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Grain aphids (*Sitobion avenae*) with knockdown resistance (kdr) to insecticide exhibit fitness trade-offs, including increased vulnerability to the natural enemy *Aphidius ervi*

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Abstract

The development of insecticide-resistance mechanisms in aphids has been associated with inhibitory, pleiotropic fitness costs. Such fitness costs have not yet been examined in the UK's most damaging cereal aphid, *Sitobion avenae* (grain aphid) (Hemiptera: Aphididae). This study aimed to evaluate the fitness trade-offs of the insecticide-resistant *S. avenae* clone versus an insecticide-susceptible *S. avenae* clone. Additionally, the parasitoid, *Aphidius ervi* (Hymenoptera: Braconidae), was introduced to examine its potential as a biological control agent. This study found that insecticide-resistant clones had significantly lower population growth and individual relative growth rate. Furthermore, insecticide-resistant clones suffered from a significantly greater rate of parasitisation (mummification) compared to their insecticide-susceptible counterparts. The successfulness of the parasitoid as a biological control agent could prevent the spread of the insecticide-resistant genotype. However, for this to be possible, insecticide spraying regimes need to be moderated, and habitat modification and parasitoid manipulation must be considered.

Introduction

The evolution of organisms occurs via genetic variation and selection imposed by many abiotic and biotic environmental factors. Each factor can exert an opposing selection pressure, resulting in the variation of optimal levels of defence or immunity depending on the environmental conditions. The establishment of trade-offs occur when opposing selection pressures cause the defence/immunity level to be lower than the maximum [1]. In areas where selection pressures vary over time, the balance between trade-offs can shift, which may lead to the optimal defence/immunity level changing. Environmental fluctuations can lead to organisms mutating to better suit their new environment; however, these mutations can be limited if they incur pleiotropic fitness costs which affect physiological or behavioural traits [1].

this author is articulated in the 'author contributions' section.

Competing interests: Louise McNamara, is employed by a commercial company: Teagasc, Crops Research centre. I can state that: The funder provided support in the form of salaries for author [LM], but did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The specific role of this author is articulated in the 'author contributions' section. With regards to the Competing Interests Statement we are also able to state that: "This does not alter our adherence to PLOS ONE policies on sharing data and materials."

In response to strong, unambiguous selection pressures caused by intense, widespread agricultural activity, some pests have developed adaptive traits including pesticide resistance. A mechanism known as 'knockdown resistance' (kdr) has allowed cross-resistance in pests to DDT and pyrethroids. This mechanism is characterised by a reduction in the sensitivity of the nervous system caused by a single amino acid substitution (L1014F) in the insect's voltage gated sodium channel gene [2, 3]. Intuitively, kdr resistant individuals should experience fitness costs in areas where there is no insecticide pressure compared to susceptible ones. If this was not the case, the frequency of resistant alleles would be higher prior to exposure to pesticides [4]. Therefore, the resistant genes are likely to have deleterious pleiotropic costs, which have constrained the adaptive trait [5]. In support of this theory, there is growing evidence detailing the maladaptive side-effects of fitness changes on other seemingly unrelated traits [6, 7].

During the late summer of 2011, growers in England began reporting that *Sitobion avenae* (grain aphid) (Hemiptera: Aphididae) were becoming less susceptible to pyrethroids sprayed on cereal crops. *Sitobion avenae* is one of the most damaging cereal aphids in Western Europe, feeding on all cereals including barley, wheat and rice [8]. *Sitobion avenae* show a strong preference for the ear of cereals, which generally stay physiologically active for longer than the leaf. This allows *S. avenae* to maintain itself for considerably longer than other aphid species [9]. Foster *et al.* [3] identified that the kdr mechanism had resulted in clonal variation in the *S. avenae* sample with resistant clones exhibiting a 40-fold Resistance Factor. Currently, most studies investigating fitness trade-offs caused by insecticide-resistance have involved *Myzus persicae* (Hemiptera: Aphididae) (peach-potato aphid). There are no studies into the effect of the kdr mechanism on *S. avenae* and the maladaptive fitness traits that may be incurred. Existing literature suggests that the kdr-resistant *S. avenae* clone may have invested in the kdr mutation at the cost of pleiotropic performance traits. Malloch *et al.* [10] show that the frequency of the kdr resistant *S. avenae* clone in UK suction trap catches has stabilised at around 30%, which provides further evidence for the likelihood of some fitness costs associated with the kdr mutation. Currently the kdr mechanism is heterozygous (kdr-SR), but if homozygous resistance (kdr-RR) were to evolve, the levels of resistance would be expected to further increase [3].

