Larval antibiosis to cabbage stem flea beetle (*Psylliodes chrysocephala*) is absent within oilseed rape (*Brassica napus*)

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Abstract

BACKGROUND: Insect pests present a global threat to crops, with plant resistance representing a key breeding goal. The cabbage stem flea beetle (*Psylliodes chrysocephala*; CSFB) is the most damaging pest of oilseed rape (*Brassica napus*; OSR) within Europe; however, CSFB resistance is yet to be found within *B. napus*. To address this, we examine CSFB larval development over time, explore antibiosis across a diverse Brassica panel, and test whether larvae can develop within model Brassica relatives (*Brassica rapa* and *Arabidopsis thaliana*).

RESULTS: CSFB larvae completed development from four weeks post-infestation, undergoing a 20-fold size increase, with larval recovery after two weeks allowing semi-high throughput resistance phenotyping. Applying this method to 98 Brassica genotypes (97 *B. napus* and a single *Sinapis alba*), we found weak evidence for genotype effects on larval survival, however phenotype validation with 'resistant' and 'susceptible' *B. napus* genotypes showed no differences in larval survival or adult emergence. Larval antibiosis was consistently observed in *S. alba*. Finally, we showed that *B. rapa* and *A. thaliana* represent suitable hosts for CSFB, with larvae increasing 8-10× in size after two weeks.

CONCLUSION: CSFB larval antibiosis appears absent within *B. napus*, possibly due to bottlenecks experienced during domestication. However, larval antibiosis is present within *S. alba*, and future work should study the basis of this resistance. Further, CSFB larval screening in Brassica relatives presents an opportunity to explore CSFB resistance genetics, informing breeding progress for insect resistance in *B. napus*.

Keywords

Oilseed rape, cabbage stem flea beetle, Brassica, host plant resistance, integrated pest management, antibiosis, entomology

1. Introduction

Insect herbivory is one of the most common trophic interactions on earth and represents a major selection pressure for plant defence traits.[1–3] In food crops, selective breeding for high yield, quality traits, and uniform growth has reduced crop genetic diversity and altered plant-insect interactions,[4–6] such that modern crops are generally more insect-susceptible than their wild relatives.[7,8] In addition, agricultural ecosystems often comprise monocultures of crop genotypes, further increasing insect susceptibility.[9] Insect pests therefore represent a global threat to food production, causing yield losses of up to 40% a year.[10,11]

Historically, pesticides have limited pest impacts on crop yield and enhanced agricultural productivity.[12,13] However, the adverse environmental effects of pesticide use[14,15] coupled with the evolution of pesticide-resistant insect populations[16] mean more sustainable pest management approaches are required. Host plant resistance, defined as plant traits influencing insect-associated damage,[17] offers a promising strategy for pest management.[18–20] Plant resistance to insect herbivory can manifest through three non-exclusive pathways: (1) antixenosis, in which insects are deterred from host plant colonisation; (2) antibiosis, in which insects suffer reduced fitness on host plants; and (3) tolerance, in which plants compensate for insect damage through increased growth or reproduction.[17,20,21] A crucial first step in identifying host plant resistance requires phenotyping for resistance variation across plant species or genotypes, before using genetic mapping approaches to associate genes and traits with resistance.[19,20] However, the identification of insect resistance genes has proven challenging due to the polygenic nature of resistance and labour-intensive phenotyping methodologies.[20,22,23]

Oilseed rape (*Brassica napus*; OSR), an allopolyploid derived from the hybridisation of *Brassica rapa* and *Brassica oleracea*,[24] represents the second-largest oilseed crop by area grown globally and a key break crop in rotation systems.[25,26] An important breakthrough in OSR commercial success was the production of double-low genotypes,[24,27,28] plants yielding seeds with low levels of both erucic acid, a fatty acid with potential cardiotoxic effects,[29] and glucosinolates, a group of secondary metabolites involved in plant defence.[30] As a result, modern OSR genotypes are severely bottlenecked and express low levels of glucosinolates within tissues,[24,31–33] compromising disease and pest resistance. OSR is therefore susceptible to a range of pathogens and insects,[34,35] with insect resistance now representing a major objective for breeders.[22,36]

Within Europe, the cabbage stem flea beetle (*Psylliodes chrysocephala*; CSFB), a Brassica specialist,[37] represents the most damaging insect pest of OSR.[35] The CSFB life cycle is closely linked to the cropping cycle of winter OSR, with distinct damage symptoms associated with different life stages.[38] Adult CSFBs feed on emerging seedlings during early autumn, impacting crop establishment and reducing vigour.[38] Following adult reproduction, CSFB larvae mine OSR stems and petioles during autumn and winter, passing through three larval instars before leaving the plant to pupate in spring.[38] CSFB larvae are the more economically damaging life-stage, with mining leading to stunted growth, frost and pathogen susceptibility, and plant mortality, causing severe yield penalties.[39–42]

CSFB control within OSR is heavily pesticide reliant. Previously, management involved the use of neonicotinoid seed treatments that provided systemic protection throughout the growth cycle.[43,44] However, concerns over the environmental effects of neonicotinoids led to their 2013 withdrawal within the European Union.[45,46] In the absence of neonicotinoids, growers have become increasingly reliant on pyrethroid insecticides,[43,44] driving the evolution of pyrethroid resistance within European CSFB populations.[47–49] Limited control options have therefore led to increased CSFB abundance and subsequent reductions in OSR growth.[44,50,51] In the UK, a ten-fold increase in CSFB larval loads[50] has been accompanied by a 67% reduction in OSR land use[52] (from 621,000ha in 2014 to 204,000ha in 2025) following the neonicotinoid ban. Given policies aimed at reducing pesticide use,[53] the development of CSFB-resistant OSR genotypes represents a key target for the European OSR industry.[22,36]

Attempts to phenotype Brassica diversity for CSFB resistance have been extremely limited to date, with just a single study comparing larval resistance between *B. napus* and four relatives (*B. rapa*, *B. oleracea*, *Sinapis alba*, and *Raphanus sativa*).[54] This is mirrored for other insect pests of *B. napus* (reviewed in Hervé[22] and Obermeier[36]), reflecting the challenging nature of exploring insect resistance. Despite phenotyping relatively small Brassica collections, these studies have highlighted a lack of insect resistance across *B. napus* genotypes, partial resistance in some diploid relatives (*B. rapa* and *B. oleracea*) and resynthesised *B. napus* genotypes, and resistance and/or tolerance in more distantly related Brassica species.[22,36] CSFB larval antibiosis has been shown within *Sinapis alba*, with larvae experiencing lower survival and slower development, while such effects were lacking across four tested *B. napus* genotypes.[54] However, whether CSFB larval antibiosis exists across a broader diversity of *B. napus* remains to be seen.

In the present study, we aimed to explore CSFB larval antibiosis (defined as reduced survival and development) across a diverse *B. napus* population, with the goal of identifying resistant genotypes that may provide trait and gene discovery for future breeding efforts. We outline a method for CSFB larval screening[55] and investigate larval development under laboratory conditions, with our results informing a semi-high throughput approach for phenotyping CSFB larval antibiosis across 98 Brassica genotypes (97 *B. napus* and a single *S. alba*). Importantly, this represents the largest *B. napus* collection phenotyped for insect resistance to date, containing a range of diverse morphotypes (OSR, swedes, and fodders). Given the diversity of other traits within this collection,[56–58] we hypothesised that larval antibiosis would exist within some *B. napus* genotypes, providing a foundation for association mapping of genes involved in CSFB resistance. Finally, to broaden the genetic resources available for CSFB-Brassica interactions, we applied CSFB larval screening to two model *B. napus* relatives, *B. rapa* and *Arabidopsis thaliana*.

