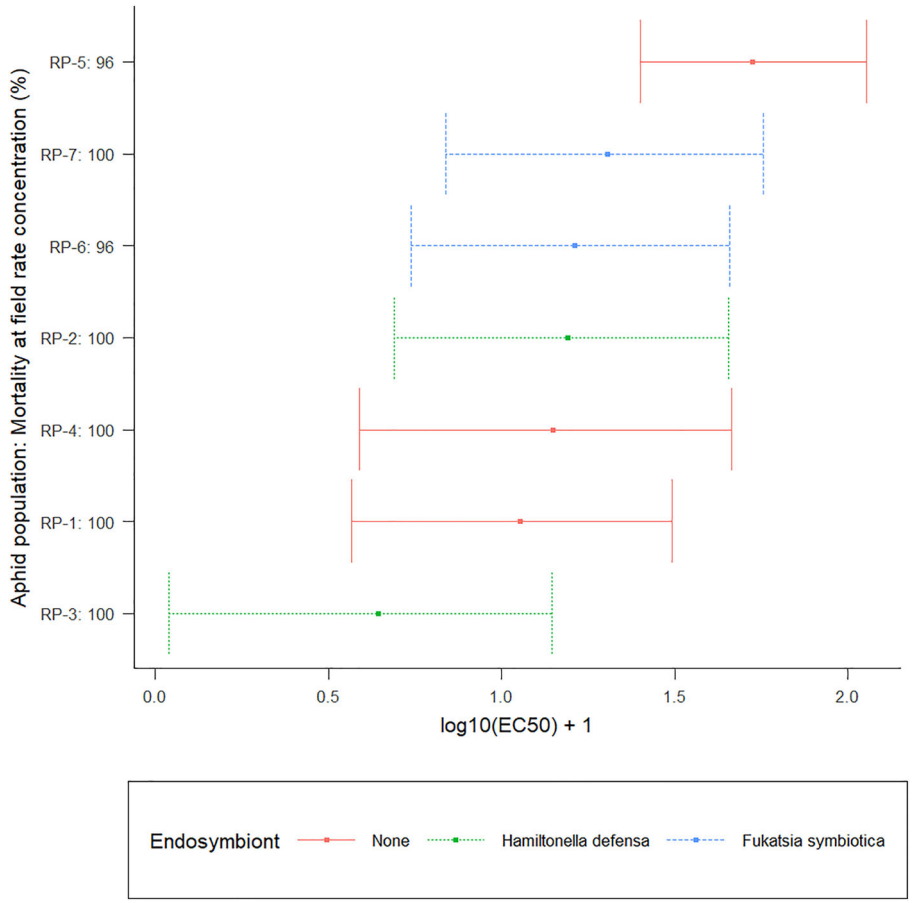

FIGURE 3 Estimated EC50 values and 95% CI for *Rhopalosiphum padi* populations. EC_{50} values are shown on the $\log_{10}+1$ scale to aid interpretation. Aphid population on the y-axis is followed by the percentage population control at field rate concentration