With increased resistance to pesticides, it has become imperative to develop other pest management techniques, such as, exploiting and manipulating the natural enemies of pests to act as a biological control [11]. The use of natural enemies to supress specific pest organisms has evolved into an important facet of integrated pest management (IPM) (e.g. [12, 13]). The effectiveness of natural enemies as a biological control depends on several characteristics. These include high reproductive potential, a short development time in relation to prey and a high level of prey specificity [14]. Such characteristics are exemplified in the parasitoid Diptera and Hymenoptera. Adult females belonging to these orders are generally highly fecund, develop inside their prey making generation time similar to that of the host and only specialise in attacking a small number of prey species [14]. With over 400 species recorded [15], the use of aphid-specific parasitoids (Hymenoptera: Braconidae) in controlling aphid populations has been well documented in various cropping systems [15, 16]. *Aphidius ervi*, used in this study is a solitary endophagous parasitoid, with an overall time from oviposition to wasp emergence of 14 ± 3 days [17, 18]. To locate hosts, *A. ervi* use chemical cues such as aggregation and sex pheromones, and plant volatiles [19]. After locating the aphid, female *A. ervi* rapidly attempt to parasitise it by penetrating its exoskeleton with an ovipositor [20].

The present study was designed to determine if the kdr-resistant *S. avenae* clone has developed any maladaptive behavioural or physiological characteristics because of the kdr mechanism. The study compared and assessed differences in performance traits in the kdr-resistant and kdr-susceptible clones. It was hypothesised (i) that the kdr-susceptible clone would have a significantly greater aphid population growth rate and individual relative growth rate than the

kdr-resistant clone, (ii) the kdr-susceptible clone would be able to deter the parasitic wasp *Aphidius ervi* more successfully than the kdr-resistant clone and (iii) a greater proportion of the kdr-resistant clone would be parasitised, and the parasitoid emergence rate would be greater in the kdr-resistant colonies.

Materials and methods

Study species

Barley (*Hordeum vulgare* cv. Sienna) sourced from Bairds Malt (Witham, UK) was used as the host plant. Four barley seeds were planted in to each of 24 2 L pots containing Levington M3 High Nutrient Compost (Everris, Ipswich, UK). Plants were grown in a glasshouse at 21 ± 2˚C, under a 16:8 h light:dark photoperiod and watered twice weekly throughout the study. After 21 days plants were thinned to leave one plant per pot, which were grown on for a further 40 days until they reached GS23 (AHDB Cereal growth stages).

Two clonal lines of the grain aphid, *S*. *avenae*, were sourced from long-term colonies reared by the James Hutton Institute in Dundee: (1) homozygous fully insecticide susceptible of SA12A lineage (kdr-SS) and (2) kdr heterozygous insecticide resistant of SA3 lineage (kdr-SR). Aphid colonies were reared on three barley plants in separate mesh cages (50 cm by 50 cm by 50 cm) in an insectary (17 ± 3˚C; 65 ± 5% RH; LD 16:8 h, 150 μmol m^{-2} s^{-1}). Plants were replenished each week. Clonal integrity was verified at the beginning and end of the experiment through DNA genotyping. DNA was extracted from single adult *S*. *avenae* using a sodium hydroxide method described in Malloch et al. [21]. Five microsatellite loci were examined: Sm10, Sm12, Sm17, SaΣ4, and S16b using published primer pair sequences [22, 23]. PCR was carried out in 8 μl volumes using Illustra^{TM} Ready to Go PCR beads (GE Healthcare). When the bead is reconstituted the concentration of each dNTP is 200 μM in 10 mM Tris HCl, 50 mM KCl and 1.5 mM $MgCl_2$. Each bead contains 2.5 units of Taq polymerase. PCR was carried out on a Techne 5 Prime /02 thermal cycler using the Touchdown programme described in Sloane et al. [24]. Genotyping was carried out on an ABI 3730 DNA analyser and the results interpreted using GeneMapper software. Genotypes were assigned using a reference data set for the SA12A and SA3 colonies held at the James Hutton Institute.