2. Materials and Methods

2.1. Cabbage stem flea beetle rearing

We established laboratory CSFB populations by rearing larvae from OSR (KWS Barbados) collected at the John Innes Centre Field Station (Bawburgh, Norfolk, UK) in the winters of 2021/2022 and 2022/23. Larvae were recovered from OSR plants using the Berlese method[59] and placed onto pak choi (*B. rapa pekinensis* Hanakan, Suttons Seeds, Paignton, UK) to complete development. On emergence, adult beetles were kept in populations of up to 70 individuals inside ventilated boxes (L17×W12×H6cm) lined with moist tissue and fed Chinese cabbage (*B. rapa pekinensis* F1 Hilton, Suttons Seeds) *ad libitum*. Fresh cabbage was provided every 7-12 days, during which dead adults were removed, and eggs were collected. Collected eggs were placed on pak choi to undergo development, with newly emerged adults used to setup new populations, thereby completing the CSFB lifecycle under laboratory conditions. All rearing steps were carried out under constant conditions (22±1°C, ~60% RH, L16:D8). Captive CSFB populations would have cycled through approximately 3-9 generations when the below experiments were carried out, with no further addition of field-collected individuals since winter 2022/23.

2.2. Larval development time course

To understand CSFB larval development under laboratory conditions, we conducted a time course experiment across four timepoints (1-4 weeks post-larval infestation) in a single *B.*

napus genotype (Apex-93_5×Ginyou_3 DH; Warwick Gene Bank accession = 12841-mc-6). Seeds were sown into Levington Advance F2 compost (ICL, Amsterdam, NL) and ten days post-sowing, 20 seedlings were individually repotted into 9cm pots containing JIC Cereal Mix (65% peat, 25% loam, 10% grit, 3kg/m^3 dolomitic limestone, 1.3kg/m^3 PG Mix, 3kg/m^3 osmocote exact), assigned to a timepoint, and randomised (**Figure S1**). Plants were grown under constant conditions (18±1°C, ~60% RH, L10:D14) and watered regularly. The same growth procedure and conditions were used for all other experiments conducted.

Larval infestation began four-weeks post-sowing, when plants possessed 5-6 true leaves (mean = 5.3). Infestation was based on Döring & Ulber[54] and Coston *et al.*[41], with 12, <24 hour-old CSFB larvae added to each plant over a four-day period (i.e. three larvae per day),[55] reflecting field-realistic UK infestation levels expected to lower harvest yield.[41,50,59] Adding larvae across four-days allows sufficient larvae to hatch, is field-relevant (whereby larvae enter plants across multiple months),[40] and ensures plants are not overly stressed from multiple larvae entering concurrently.

To stimulate hatching, approximately 1000 CSFB eggs were placed at constant conditions (22±1°C, ~60% RH, L16:D8) 1-4 days prior to infestation. Each infestation day, three <24 hour-old larvae were transferred to the base of each plant (**Figure 1A**) and observed for 10 seconds to ensure movement. Any larvae that did not move were removed and replaced with a new larva. Each plant was sealed within a microperforated bag (L70×W38cm), ensuring that larvae could not move between plants. To account for circadian effects on plant defence compounds,[60] larval infestation was carried out at the same time every day, with plants infested in a different order each day. On the final infestation day, plants were checked for successful larval penetration, defined as entry holes and mines visible on the stem or petioles (**Figure 1A**) and/or visible larvae under the plant epidermis.

Five infested plants were harvested and weighed at each timepoint, with the leaves, petioles and stem dissected to recover larvae. Location and instar of all live larvae within each plant were recorded, with recovered larvae stored in 70% ethanol until imaging. Dead larvae found during dissection were also recorded. Twenty <24 hour-old larvae were also collected for imaging. All collected larvae were photographed (**Figure 1E**; **Figure S2**), and the area of each larva was measured using ImageJ.[61] Occasionally, larvae were damaged during dissection and therefore unavailable for measurement. Hence, of the 131 live larvae recovered during dissection, 126 were measured.

2.3. Diversity screening for larval antibiosis

To explore variation in CSFB larval antibiosis across *B. napus*, we selected 96 genetically fixed genotypes from the Renewable Industrial Products from Rapeseed[56] diversity panel (**Table S1**) for larval phenotyping. Genotypes were randomised across a temporal 12-block alpha design (plots per block (k) = 24, replicates per genotype (r) = 3, blocks per replicate (s) = 4) and grown across two CER shelves, with a new block beginning every 1-9 weeks (**Table S2**; **Figure S3**). Each block also contained a negative *B. napus* control (KWS Campus) and a positive *Sinapis alba* control (Elsoms G1), with CSFB larval antibiosis having been previously demonstrated in *S. alba*.[54] In a single block (B8), two genotypes (57 and 72) died before infestation and were therefore regrown in later blocks (**Table S2**; **Figure S3**). Hence, each block contained 24-27 plants, giving a total of three replicates for all 96 *B. napus* genotypes and 12 replicates for both control genotypes.

Larval infestation[55] was performed at four-weeks post-sowing, when plants possessed 1-9 true leaves (mean = 4.32). Our larval development experiment showed highest larval recovery two-weeks post-infestation, with larvae increasing eight-fold in size (**Figure 1B-C**). Hence, two weeks post-infestation, all plants were harvested and weighed, with larvae recovered using the Berlese method[59] at 22±1°C for up to 17 days. Larvae damaged during collection were unavailable for imaging; therefore, of the 2,229 larvae recovered, 2,201 were measured. Larval antibiosis was defined from both the number of larvae recovered (i.e. larval survival) and the size of recovered larvae (i.e. larval development).

2.4. Phenotype validation

Our larval diversity screen identified potential variation in CSFB larval antibiosis across genotypes (**Figure 2A**). Therefore, to confirm the larval antibiosis variation, we selected six genotypes (three 'resistant' – 33, 61 and 67 – and three 'susceptible' – 14, 84 and 89; **Figure 2A**) for phenotype validation, along with the two control genotypes used in the diversity screen. A total of nine replicates per genotype were phenotyped across three temporal blocks (i.e. three genotype replicates per block, giving 24 plants per block and 72 plants overall). Plants were grown in randomised blocks across two CER shelves, with a new block starting every 1-3 weeks (**Figure S4**). Larval infestation[55] was performed three weeks post-sowing, when plants possessed 2-6 true leaves (mean = 3.88). Two weeks post-infestation, plants were harvested, weighed, and larvae were collected using the Berlese method,[59] with all recovered larvae (n = 561) imaged and measured.

To link larval recovery to adult emergence, we also explored adult emergence across the selected genotypes. Patterns of larval recovery between the control genotypes were similar across the diversity screen and phenotype validation experiment (**Figure S5**); therefore, to increase throughput, the adult emergence experiment only contained the six genotypes of interest. A total of 10 replicates for each genotype were phenotyped across two temporal blocks (i.e. five genotype replicates per block, giving 30 plants per block and 60 plants overall). Plants were grown in randomised blocks across two CER shelves, with a new block starting each week (**Figure S6**). Larval infestation[55] was carried out four weeks post-sowing, when plants possessed 3-6 true leaves (mean = 4.25), and larvae were left to develop until adult emergence.