endosymbiont communities (Alkhedir et al., 2013; Guo et al., 2019; Leybourne, Bos, et al., 2020; Malloch et al., 2016). This intra-species diversity can affect the aphid phenotype, and there is evidence that this could include heightened aphid susceptibility to insecticides (Li et al., 2021). In order to examine whether natural occurrence of facultative endosymbionts influences aphid sensitivity to pyrethroids, we characterized the facultative endosymbiont community of the 32 aphid populations and related this to the results of the dose–response assays. We detected facultative endosymbionts in 92% of the *S. avenae* populations and 57% of the *R. padi* populations. These natural levels of endosymbiont infection are similar to those reported in previous endosymbiont surveys (Alkhedir et al., 2013; Fakhour et al., 2018; Guo et al., 2019; Łukasik et al., 2013). Our *S. avenae* populations showed a high prevalence of *R. insecticola* infection (72%). This is comparable with infection levels detected in Morocco, 75% *S. avenae* population infection with *R. insecticola* (Fakhour et al., 2018), and above levels previously observed in *S. avenae* sampled from Germany, 50% *R. insecticola* infection (Alkhedir et al., 2013). Similarly, *H. defensa* usually occurs at low-to-moderate frequency in *R. padi* populations, with previous studies reporting infection frequencies between 10–38% (Guo et al., 2019; Leybourne, Bos, et al., 2020), comparable with 28% of *R. padi* populations detected to be infected with *H. defensa* in our aphid populations. We detected *F. symbiotica* in a small proportion of our *S. avenae* and *R. padi* populations. *F. symbiotica* can occur at high levels in *A. pisum* populations (Zytynska & Weisser, 2016) but rarely infects other aphid species, occurring at low frequencies where it is detected (Łukasik et al., 2013; Zytynska & Weisser, 2016).

Although we detected variation in endosymbiont infection frequencies across our populations, with 84% of populations carrying at least one facultative endosymbiont and 9% carrying two facultative endosymbiont species, we did not detect any link between endosymbiont infection and heightened susceptibility to insecticides. This is in contrast with recent lab studies, where an association between endosymbiont infection and heightened insecticide susceptibility has been reported (Li et al., 2021; Skaljac et al., 2018). However, it should be noted that these studies have only examined the influence of *H. defensa* (Li et al., 2021) and *S. symbiotica* (Skaljac et al., 2018), which were only found in one of our aphid species (*H. defensa* infection in *R. padi*) or not detected in our populations (*S. symbiotica*). Recent research has shown that artificial inoculation with *H. defensa* in the grain aphid *S. miscanthi* increases aphid sensitivity to a range of insecticides at low concentrations, including neonicotinoids and diamides (Li et al., 2021). Similar observations have been made in pea aphids

TABLE 2 Endosymbiont profiles of the 32 aphid populations

Clone name	Species	*B.a (primary)*	*Spi*	*R.i.*	*H.d.*	*R-siella*	*F. s.*	*S.s.*	*R-tsia*	*Ars.*
SA-1	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-2	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-3	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-4	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-5	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-6	*S. avenae*	+	−	−	−	−	−	−	−	−
SA-8	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-9	*S. avenae*	+	−	+	−	−	+	−	−	−
SA-10	*S. avenae*	+	−	+	−	−	+	−	−	−
SA-11	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-12	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-13	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-14	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-15	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-16	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-17	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-18	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-19	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-20	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-21	*S. avenae*	+	−	−	−	−	−	−	−	−
SA-22	*S. avenae*	+	−	+	−	−	+	−	−	−
SA-23	*S. avenae*	+	−	−	−	−	+	−	−	−
SA-24	*S. avenae*	+	−	−	−	−	+	−	−	−
SA-25	*S. avenae*	+	−	+	−	−	−	−	−	−
SA-26	*S. avenae*	+	−	+	−	−	−	−	−	−
RP-1	*R. padi*	+	−	−	−	−	−	−	−	−
RP-2	*R. padi*	+	−	−	+	−	−	−	−	−
RP-3	*R. padi*	+	−	−	+	−	−	−	−	−
RP-4	*R. padi*	+	−	−	−	−	−	−	−	−
RP-5	*R. padi*	+	−	−	−	−	−	−	−	−
RP-6	*R. padi*	+	−	−	−	−	+	−	−	−
RP-7	*R. padi*	+	−	−	−	−	+	−	−	−

Note: Symbiont abbreviations: *B.a (B. aphidicola; essential primary endosymbiont)*, *Spi (Spiroplasma spp.)*, *R.i. (Regiella insecticola)*, *H.d. (Hamiltonella defensa)*, *R-siella (Rickettsiella sp.)*, *F.s. (Fukatsia symbiotica)*, *S.s. (Serratia symbiotica)*, *R-tsia (Rickettsia spp.)* and *Ars. (Arsenophonus spp)*.