The aphid parasitoid *A*. *ervi*, was acquired as mummies (Fargro Ltd., West Sussex) and used immediately upon receipt.

Experimental setup

The experiment was conducted in Scotland's Rural College (SRUC) insectary (17 ± 3˚C; 65 ± 5% RH; LD 16:8 h, 150 μmol m^{-2} s^{-1}) at the King's Buildings campus at the University of Edinburgh between the 11th February 2019 and the 5th April 2019.

61 days after planting (at GS23), 24 barley plants were randomly assigned to eight mesh chambers (50 cm by 50 cm by 50 cm), with three plants per chamber. The kdr-SS and kdr-SR clones were randomly allocated to each chamber so that there were four chambers of each genotype. Each plant was inoculated with six apterous adult aphids. They were distributed evenly between the first and second longest tiller of the plant (three aphids per tiller).

Aphid performance traits

Tiller-level aphid abundance. Aphid counts were conducted twice a week for five weeks on the first and second longest tiller of each plant. All aphids from the base of the tiller to the ear were counted. This acted as a proxy of aphid population growth.

Aphid Relative Growth Rate (RGR). RGR was calculated for one aphid per plant. A clip cage [25] was placed over a healthy apterous adult aphid. After 24 h the clip cage was removed and the adult aphid and all but one of the nymphs were removed. The clip cage was then replaced over the nymph, and after a further 48 hours, it was weighed using a Mettler Toledo XP6 Analytical Balance (Mettler Toledo Ltd., Leicester, UK). After weighing, it was transferred back to the barley leaf and covered by the clip cage again. Exactly 72 h later, the nymph was reweighed, and RGR calculated using van Emden and Bashford's [26] formula:

$$\mathrm{RGR} = \frac{[\ln(\mathit{Final\ Weight}) - \ln(\mathit{Initial\ Weight})]}{[\mathit{Growth\ Period}\ (\mathit{days})]}$$

The RGR values were then averaged for each genotype to create a Mean Relative Growth Rate (MRGR). On the occasion that the aphid final weight was less than the initial weight, the data were discarded due to the assumption that the aphid had been damaged [27].

Parasitoid-aphid interactions. Aphid behaviour responses initiated by parasitoid wasps were observed under a binocular microscope. A 5 cm length of barley leaf with one apterous adult aphid attached was placed inside a Perspex Petri dish along with one female wasp. Following first physical contact between the aphid and wasp, aphid behavioural responses were recorded for one minute. First contact occurred when the parasitoid walked over the aphid, or touched it with its ovipositor or antennae [7]. During this time, the 'warding behaviour' recorded as the number of kicks and drops were counted. A kick was defined as the aphid moving its body vigorously whilst kicking its hind legs in the direction of the wasp [28]. A drop was recorded when the aphid did a short jump away from the feeding site and the wasp. This normally resulted in the aphid detaching itself from the leaf [29]. A new wasp, aphid, barley leaf and Perspex Petri dish was used for each observation to avoid pseudoreplication.

Mummification of *Sitobion avenae* clones. Thirty one days after aphids were placed on the experimental plants (92 days after planting), thirty-five *A*. *ervi* wasp mummies were placed into each of eight Perspex Petri dishes, one of which was added to the centre of each chamber. The emergent wasps were left in the chambers for 21 days to parasitise the aphids. Each barley plant was then harvested along with its aphid population, and the number of new mummies per plant counted, removed and placed into sealed Petri dishes. Each plant was then bagged and placed into a freezer (-20˚C) for three days. The number of aphids per plant was then counted and the proportion of mummified aphids to the total number of aphids calculated for each plant.