Based on results from the larval development experiment (**Figure 1B-D**), we stopped watering plants at six weeks post-infestation (since all larvae should have left the plants to pupate in the soil) and began checking for adult emergence every 2-7 days. Seven weeks post-infestation (when adults were first observed), plant vegetation was disposed, allowing easier collection of adults. At each check, collected adults were counted and removed using an insect aspirator, with the experiment terminated when no new adults had emerged for two weeks. Hence, this experiment provided two extra measures of larval antibiosis: total adult emergence (i.e. the number of larvae that successfully completed development) and time to first adult emergence (i.e. larval development time). A single plant (61.10) died between infestation and adult collection and so was excluded from final analyses.

A full protocol for CSFB larval resistance phenotyping can be found in Brock & Wells.[55]

2.5. Larval screening in Brassica relatives

Brassica napus relatives may offer novel insights into CSFB resistance.[22,54] Hence, we assessed whether CSFB larval screening worked in *B. rapa* and *A. thaliana*.

Brassica rapa larval screening was carried out using the R-o-18 genotype.[62] Plants were grown as per *B. napus*, with a total of 15 plants grown for larval screening. Infestation[55] was carried out at three-weeks post-sowing (**Figure 4A**) when plants possessed 4-5 true leaves (mean = 4.20). Post-infestation, a single plant died, leaving 14 plants for larval screening. Two weeks post-infestation, plants were harvested and larvae were recovered using the Berlese method.[59]

Arabidopsis thaliana larval screening was carried out using the Ws-0 genotype. Seeds were sown into Levington Advance F2 and stratified at 4°C for one week, before being transferred to the same growth conditions used for *B. napus*. Three weeks post-stratification, 15 *A. thaliana* plants were repotted into 5cm pots, and left to grow for a further week. Larval infestation was

carried out four weeks post-stratification, when rosette stage plants possessed 11-18 true leaves (mean = 14.13). Approximately 100 CSFB eggs were setup two days before larval infestation. Plants were infested by introducing a single <24 hour-old larva to the centre of the rosette (**Figure 4C**), with the introduced larva observed for 10 seconds to ensure movement. Plants were enclosed within sealed perforated bags (L30×W15cm), ensuring larvae could not move between plants. One-week post-infestation, plants were checked for successful larval infestation, defined as visible larval entry holes and/or mines on the petioles (**Figure 4D**). Two-weeks post-infestation, plants were harvested and larvae were collected using the Berlese method.[59]

Recovered larvae (*B. rapa n* = 137; *A. thaliana n* = 9) and <24 hour-old larvae (*B. rapa n* = 12; *A. thaliana n* = 15) from both experiments were imaged and measured as before.

2.5. Statistical analyses

All analyses were conducted using R v4.3.2 in RStudio v2023.09.01.[63,64] A mix of linear and linear mixed models, respectively fitted using the 'stats' and 'lmerTest' packages,[65] were used to conduct statistical analyses and the 'ggplot2' package was used to create plots.[66] Linear model effects were assessed using the *Anova()* function (type II) from the 'car' package,[67] while linear mixed model fixed effects were assessed using the *anova()* function (type II, Kenward-Roger denominator degrees of freedom) from the 'lmerTest' package.[65] Maximal models comprising the response variable and all possible effects were initially fitted before non-significant effects were sequentially dropped to produce a final model. Formulas for each fitted model and associated sample sizes can be found in **Table S3**. Justification of effects included and retained within models can be found in the **Supplementary Methods**. Estimated marginal means (EMMs) for genotypes and Tukey post hoc comparisons of main effects of interest within final models were performed using the 'emmeans' package.[68] Unless otherwise stated, values in the results section represent the raw mean ± standard error. All data and code required to recreate analysis are available from Zenodo.[69]

3. Results

3.1. Larval development occurs across four weeks under laboratory conditions

All infested plants (*n* = 20) showed CSFB larval herbivory symptoms on the last day of infestation (**Figure 1A**). Live larval recovery was significantly influenced by both plant weight at dissection ($F_{(1, 15)} = 5.16$, $p = 0.04$) and dissection timepoint ($F_{(3, 15)} = 17.07$, $p < 0.001$), with consistent larval recovery for the first three weeks (1W: 8.00±0.32; 2W: 8.20±0.49; 3W:

7.00±0.63) before a sharp decrease to 3.00±0.95 at four weeks post-infestation (**Figure 1B**). A similar pattern was also observed when including both live and dead larvae recovered during dissections (1W: 10.80±0.58; 2W: 9.80±0.37; 3W: 8.00±0.32; 4W: 4.00±1.14; **Figure S7**).

Larvae significantly increased in size over time ($F_{(4, 12.95)} = 134.65$, $p < 0.001$), undergoing a ~20.5× size increase from 0.44±0.02mm^2 at hatching to 9.05±0.27mm^2 at four weeks post-infestation (**Figures 1C & 1E**). Instar composition also changed significantly over time ($F_{(3, 16)} = 368.51$, $p < 0.001$), with an L1 majority (90.56±5.80%) at one week, an L2 majority (87.62±5.78%) at two weeks, and an L3 majority at three (87.50±6.85%) and four weeks (100%) post-infestation (**Figure 1D**). Larval size significantly increased with instar ($F_{(2, 118.08)} = 63.19$, $p < 0.001$), from 1.01±0.06mm^2 at L1, to 3.46±0.14mm^2 at L2, and 7.78±0.23mm^2 at L3 (**Figure S8**). Taken together, these results suggest that larvae complete development and leave the plant to begin pupation from four weeks post-infestation under our laboratory conditions.

Plant dissections also revealed changes in larval location over time (**Figure S9**). One-week post-infestation, larvae were distributed within the main stem, cotyledons, and true leaf petioles. However, 2-4 weeks post-infestation, larvae were rarely found within the stem, cotyledons, or petioles towards the plant apex, and were instead distributed within the mid-petioles (**Figure S9**). Petiole sharing (>1 larvae per petiole) was also frequently observed (**Figure S10**). Of the 223 petioles dissected across all timepoints, 73 (32.74%) contained larvae and, of the infested petioles, 31 (42.47%) contained two or more larvae, with five larvae sharing a single petiole on one occasion (**Figure S10**).

3.2. Diversity screening identifies potential larval antibiosis in *B. napus*

All infested plants ($n = 312$) showed CSFB larval herbivory symptoms on the final infestation day. Along with plant weight at evacuation ($F_{(1, 144.44)} = 6.72$, $p = 0.01$), larval survival was significantly influenced by genotype ($F_{(97, 194.60)} = 1.83$, $p < 0.001$; **Table 1**), with lowest survival in *S. alba* G1 and highest survival in *B. napus* 84 (**Figure 2A**; **Figure S11A**). Importantly, larval survival was significantly lower ($t_{(1, 202.24)} = -5.33$, $p < 0.001$) in the *S. alba* positive control compared to the negative control (**Figure S12A**; **Figure S13**), demonstrating expected differences in CSFB larval survival between *S. alba* and *B. napus*.[54] Excluding *S. alba*, EMM (95% CI) larval survival ranged from 4.24 (2.18-6.31) to 10.40 (8.34-12.47) across the 97 *B. napus* genotypes (**Figure 2A**), suggesting that genetic variation for CSFB larval antibiosis may exist within *B. napus*. Using our larval survival distribution (**Figure 2A**), we assigned three genotypes as resistant (EMM (95% CI) larval survival: **67** = 4.24 (2.18-6.31); **61** = 4.40 (2.33-6.47); **33** = 4.62 (2.55-6.68)) and three as susceptible (EMM (95% CI) larval survival: **14** = 9.35 (7.28-11.41); **89** =

9.97 (7.91-12.03); **84** = 10.40 (8.34-12.47)). Importantly, larval survival was not significantly different between the three resistant genotypes and *S. alba*, but larval survival was significantly lower in the three resistant genotypes compared to the three susceptible genotypes (**Figure S13**).

