John Innes Centre

Phenotypic variation in feeding by the cabbage stem flea beetle (*Psylliodes chrysocephala*) in white mustard (*Sinapis alba*), and investigation of its genetic basis

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Abstract

Oilseed rape (*Brassica napus*) is one of the worlds most economically important oilseed crops. The cabbage stem flea beetle (CSFB) is a major pest of oilseed rape, and there are currently limited control measures available. Genetic resistance would allow effective management of CSFBs, but no resistance genes have been identified. This thesis utilised a captive colony of CSFBs to screen *Brassicaceae* for resistance, resulting in the discovery of a potential quantitative trait loci linked to CSFB resistance within a wild relative, white mustard (*Sinapis alba*).

The dynamics of a captive colony of CSFB were studied which facilitated experimentation utilising the insect. A pipeline was developed to quantify adult CSFB feeding for 49 lines of *B. napus* and 14 lines of *S. alba*. While all *B. napus* were equally susceptible, two *S. alba* lines had a nine-fold difference in resistance.

No clear resistance mechanisms were observed during the limited characterisation performed, though the defence trait was found to be constitutively expressed. Transcriptome analysis using RNAseq associated wounding and chitin responses with the susceptible line, and salicylic acid catabolism, redox state, and selenium metabolism with the resistant line. Bulked segregant analysis identified a potential quantitative trait loci (QTL) associated with resistance on chromosome 11 containing 56 genes, five of which were differentially expressed. Genes in this region were discussed for their potential roles in resistance.

These results increase knowledge of performing experiments using adult CSFBs, and associate genes and metabolic pathways to CSFB resistance. Future experiments comparing these contrasting varieties could aid in identification of a specific resistance mechanism. Additional experiments for mapping QTL could also substantiate which regions are associated with resistance. In future work, gene candidates can be tested further to understand their mechanism with the aim to eventually develop oilseed rape varieties with increased resistance to the CSFB.

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List of Abbreviations

ANOVA	Analysis of variance
B	*Brassica*
BSA	Bulked segregant analysis
CER	Controlled environment room
CSFB	Cabbage stem flea beetle
DAMP	Damage associated molecular pattern
ESE	Estimated standard error
FID	Flame ionisation detection
GC	Gas chromatography
HP	High performance
JA	Jasmonic acid
KO	Knock out
LOESS	Locally weighted least squares regression
LRR	Leucine rich repeat
MS	Mass spectrometry
NGS	Next generation sequencing
OSR	Oilseed rape
P	*Psylliodes*
PAMP	Pathogen associated molecular pattern
GWAS	Genome wide association study
QTL	Quantitative trait loci
R^2	Coefficient of determination
RLK	Receptor like kinase
RLP	Receptor like protein
ROS	Reactive oxygen species
S	*Sinapis*
SA	Salicylic acid
SD	Standard deviation
SE	Standard error
Se	Selenium
SEM	Scanning electron microscopy
TF	Transcription factor
WOSR	Winter oilseed rape

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1. An introduction to the cabbage stem flea beetle as a pest of oilseed rape

Oilseed rape (*Brassica napus*; OSR) is the worlds second most important vegetable oilseed crop, grown across the globe in regions including Europe, China, India, Canada and Australia. The species originated from domestication of interspecific hybrids of *Brassica oleracea* (cabbage) and *Brassica rapa* (turnip rape) approximately 7500 years ago (Chalhoub et al., 2014). Winter OSR (WOSR) is a valuable crop for UK agriculture in particular. In the last decade, the oilseed rape crop was worth a mean of £ 700 million/year to the UK economy (DEFRA, 2019). Food grade oil, biodiesel feedstock, and high-protein animal feed are the major products. WOSR also acts as a profitable break crop for cereal rotations by reducing the natural build up of cereal pests and disease from continuous growing of cereals in the same field. It is also an important cover crop, preventing soil erosion while improving tilth. Despite the importance and high-demand of WOSR, the sown area has declined rapidly in both the UK and Europe. In 2022, WOSR growing area in England has decreased to 50 % of 2013 levels, and this decline is mostly a consequence of a problematic insect pest; the cabbage stem flea beetle (CSFB), *Psylliodes chrysocephala* (DEFRA, 2022).