***Aphidius ervi* emergence success.** The Petri dishes containing the collected mummies remained in the insectary (conditions as detailed above) for seven days to allow the parasitoids to emerge freely, in accordance with development times determined by Ives *et al*. [18]. The number of hatched mummies was then counted. Each mummy was examined for an emergence hole, and the proportion hatched to unhatched represented parasitoid emergence success.

Data analysis

The influence of aphid clonal line on population size over time was explored using repeated measures ANOVA in the GenStat statistical package (19th edition, VSN International Ltd., Hemel Hempstead, UK). Aphid clonal line (kdr or non-kdr) was fitted as a fixed factor with two levels and with the numbers of aphids on each leaf at each observation time as the response variable. The identity of the cage in which observations were made was fitted as a random factor. Due to repeated observations being made on each leaf, the degrees of freedom were multiplied by the Greenhouse-Geisser epsilon correction factor before F probabilities were calculated.

All other statistical analyses were conducted using R version 3.5.1 statistical software [30]. All data were checked for normal distribution using a Shapiro-Wilk test. After this assumption

was met, a Bartlett test was carried out on categorical data to ensure there was equal variance across all samples (homoscedasticity). All the data also met this assumption.

A single-factor ANOVA was also used to determine differences between genotypes for MRGR, proportion of mummified aphids and emergence success. The interaction data was normally distributed count data. Therefore, a generalised linear model with Poisson distribution was used to assess the effect of genotype.

Graphs were made using Microsoft Excel 2016 or SigmaPlot 13.0.

Results

Tiller-level aphid abundance

Fig 1 shows that the kdr-SS clone had greater tiller-level abundance throughout the experiment than the kdr-SR clone. From day 7 onwards this was statistically significantly different. The kdr-SR clone increased, on average, by 3.2 aphids per day, whereas the kdr-SS clone increased by an average of 4.4 aphids per day. The significant difference in abundance between clones continued until day 28, by which point the kdr-SS abundance had reached a plateau of approximately 119 ± 2 individuals. The kdr-SR population was still increasing at the end of the 31 day period.

Mean relative growth rate

Fig 2 illustrates that the Mean Relative Growth Rate (MRGR) of individual kdr-SR aphids was significantly lower than that of individual kdr-SS clone ($F_{1,14} = 4.8$, $P < 0.0466$). Sample size varied between genotype due to aphid damage or mortality.