Larval size was not influenced by plant weight or larval density within plants (**Table S3**) but did vary significantly by genotype ($F_{(97, 170.64)} = 1.73$, $p < 0.001$; **Table 1**), with smallest larvae in *B. napus* 67 and largest larvae in *B. napus* 39 (**Figure 2B**; **Figure S11B**). There was no significant difference in larval size between control genotypes (**Figure S12B**) nor between any of the resistant and susceptible genotypes identified by larval survival (**Figure S14**). Using per genotype raw means, we found a weak correlation between larval survival and larval size ($F_{(1, 96)} = 4.11$, adjusted $R^2 = 0.03$; $p = 0.05$; **Figure S15A**), suggesting that larvae may develop faster within genotypes in which survival is higher. However, this correlation was not present when using per genotype EMMs ($F_{(1, 96)} = 4.11$, adjusted $R^2 = 0.02$; $p = 0.09$; **Figure S15B**). Raw and estimated means for larval survival and size across all genotypes can be found in **Tables S4-S7**.

3.3. Phenotype validation for resistance did not confirm the presence of antibiosis

All infested plants in the phenotype validation experiment ($n = 72$) showed CSFB larval herbivory symptoms. Larval survival was again influenced by both plant weight at evacuation ($F_{(1, 58.85)} = 6.02$, $p = 0.02$) and genotype ($F_{(7, 59.39)} = 17.77$, $p < 0.001$; **Table 2**), with lowest survival in *S. alba* G1 and highest survival in *B. napus* 84 (**Figure 3A**). However, no significant differences in larval survival were found across any of the initially identified resistant and susceptible genotypes (**Figure 3A**), with the plant genotype effect driven entirely by lower larval survival within *S. alba* compared to *B. napus*.

There was also a significant effect of genotype on larval size ($F_{(7, 59.39)} = 17.77$, $p < 0.001$; **Table 2**), with smallest larvae in *S. alba* G1 and largest larvae in *B. napus* 89 (**Figure 3B**). Unlike the diversity screen, larvae were significantly smaller ($t_{(1, 94.89)} = -3.73$, $p < 0.01$) in the *S. alba* positive control compared to the negative control (**Figure 3B**), corroborating previous observations of slower larval development in *S. alba*.[54] Interestingly, we also observed significant differences in larval size between *B. napus* genotypes, with genotypes 84 and 61 hosting smaller larvae than genotype 89 (**84 – 89**: $t_{(1, 51.59)} = -3.90$, $p < 0.01$; **61 – 89**: $t_{(1, 56.36)} = -3.81$, $p < 0.01$) and no larval size differences between *S. alba* and *B. napus* genotypes 84, 61, and 67 (**Figure 3B**).

All infested plants in the adult emergence ($n = 60$) experiment showed larval herbivory symptoms. A mean of 6.36±0.30 adult beetles successfully emerged across all plants

(equivalent to a 53% larvae-to-adult success rate; **Figure 3C**), with first adult emergence beginning at 8.31±0.08 weeks post-infestation (**Figure 3D**). Consistent with the phenotype validation experiment, there was no significant effect of *B. napus* genotype on either total adult emergence ($F_{(5, 52)} = 1.08$, $p = 0.38$) or weeks to first adult emergence ($F_{(5, 52)} = 1.26$, $p = 0.29$; **Table 2**; **Figure 3C-D**). Per genotype raw means for all response variables measured for both experiments can be found in **Tables S8-S11**.

3.4. Larval screening can be successfully performed in model species

All 15 infested *B. rapa* plants showed larval herbivory symptoms on the final day of infestation (**Figure 4A**). Two-weeks post-infestation, a mean of 9.79±0.30 larvae were recovered from the 14 surviving plants (**Figure 4B**). Larvae significantly increased in size in *B. rapa* ($t_{(1, 11.73)} = 5.19$, $p < 0.001$), undergoing a ~10.5× size increase from 0.45±0.01mm^2 at hatching to 4.71±0.13mm^2 as recovered larvae (**Figure 4C**).

One-week post-infestation, 14 of the 15 infested *A. thaliana* plants (93.33%) presented larval herbivory symptoms (**Figure 4E**) and, two-weeks post-infestation, larvae were recovered from nine plants (60%; **Figure 4F**). Larvae significantly increased in size ($t_{(1, 7.99)} = 2.28$, $p = 0.05$), developing ~8.5× in size from 0.50±0.01mm^2 at hatching to 4.25±0.52mm^2 as recovered larvae (**Figure 4F**).

4. Discussion

CSFB larval herbivory imposes severe yield penalties on OSR growth, with larval antibiosis representing a key breeding target. Despite this, the challenging nature of insect resistance phenotyping has limited attempts to explore CSFB resistance.[54] Our research aimed to address this by producing a laboratory-based method for controlled larval antibiosis screening,[55] before: (1) tracking larval development over time within *B. napus*; (2) exploring larval antibiosis across a large Brassica population containing 97 *B. napus* genotypes and a single *S. alba*; and (3) testing larval screening in two further model *B. napus* relatives – *B. rapa* and *A. thaliana*. We showed that, under our growth conditions, larvae complete development from four weeks post-infestation, with larval recovery at two weeks post-infestation providing a faster way of scoring larval antibiosis (defined as reduced survival and development). Larval screening of the Brassica population revealed limited evidence for resistance within *B. napus* but showed consistent support for larval antibiosis in *S. alba*. Finally, larvae were also able to develop in both *B. rapa* and *A. thaliana*, broadening the genetic resources available to understand CSFB-Brassica interactions.

4.1. CSFB larval development

Under controlled conditions, CSFB larvae completed development within *B. napus* from four weeks post-infestation, as evidenced by a sharp decrease in larval recovery at the four-week timepoint, coupled with a 20× size increase and progression through three instars (**Figure 1**). Given that we did not search the soil for pupae at four weeks, an alternative explanation may be that larval death occurred at this time. However, given that later instars are more robust,[41,70] and that adults were first collected at seven weeks post-infestation in our adult emergence experiment (**Figure 3D**), this seems unlikely. Importantly, larval recovery remained high at two-weeks, with larvae significantly increasing in size. Hence, recovery at two-weeks post-infestation provides a faster method to phenotype larval antibiosis across Brassica populations.

Despite the controlled nature of larval infestation, larval size was highly variable within plants. There are several interacting reasons that may explain this. Firstly, larvae were introduced to plants over the course of four days. Hence, the first introduced larvae infested plants with no prior herbivory experience, while the final introduced larvae entered plants that had likely mounted herbivory-triggered defence responses,[71] possibly slowing development. Indeed, glucosinolate abundance is altered in Brassica species following CSFB larval herbivory but effects on larval development are unclear.[54] Further studies on induced responses to CSFB larval herbivory at both the transcriptome- and metabolome-level may therefore provide a better understanding of plant defence strategies. Secondly, larval development may be influenced by feeding location within a plant, given variable distribution of plant defence compounds across tissues.[72,73] We found that larvae generally fed in the stem and low- to mid-petioles, avoiding petioles towards the plant apex, where defence compounds are likely more abundant.[73] Such patterns have previously been shown in the generalist pest *Spodoptera littoralis*[72] but whether the same effects exist for Brassica specialists such as CSFB is unknown. Finally, larval density within plants may also influence development, with density-dependence influencing key life-history traits in insects.[74,75] Interestingly, we found that larvae frequently shared petioles despite there being many other non-infested petioles to feed in. Given that CSFB larvae feed internally and movement between plants is probably rare, there are likely adverse fitness costs to developing under high densities and strong selection on females to oviposit on lower density plants. Pre-diapause CSFB females can actively respond to seedling volatiles,[76] but the volatile profiles of infested plants and whether females respond to these cues remains to be seen. Density-dependent effects on CSFB oviposition, larval development, and life-history presents an interesting avenue for future research.