The CSFB (Coleoptera: *Chrysomelidae*) is a specialist insect pest of the mustard family (*Brassicaceae*). It is found across the northern hemisphere, and is especially damaging to OSR in Europe, Canada and China (Zheng et al., 2020). In addition to the CSFB, the crucifer flea beetle *Phyllotreta cruciferae* is also particularly voracious. Both species have annual life cycles in Europe, and are black (or occasionally brown in the case of the CSFB), and have a metallic green/blue sheen (Bartlet et al., 1999b). Their pronounced hind femora enable them to jump powerfully as an escape response, like their namesake. Although their wings do allow flight, they rarely fly outside of their migration in the middle of the adult life cycle, and it is believed that the flight muscles atrophy and are reabsorbed (Bonnemaison, 1965). Adult CSFBs measure 4–5 mm in length, whereas the adults of the crucifer flea beetle are significantly smaller at just 2-3 mm in length. Furthermore, the stages of the life cycle found throughout the year are different between the two species. Crucifer flea beetle adults eclose around August and are the stage that overwinter, whereas CSFB adults appear around May and it is the larvae that overwinter (Vig, 2002). More research has been published on the crucifer flea beetle, which can be used to extrapolate to the CSFB due to their similar habits. Although both species have challenged farmers and agronomists for decades, it is the CSFB which has become particularly problematic in recent years.

The annual life cycle of the CSFB in Europe is outlined in Fig. 1.1. Adult beetles eclose around June and begin feeding on the foliage of Brassicaceae crops, mainly WOSR but also cabbage, mustard, and turnip (Bodnaryk, 1992a). After the harvest, they can continue to feed on wild *Brassicaceae*, such as *Sinapis* spp. and *Raphanis* spp. (Vig, 2003). In

mid-summer, they undergo a period of aestivation which lasts until late August (Såringer, 1984). During this period, activity reduces significantly. From early autumn (around September), adults exit aestivation and migrate to a newly sown WOSR crop and feed heavily. Females lay upwards of 100 eggs each at the base of each plant above temperatures of 4 °C (Mathiasen et al., 2015b). Egg laying continues throughout winter, especially if the climate is mild (Emery et al., 2022). This is a concern for future pest pressure on WOSR, as warmer winters due to climate change could increase CSFB geographical distribution and population density (Olfert et al., 2017). This impact may already been observed in the UK; for example, in 2015 larval populations between autumn and spring increased by 243 %, which may due to extensive egg laying during that years mild winter (White and Cowlrick, 2016). After the mating and laying period in autumn, adults mostly die back during winter (Vig, 2003). Eggs hatch in the autumn, and larvae mine petioles and stems from mid-October through to early spring, over-wintering inside the plants.