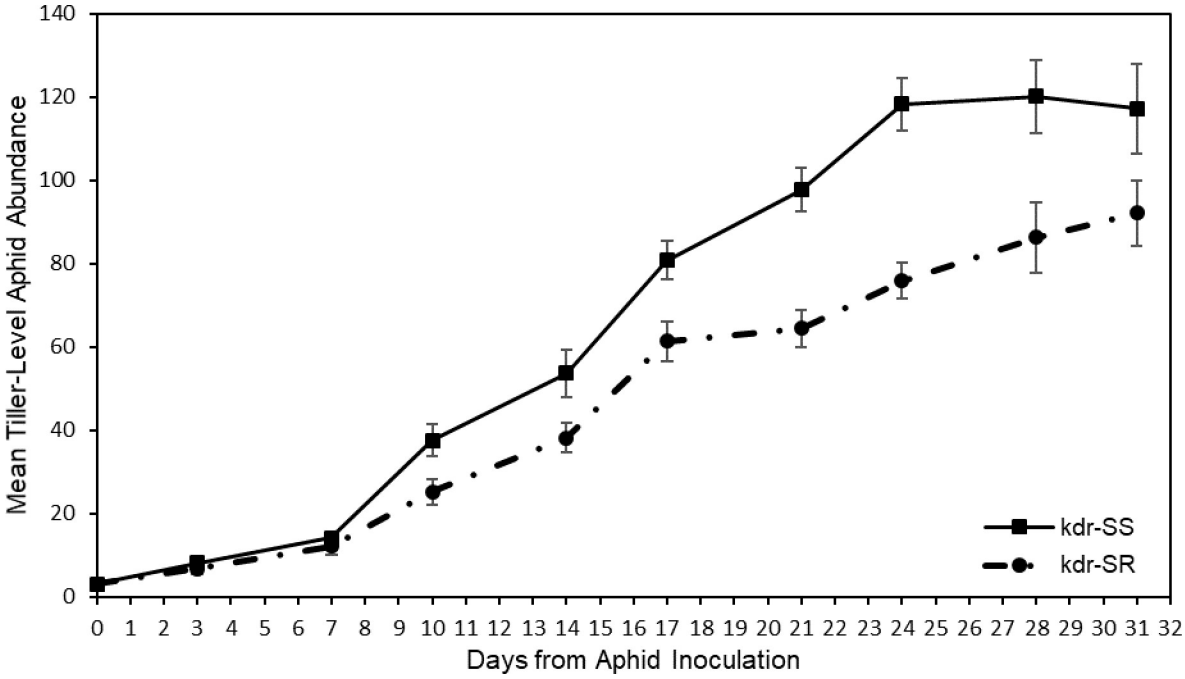

Fig 1. Mean tiller-level aphid abundance over time. Error bars represent ± SE for each category for each day of measurement. The number of aphids observed differed significantly between clonal lines ($F_{1,6} = 11.55$. P = 0.015, $r^2 = 0.089$, Greenhouse-Geiser epsilon = 0.1412) with the kdr-SS aphids being more numerous. From day 10 onwards one-way ANOVAs, undertaken for each day of measurement, indicated a significant difference ($p < 0.05$) between kdr-SS and kdr-SR aphids on each measurement day.

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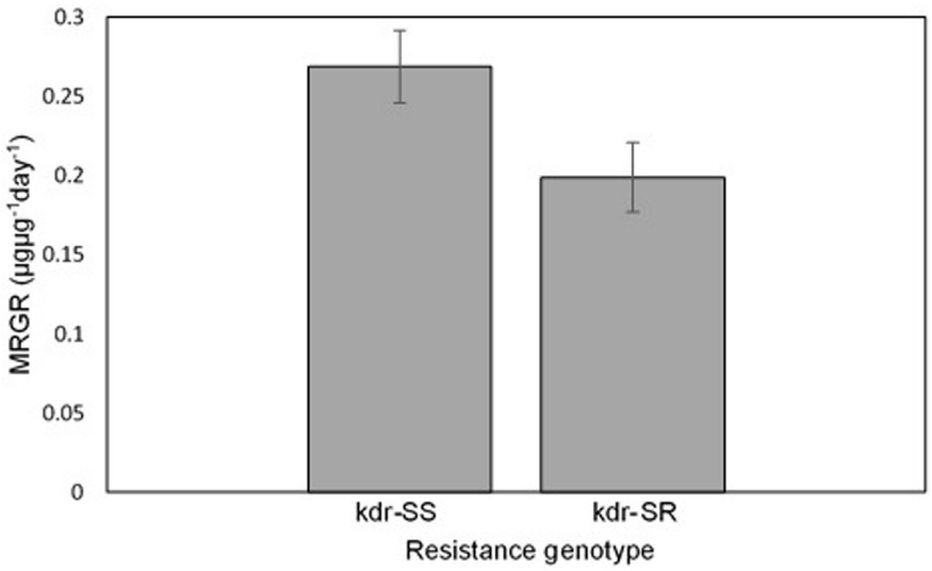

Fig 2. Mean Relative Growth Rate (MRGR) of the kdr-SS and kdr-SR clones. Error bars represent ± individual SE for each category. Aphids that were damaged were not included in the analysis. (kdr-SS $n = 7$, kdr-SR $n = 9$. $p < 0.05$).

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Parasitoid-aphid interaction

Fig 3A illustrates that there was no difference in the mean number of 'warding behaviours' between the kdr-SR and kdr-SS clones when attacked by a wasp. (GLM—z-value = 0.29, $df = 39$, $P < 0.77$). The mean number of kicks per minute was 10.5 ± 0.5 for both genotypes.