4.2. Absence of CSFB larval antibiosis across *Brassica napus*

Our initial larval diversity screen identified weak genotype effects on larval survival across the *B. napus* population (**Figure 2**; **Table 1**). However, in follow-up experiments with larger per-genotype sample sizes, differences between initially identified 'resistant' and 'susceptible' genotypes were no longer present, with similar larval survival, larval size, adult emergence, and emergence timing found across genotypes (**Figure 3**; **Table 2**). Importantly, the screened *B. napus* population shows variability across other traits.[56–58] Hence, if CSFB larval antibiosis did exist within this panel we should have been able to identify it. This is a crucial finding for the field, since researchers and breeders alike invest substantial resources trying to identify CSFB resistance under field conditions. However, if we cannot find larval antibiosis across a large population with highly controlled phenotyping approaches, it seems unlikely that field trials will provide any promising candidates. A lack of CSFB larval antibiosis in *B. napus* was also shown in Döring & Ulber,[54] albeit within a much smaller population (four genotypes versus 97 in the present study). Further, *B. napus* appears to lack resistance to a broad range of insect pests,[22,36] presumably due to genetic bottlenecking and altered defence traits experienced during domestication.[24,31–33] However, a thorough comparative investigation between *B. napus* and insect-resistant relatives would be required to confirm this.

Our study prioritised larval survival over larval size as a measure of larval antibiosis. This was because our phenotyping method involved larval recovery using the Berlese method.[59] As a result, we were able to screen large numbers of plants at the expense of accurate larval size, given larvae can continue developing within plants before recovery.[59] We are unable to distinguish between larval death within the plant versus larvae failing to penetrate the plant. However, given that all infested plants showed signs of larval herbivory symptoms on the final day, and desiccated larvae were never found on the plant exterior, we are confident that larval survival represents mortality experienced within the plant. Larval antibiosis may take place later than two weeks post-infestation but, given that antibiosis within *S. alba* was consistently observed at two-weeks, we deem this unlikely.

In the present study, we only assessed the presence of larval antibiosis across *B. napus* genotypes. However, alternative measures of CSFB resistance such as antixenosis and tolerance[17,20,21] across *B. napus* diversity remain untested. For instance, differences in cabbage butterfly (*Pieris brassicae* and *P. rapae*) oviposition preference have been shown across Brassica plants.[77] Oviposition preferences for CSFB are currently unknown; however, oviposition rate may depend on the quality of the host plant and availability of digestible carbohydrates.[78]

Further, tolerance to low larval loads (infestation of less than five larvae per plant) has been demonstrated in a single *B. napus* genotype, with yield declines observed with increasing larval density.[41] However, per-genotype relationships between larval density and plant yield are unexplored. Hence, while larval antibiosis is unlikely to exist with *B. napus*, alternative mechanisms of plant resistance may yet be discovered.

4.3. Brassica relatives can inform CSFB larval resistance

Sinapis alba, a relative of *B. napus*, consistently demonstrated CSFB larval antibiosis, with lower larval survival and smaller larvae compared to the screened *B. napus* genotypes. Promisingly, CSFB larval antibiosis has also been reported in a separate *S. alba* genotype,[54] suggesting that larval resistance may be a shared trait across *S. alba* diversity. Future work should look to confirm whether larval antibiosis is the rule within *S. alba* by applying our screening methodology[55] to a genetically diverse population. Additionally, since we only measured larval survival and size at two-weeks post-infestation, it would be interesting to see if adults can successfully emerge from *S. alba* and whether adults suffer any adverse effects, such as reduced fecundity or lifespan.[79] *Sinapis alba* shows resistance to a range of other insect pests,[80–82] and, in some cases, this resistance has been successfully tracked to QTLs and introgressed into OSR.[83,84] For instance, a mapping population derived from a resistant × susceptible *S. alba* cross allowed the identification of a QTL explaining 10% of heritable variation associated with cabbage seedpod weevil (*C. obstrictus*) resistance.[83] Hence, identifying the metabolic and genetic factors associated with CSFB resistance in *S. alba* and introgressing these traits into *B. napus* may represent a key strategy in breeding CSFB-resistant OSR.

In the present study, we showed that CSFB larvae can successfully infest and develop within both *B. rapa* R-o-18 and *A. thaliana* Ws-0 (**Figure 4**). Alongside *S. alba*, both *B. rapa* and *A. thaliana* may also provide insights into CSFB resistance. As the progenitors of *B. napus*, genetic diversity is far higher in both *B. rapa* and *B. oleracea*,[24] and both species may contain sources of CSFB resistance. An interesting approach could be to screen populations of both species for CSFB resistance before performing crosses to resynthesise 'ultra-resistant' and 'ultra-susceptible' *B. napus* genotypes. Such approaches have previously been applied to resynthesise *B. napus* with resistance to the fungal pathogen *Verticillium longisporum*,[85,86] while resynthesised *B. napus* has also shown increased resistance to rape stem weevil (*C. napi*).[87] In addition, *B. rapa* R-o-18 offers a selection of TILLING mutants,[62] providing a rich resource for testing gene function in relation to larval resistance.

CSFB larval screening in *Arabidopsis* also offers a promising alternative to identify resistance genes. Alongside the huge genetic diversity available in *Arabidopsis*, larval screening could be far higher throughput than in *Brassica* species, given the requirement for fewer larvae (one larva per plant in *Arabidopsis* versus 12 in *Brassica*), fewer infestation days (one day in *Arabidopsis* versus four in *Brassica*), and less space. Plant-insect association studies across diverse *Arabidopsis* populations have identified candidate genes for cabbage butterfly (*P. brassicae*) oviposition,[88] cabbage whitefly (*Aleyrodes proletella*) survival and fecundity,[89] and diamondback moth (*Plutella xylostella*) larval herbivory.[90] Such approaches could therefore be used to identify CSFB resistance candidates in *Arabidopsis* before searching for homologs within *Brassica* genomes. Further, the rich diversity of knock-out and over-expression lines in *Arabidopsis* offers a convenient method for testing CSFB resistance gene function.

4.4 Conclusions

We outline a method for highly controlled CSFB larval screening within Brassica and, by applying this method to a diverse collection of Brassica genotypes, show limited evidence for larval antibiosis within *B. napus* but consistent antibiosis in white mustard (*S. alba*). These findings highlight the need for researchers and breeders to look beyond *B. napus* genetic diversity to uncover genes involved in CSFB resistance, with Brassica relatives representing a promising resource. Additionally, the suitability of *B. rapa* and *A. thaliana* for larval screening provides further genetic diversity to mine for CSFB resistance, along with the possibility of candidate gene testing. Finally, beyond the use of these methods for gene and trait discovery in OSR breeding, we also suggest that the CSFB-Brassica system could provide fundamental insights into plant-insect interactions for insects with larval mining strategies.