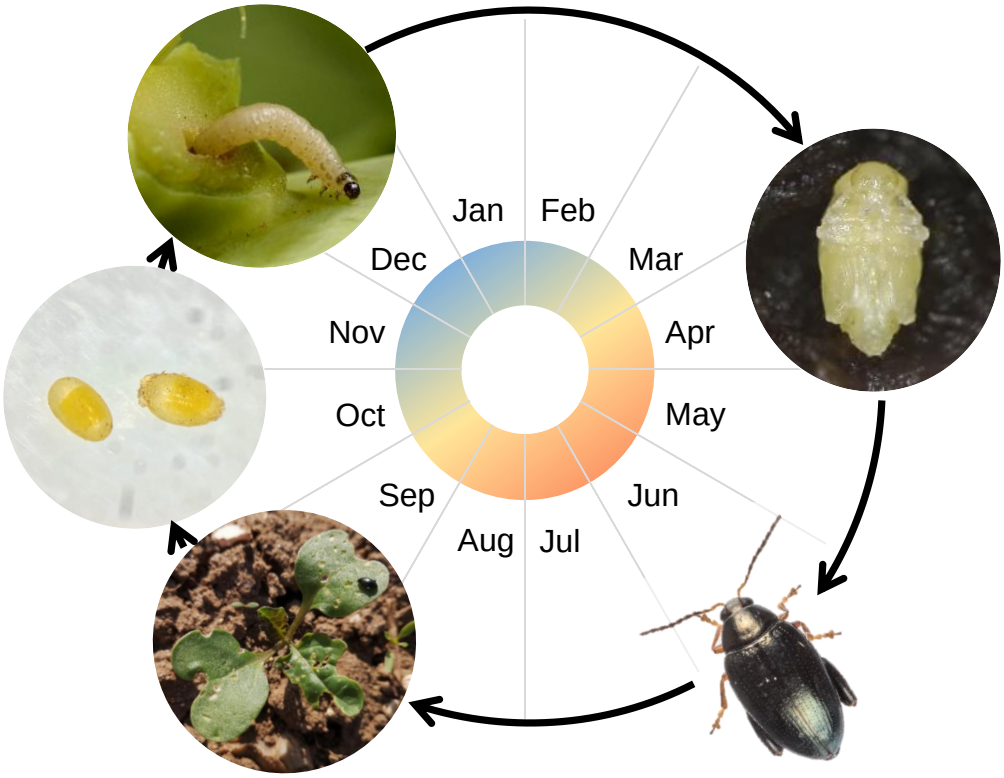

Figure 1.1: Life cycle of the cabbage stem flea beetle, *Psylliodes chrysocephala*, in UK populations. Adult beetles eclose from pupae in early summer, around June. The adults feed on the seed pods and leaves of the mature crop before aestivating over the summer. Once the new oilseed rape crop is sown in August, the adults migrate and feed heavily on the emerging seedlings from September. Adults begin laying eggs in October, and continue through the winter if the temperature is warm enough. Larvae hatch in late autumn and mine inside the stems and petioles of the growing oilseed rape crop. They shelter inside the crop through the winter, before dropping into the soil around April to pupate. Pupae image credit: Ryan Brock. Larvae image credit: Gilles San Martin via Flickr.

CSFB are severely damaging during both their adult and larval stages. Adults feed on the foliage of the young crop around September, and cause characteristic "shot-holing" damage which can kill seedlings by destroying the growing point, especially if the weather is dry (Nicholls, 2016; Williams, 2010). Plants that manage to escape must then contend with CSFB larvae, which are miners of the stems and petioles (Döring and Ulber, 2020). Larvae mining further exacerbate damage to surviving plants by reducing vigour, increasing risk to disease and frost damage, and in extreme cases causing stem collapse (Bonnemaison and Jourdheuil, 1954).

Historically, synthetic insecticide treatments have been used to effectively manage CSFB. Synthetic chemical sprays or dusts of camphechlor, diazinon, dichlorodiphenyltrichloroethane (DDT), dieldrin, endosulfan, heptachlor, lindane, or parathion were used from the mid-20th century to control CSFB (Bonnemaison, 1965). However, over the last few decades there has been increased concern over the damage these chemicals pose to both human and environmental health. Consequently, the use of these insecticides has gradually been repealed (European Commission, 2022). An alternative class of synthetic insecticides were introduced in the 1990s, and were highly effective for controlling CSFB. These were the neonicotinoids, which includes imidacloprid, clothianidin, and thiamethoxam.

Neonicotinoids are highly effective at controlling a broad range of commercially important insect pests, and are effectively taken up by the roots and leaves of plants and translocated to all plant parts (Maienfisch et al., 2001). Furthermore, neonicotinoids have a favourable safety profile as they possess high affinity for insect nicotinic acetylcholine receptors, but little to no affinity for the mammalian receptors (Matsuda et al., 2001). These receptors are confined to the insect nervous system, and neonicotinoids act as agonists, causing insect paralysis and death (Lu et al., 2022). Imidacloprid was the dominant neonicotinoid used on OSR between 2000 and 2008, replacing the previously-favoured gamma-hexachlorocyclohexane synthetic insecticide at the turn of the century (Budge et al., 2015). The systemic activity of neonicotinoids means that they provide protection to all tissues at an early growth stage, protecting the crop when it is most vulnerable. They also negate the need for foliar sprays, which reduces application rates and therefore hazards to non-target organisms. Consequently, neonicotinoids were the insecticide class of choice for CSFB control. In 2012, neonicotinoids seed treatments were applied to 83 % of WOSR seed in the UK, and the cabbage stem flea beetle motivated the majority of this insecticide use (Garthwaite et al., 2013; Zhang et al., 2017). However, in 2013 a report published by the European Food Safety Authority led to a moratorium on the use of the three key neonicotinoids due to their potential harmful effects on pollinators (EFSA, 2013). Although field studies were inconclusive (Lundin et al., 2015), this report resulted in a complete ban of these neonicotinoids in Europe in 2018. Since the moratorium in 2013, few control methods for CSFB remain. The loss of neonicotinoids has seen a significant increase in the flea beetle population in the years since [Fig. 1.2]. This increase

has been seen across Europe; for example the most comprehensive CSFB population records have been collected in Sweden over a period of 50 years, and the highest larvae densities on record were seen in the mid-2010s (Emery et al., 2022).