Mummification rate

Fig 3B illustrates the reduced proportion of mummified aphids of the kdr-SS clone compared with the kdr-SR aphids ($F_{1,6} = 6.04$, $P < 0.049$). The proportion of mummified kdr-SR aphids was 35% greater than that of the kdr-SS clone.

Aphidius ervi emergence success

Fig 3C illustrates that there was no significant difference in the emergence rate of kdr-SR parasitoids from aphid mummies in comparison with the kdr-SS clone ($F_{1,6} = 0.9$, $P < 0.38$).

Discussion

Throughout the course of the experiment, the pyrethroid resistant kdr-SR clone had a significantly lower tiller-level abundance than the pyrethroid susceptible kdr-SS clone. This may have been a consequence of the significantly reduced MRGR of the kdr-SR clone individuals compared with the kdr-SS clone. Reproductive rate is positively correlated with aphid size [31, 32] and evidence shows that larger *S. avenae* individuals have greater fecundity than smaller ones [33]. The lower population growth rate of the kdr-SR clone compared with the kdr-SS clone suggests either that the kdr-SR clones are less fecund than their counterparts and/or that they have increased mortality. A further possibility to explain the reduced kdr-SR abundance is also that they took longer to reproduce. Dixon and Wratten [34] showed that smaller aphids take longer to produce their progeny. In their study, by the tenth day of adult life, large apterous aphids had produced approximately 60% of their offspring, whereas small apterous aphids had only produced 44%. The lower MRGR of the kdr-SR individuals may therefore have led to