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6. Author contributions

Ryan E. Brock: conceptualisation (supporting); methodology (lead); investigation (lead); data curation (lead); formal analysis (lead); visualisation (lead); funding acquisition (supporting); supervision (supporting); writing – original draft (lead); writing – review and editing (equal).

Clara Courtney: methodology (supporting); investigation (supporting); writing – review and editing (supporting).

Steven Penfield: conceptualisation (supporting); funding acquisition (supporting); supervision (supporting); writing – review and editing (supporting).

Rachel Wells: conceptualisation (lead); funding acquisition (lead); supervision (lead); writing – review and editing (equal).

7. Conflict of interest

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8. Data availability statement

All data and code required for visualisation and analyses are available from Zenodo (DOI: 10.5281/zenodo.18009385).[69]

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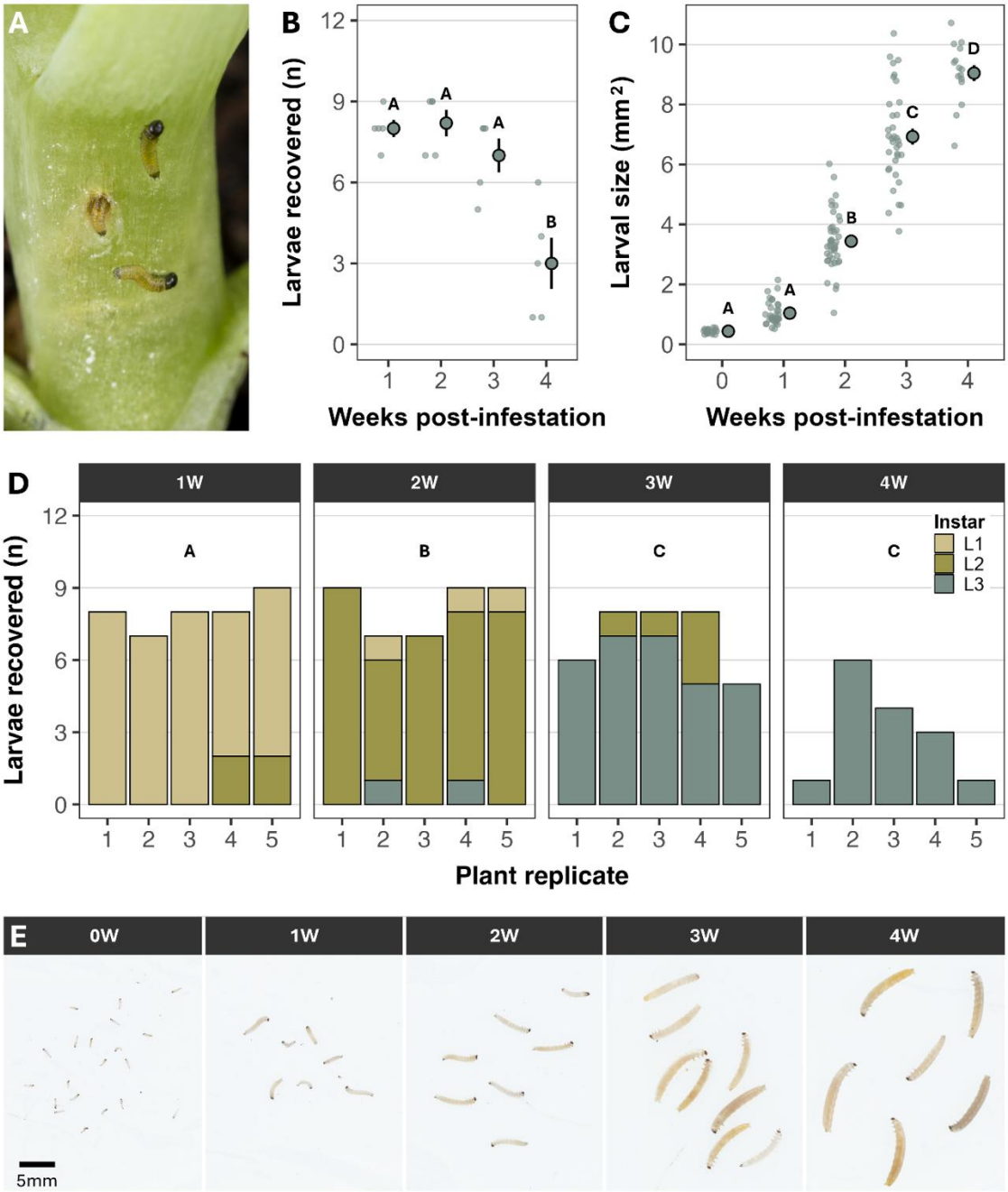

Figure 1. Cabbage stem flea beetle (*Psylliodes chrysocephala*; CSFB) larval development within *Brassica napus* plants under laboratory conditions. **(A)** Infestation of *B. napus* with <24 hour-old CSFB larvae. **(B)** Live larval recovery from infested plants at each timepoint (1-4W; n = 5 plants per timepoint). **(C)** Size of live larvae at each timepoint. Week 0 represents the size of <24 hour-old larvae. For plots **B** and **C**, large points with whiskers represent the mean ± standard error, jittered points to the left represent the raw data, and letters represent significant differences ($p < 0.001$) between timepoints based on Tukey post hoc testing. **(D)** Instar composition of live larvae within each plant across timepoints. Light green bars = first instar (L1); mid-green bars = second instar (L2); dark green bars = third instar (L3). Letters within plots represent significant differences ($p < 0.001$) between timepoints based on Tukey post hoc testing. **(E)** Representative photographs of CSFB larvae across timepoints. Scale bar represents 5mm for all larvae photos.