(*Acyrthosiphon pisum*) infected with the endosymbiont *S. symbiotica*, where symbiont-infected aphids were more susceptible to low concentrations of several insecticides, including carbamates, neonicotonoids, tetronic and tertamic acid derivatives and diamides (Skaljac et al., 2018). These studies also show that the EC_{50} values are lower for symbiont-infected aphids compared with aphid populations that do not contain facultative endosymbiont communities (Li et al., 2021; Skaljac et al., 2018). Although these studies did not examine the relationship between endosymbiont presence and susceptibility to pyrethroids, they still showcase a link between endosymbiont infection and heightened susceptibility to insecticide exposure in aphid populations. However, it should be noted that these were artificially manipulated populations developed through the endosymbiont removal and infection to establish desired endosymbiont communities under lab conditions, not comparisons of natural infections (Li et al., 2021; Skaljac et al., 2018). The next stage of research would be to examine this association under field conditions and across a broader range of insecticides, including the important pyrethroids. Future efforts should also focus on identifying the potential mechanism behind this by examining how endosymbiont density (i.e., endosymbiont titre), not just endosymbiont presence or absence, influences susceptibility to insecticides.

Caveats of the experimental approach

One caveat of our study was our lack of a characterized *kdr*-SS homozygous pyrethroid susceptible reference clone. A susceptible clone can be used as a reference baseline in order to calculate resistance ratios for each tested population and to act as an internal reference (Walsh, Ferrari, et al., 2020; Wang et al., 2020), although this is not included in every survey (Gong et al., 2021; Umina et al., 2020). The calculated EC_{50} values for our most highly sensitive aphid population for each species, *R. padi* (0.44 mg a.i. L^{-1}) and *S. avenae* (0.62 mg a.i. L^{-1}), are comparable with the EC_{50} values reported in the susceptible populations used in similar studies, including populations confirmed to contain the homozygous susceptible *kdr*-SS allele: 0.59 mg a.i. L^{-1} in deltamethrin-susceptible *R. padi* populations (Wang et al., 2020, 2021). Therefore, we are confident that our detection of decreased pyrethroid sensitivity in *R. padi* population RP-5 and our range of susceptibilities and tolerance detected in our *S. avenae* populations are comparable with susceptible clones.

In *S. avenae*, the genetic basis for resistance to pyrethroids has been well-characterized (Foster et al., 2014), and individual point mutations within the *kdr* gene can be used to identify resistant populations (Walsh, Schmidt, et al., 2020). We did not assess the genetic structure or the frequency of *kdr* point mutations in the aphid populations we tested, limiting the extent to which we can comment on whether the tolerant populations were exhibiting genetic-based *kdr*-SR or *super-kdr*-SR resistance (Foster et al., 2014) or metabolic-based resistance through the up-regulation of detoxification processes (Wang et al., 2020). We can, however, be confident that the likelihood that we collected, and therefore tested, genotypically unique populations is high: The population structure and genetic diversity of summer *S. avenae* populations from central and northern Germany, including Lower Saxony, has been characterized (Reimer, 2004). In these studies, 1172 aphids were sampled from 31 wheat fields over June and July 2001 and 504 of these aphid samples were determined to be a unique aphid genotype (Reimer, 2004). Subsequent assessments the following years indicated that the summer population of *S. avenae* in northern and central Germany can contain 43–73% unique genotypes (Reimer, 2004) and that the most common genotype (H) only comprises 5–11% of the total aphid population (Reimer, 2004). This high level of genetic diversity within summer *S. avenae* populations, including samples collected from the same field along sweep net transects (Reimer, 2004), provides confidence that genetically-distinct aphid genotypes were collected using our approach

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Longitude and Latitude coordinates for the sampling sites.

Table S2. Primers used in the endosymbiont characterization.

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