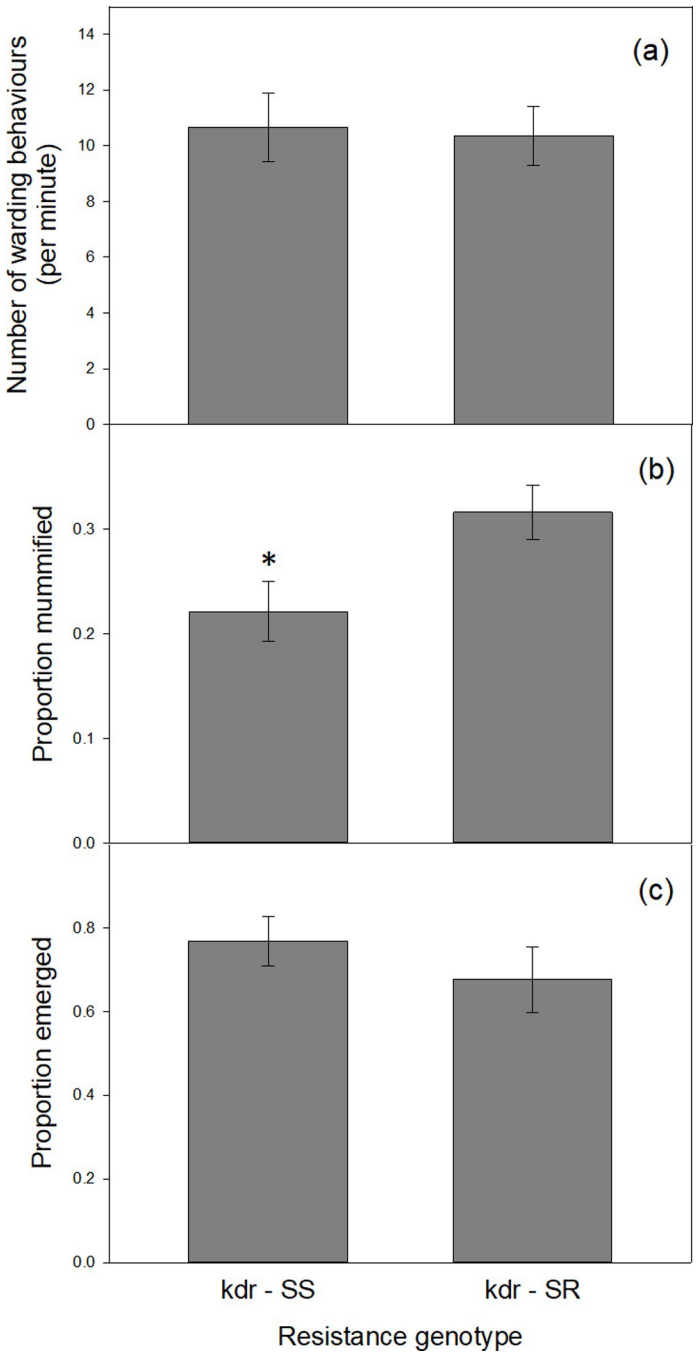

Fig 3. (a) Mean number of kicks/drops per minute carried out by the kdr-SR and kdr-SS clones when attacked by the parasitic wasp, *Aphidius ervi*. (b) The mean proportion of aphids mummified for both genotypes. Significantly more of the kdr-SR aphids were mummified compared with the kdr-SS aphids ($p < 0.05$). (c) The mean proportion of wasps that successfully emerged from aphid mummies for both genotypes. Error bars represent ± individual SE for each category.

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the increased time needed to produce all their progeny. This is supported by the observation that the kdr-SS population plateaued during the last week of the experiment, whereas the kdr-SR population was still increasing at the last count.

In addition to the slower population growth rate and reduced MRGR, the kdr-SR clone was also significantly more susceptible to parasitisation than the insecticide susceptible kdr-SS clone (31% vs 22% mummification rate, respectively). This is in agreement with Foster *et al*. [7] who showed that insecticide resistant peach potato aphids (*Myzus persicae*) also had a greater rate of mummification compared with their insecticide susceptible counterparts. Foster *et al*. [7] further went on to show that this was associated with reduced warding behaviour in the insecticide resistant clones. This study however failed to demonstrate a difference in the ability of the two clones to exhibit behaviours intended to repel parasitoid attack. Upon first contact with the parasitoid both clones exhibited kicking or dropping behaviour approximately 10 times per minute. The explanation for the increased mummification rate of the kdr-SR clone therefore cannot lie with reduced warding behaviour and may possibly be due to reduced effectiveness of warding behaviour by the smaller kdr-SR clones.

Contrary to our hypothesis there was no significant difference in parasitoid emergence success. The suitability of a host has been shown to affect parasitoid development [35] and smaller hosts are less likely to provide the nutritional quality needed for parasitoids to develop and emerge [36]. *Aphidius ervi* larvae require an intricate combination of endosymbionts and teratocytes provided by the host in order to grow exponentially within a mummy. Suboptimal teratocytes and endosymbionts provided by smaller hosts can drastically impair the physiology of parasitoid larvae [37]. The explanation for the unexpected lack of difference may lie with the relatively benign conditions found within the controlled environment, although this remains to be tested in a field situation.

The sudden appearance of the kdr mechanism in this SA3 *S*. *avenae* clone appears to be a case of 'forced evolution', in which the development of the insecticide-resistant gene has led to numerous inhibitory, pleiotropic costs. Adaptations that evolve over a long period of time are likely to be more successful than rapid forced evolution and may not appear with these significant trade-offs. It may be that the fitness trade-offs acting against pesticide resistance have been intensified [38] due to the rapid kdr-mutation. As the kdr-SR aphids performed significantly less well than the kdr-SS clone in three of the five behavioural and physiological performance traits measured in this experiment, it is likely that this is the case.

This study suggests there is further potential to incorporate parasitoids into pest management schemes. The increased rate of mummification has shown that parasitoids can exploit trade-offs in the insecticide resistant *S*. *avenae* clone which could possibly act to combat insecticide resistance. If the kdr-SR lineage acquires the ability to reproduce sexually, perhaps producing a kdr-RR genotype, it may exhibit an even greater level of immunological resistance. Should this be the case a strategy will be required to minimise the spread of this genotype and parasitoids would play a crucial part in this.

Supporting information

S1 Data.
(XLSX)

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Author Contributions

Conceptualization: Gail E. Jackson, Louise McNamara.

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Investigation: Gaynor Malloch.

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Project administration: Gail E. Jackson.

Resources: Gaynor Malloch.

Supervision: Gail E. Jackson.

Writing – original draft: Damon Little.

Writing – review & editing: Gail E. Jackson.

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