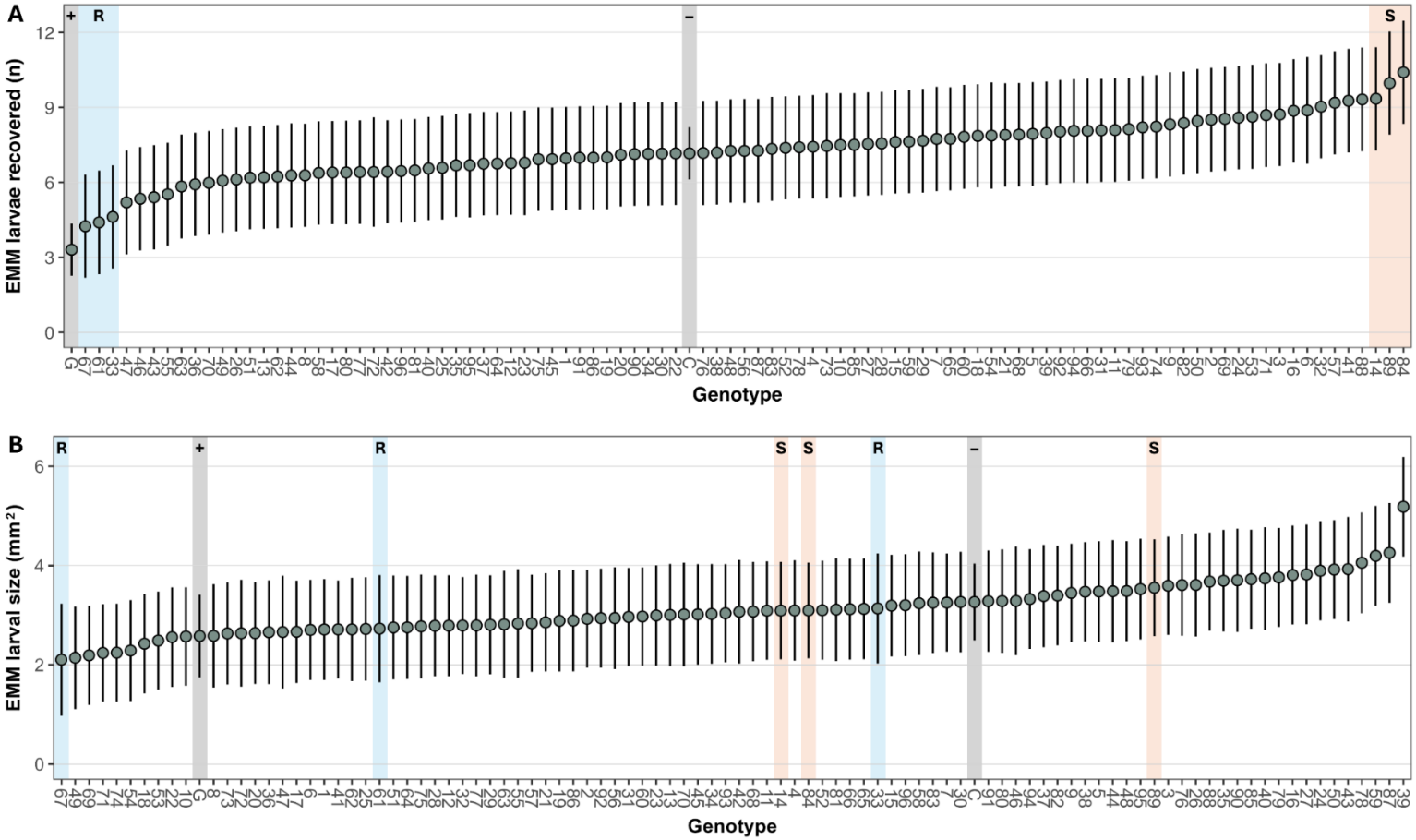

Figure 2. Per genotype estimated marginal mean (EMM) ± estimated 95% confidence intervals for cabbage stem flea beetle (*Psylliodes chrysocephala*) larval survival **(A)** and larval size **(B)** across 98 Brassica genotypes (97 *Brassica napus* and a single *Sinapis alba*; **Table S1**). Larval survival is defined as the number of larvae recovered from a plant two weeks after infestation with 12 larvae. For both plots, genotypes are arranged from lowest to highest EMM. Blue and peach highlighted bars represent genotypes identified as resistant (R) and susceptible (S), respectively. Grey highlighted bars represent the positive (G = *S. alba* Elsoms G1) and negative (C = *B. napus* KWS Campus) controls. Per genotype EMMs for larval survival and larval size can be found in **Table S5** and **Table S7**, respectively. Models used to generate EMMs can be found in **Table S3**.

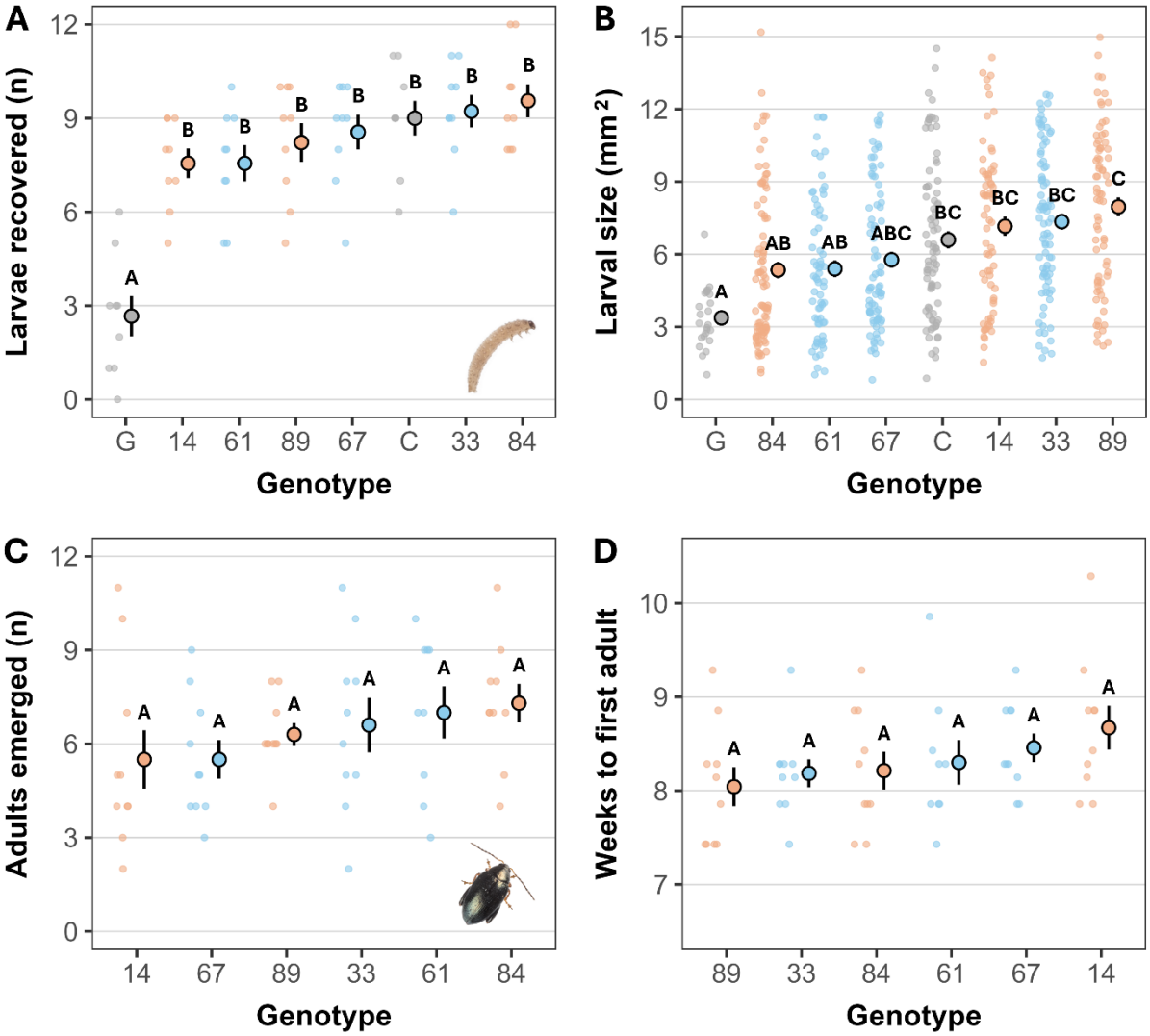

Figure 3. Cabbage stem flea beetle (*Psylliodes chrysocephala*) larval survival **(A)**, larval size **(B)**, and adult emergence **(C-D)** across eight selected Brassica plant genotypes (seven *Brassica napus* and a single *Sinapis alba*; **Table S1**). **(A)** Larval survival, defined as the number of larvae recovered from plants two weeks after infestation with 12 larvae, across eight Brassica genotypes. **(B)** Size of larvae recovered across eight Brassica genotypes. **(C)** Total number of adult beetles that emerged across six *B. napus* genotypes. **(D)** Time in weeks to first adult beetle emergence across six *B. napus* genotypes. For all plots, genotypes are ordered from lowest to highest mean. Blue and peach points represent genotypes from the diversity screen that were initially identified as resistant (R) and susceptible (S), respectively (**Figure 2**). Grey points represent the positive (G = *S. alba* Elsoms G1) and negative (C = *B. napus* KWS Campus) controls. Large points with whiskers represent the mean ± standard error, jittered points to the left represent the raw data, and letters represent significant differences ($p < 0.01$) between genotypes based on Tukey post hoc testing. Note that plots **C-D** do not include the control genotypes, since these were dropped (to increase experimental throughput) due to similar patterns of larval survival between the diversity screen and the phenotype validation experiment (**Figure S5**). Per genotype sample sizes and summary statistics for all responses can be found in **Tables S8-S11**.

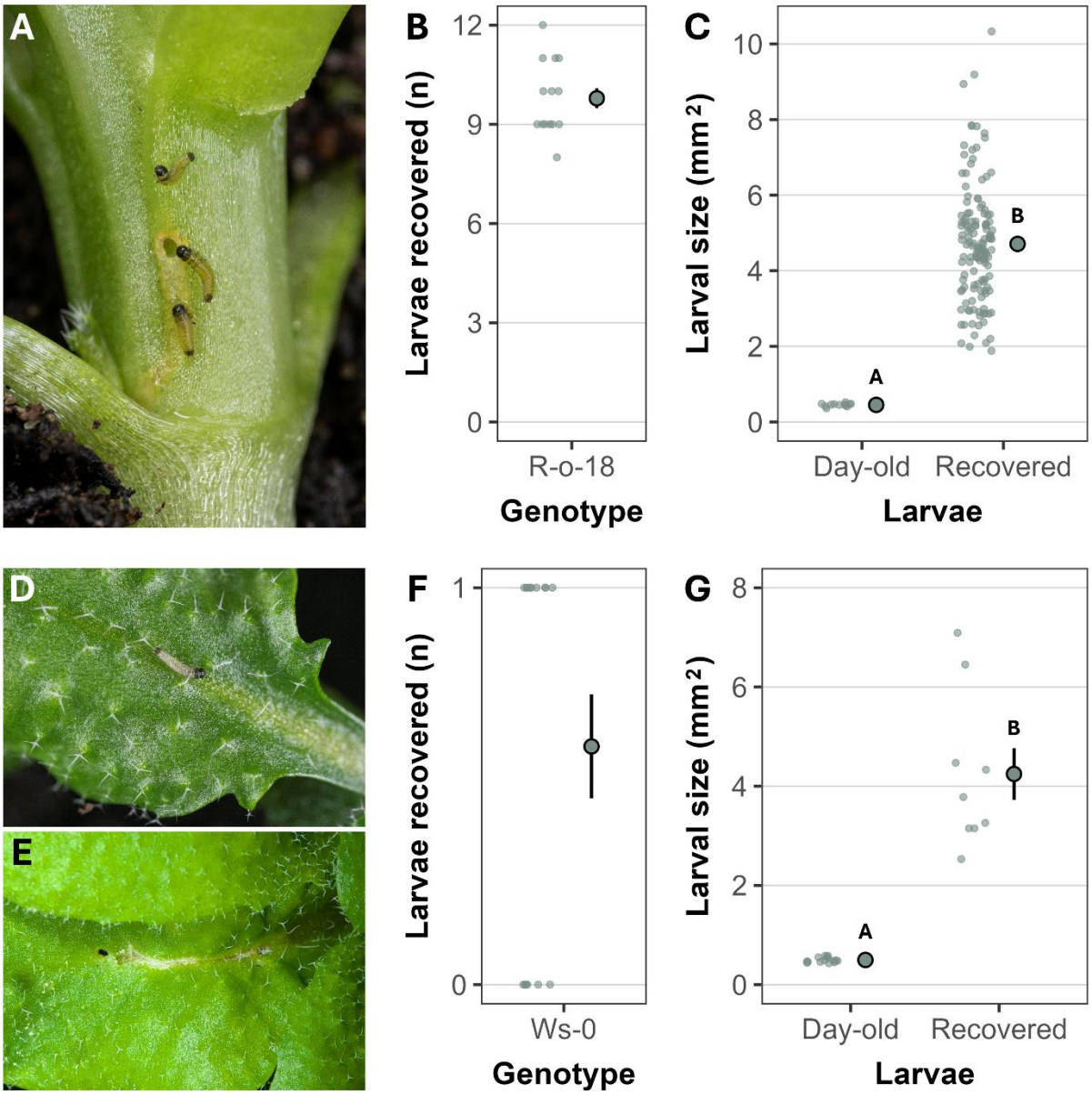

Figure 4. Cabbage stem flea beetle (*Psylliodes chrysocephala*; CSFB) larval recovery and development within *Brassica rapa* Ro-18 **(A-C)** and *Arabidopsis thaliana* Ws- 0 **(D-G)** plants. **(A)** Infestation of *B. rapa* with <24 hour-old larvae. **(B)** Larval recovery from infested *B. rapa* plants (n = 14) two weeks post-infestation. **(C)** Size of day-old larvae vs. larvae recovered from infested *B. rapa* plants. **(D)** Infestation of *A. thaliana* with a single <24 hour-old larva. **(E)** Larval herbivory damage on the midrib of an *A. thaliana* leaf one-week post-infestation. **(F)** Recovery rate (0/1) of larvae from infested *A. thaliana* plants (n = 15). **(G)** Size of <24 hour-old larvae vs. larvae recovered from infested *A. thaliana* plants. For plots **B**-**C** and **F**-**G**, large points with whiskers represent the mean ± standard error, jittered points to the left represent the raw data, and letters represent significant differences ($p \leq 0.05$) between larval status based on Tukey post hoc testing.

Table 1. ANOVA summary of fitted fixed effects for cabbage stem flea beetle (*Psylliodes chrysocephala*) larval survival and larval size across the 98 Brassica plant genotypes (97 *Brassica napus* and a single *Sinapis alba*) screened for larval resistance. Details for both fitted models can be found in **Table S3**.

Response	Fixed effect	F	Num. df	Den. df	*p*
Larval survival	Genotype	1.83	97	194.60	**<0.001**
	Plant weight	6.72	1	144.44	**0.01**
	Replicate	1.15	2	6.99	0.37
Larval size	Genotype	1.73	97	170.64	**<0.001**
	Replicate	2.51	2	8.85	0.14

Num. df = numerical degrees of freedom; Den. df = Kenward-Roger estimated denominator degrees of freedom.

Table 2. ANOVA summary of fitted fixed effects for cabbage stem flea beetle (*Psylliodes chrysocephala*) larval survival, larval size, adult emergence, and time to first adult emergence across the Brassica plant genotypes (seven *Brassica napus* and a single *Sinapis alba*) selected for larval resistance phenotype validation. Details for all fitted models can be found in **Table S3**.

Response	Fixed effect	F	Num. df	Den. df	*p*
Larval survival	Genotype	17.77	7	59.39	**<0.001**
	Plant weight	6.02	1	58.85	**0.02**
	Block	3.72	2	3.81	0.13
Larval size	Genotype	6.29	7	59.01	**<0.001**
	Block	2.60	2	2.93	0.22
Adult emergence	Genotype	1.08	5	52	0.38
	Block	2.62	1	52	0.11
Weeks to adult emergence	Genotype	1.26	5	52	0.29
	Block	0.65	1	52	0.42

Num. df = numerical degrees of freedom; Den. df = Denominator degrees of freedom (Kenward-Roger estimated for larval survival and larval size responses).