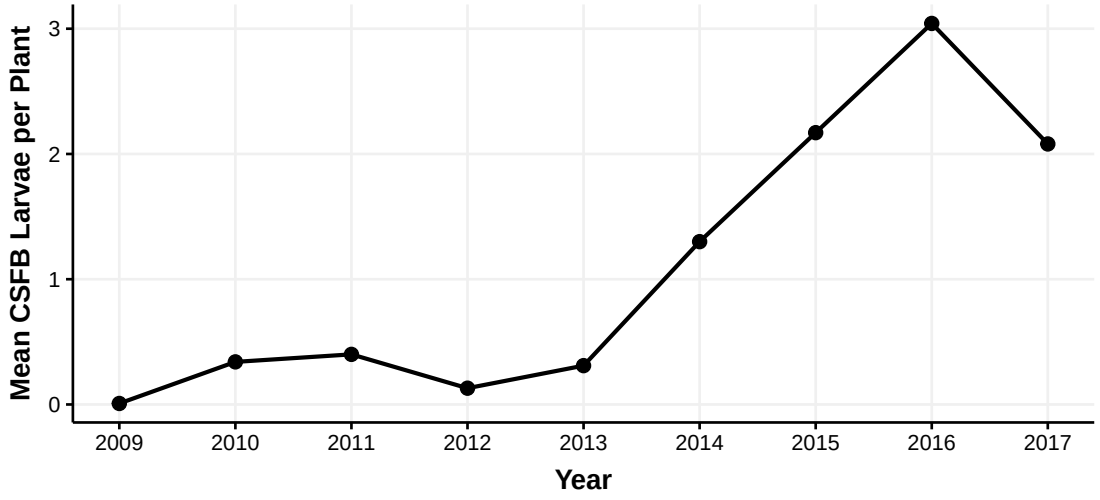

Figure 1.2: Mean number of cabbage stem flea beetle (CSFB) larvae found per plant in the UK since 2009, demonstrating the increase since the moratorium on neonicotinoid insecticides in December 2013. Figures obtained from (AHDB, 2013).

In 2014, the first year without neonicotinoids, yield losses due to CSFB in some parts of the country were as high as 14 % of the total crop, with a mean of approximately 5 % across the UK (Dewar et al., 2016). Attempts to control the CSFB population have been reflected in the increased use of alternative pesticide treatments. In 2016, 58 % of all insecticide treatments were applied to control for CSFB in the UK, doubling since 2013 (Garthwaite et al., 2019, 2013). The only remaining insecticide class to treat CSFB are pyrethroids (e.g. lambda-cyhalothrin). However, populations of *P. chrysocephala* across Europe exhibit high levels of both knock-down resistance, and metabolic resistance, hampering the effectiveness of pyrethroids against the CSFB (Højland et al., 2015; Willis et al., 2020). Nonetheless, 93 % of insecticides used on oilseed rape in the UK are now pyrethroid sprays, as these are all that remain of the chemical arsenal. As well as being a foliar spray (and so ineffective against stem-mining larvae), pyrethroids are also damaging to insect natural enemies of the CSFB, especially during the autumn when shelter is absent (Douglas and Tooker, 2016). There is a clear need for alternatives to synthetic chemical pesticides to avoid evolving resistance, and the uncertain fate of future legislation on their use.

1.1 The potential for resistant varieties

It is necessary to utilise alternative management options to combat CSFB instead of over reliance on synthetic chemical insecticides. Integrated pest management (IPM) is a desirable technique to manage CSFB, as it combines multiple methods which are adjusted for the given environment and level of pest pressure (Ortega-Ramos et al., 2022). IPM is defined under the EU Sustainable Use of Pesticides Directive (Directive 2009/128/EC) as utilisation of all available pest control techniques to reduce pest populations in a sustainable manner, while minimising risks to human and environmental health (European Parliament, 2009). IPM programs for WOSR combine cultural controls with biological and chemical treatments where appropriate, which have previously been reviewed for CSFB (Hoarau et al., 2022; Ortega-Ramos et al., 2022). For example, CSFB damage can be reduced by encouraging natural enemies, altering sowing dates, and using companion planting. However, these techniques alone have been unable to control CSFB sufficiently to allow economic viability of WOSR (Dosdall and Stevenson, 2005). Importantly, a key part of IPM is currently lacking for WOSR, which is host plant resistance.

The impact of insect pests has previously been reduced using host plant resistance. For example, the the impact of *Myzus Persicae* (the peach potato aphid) has been significantly reduced by breeding for tolerance to turnip yellow virus (TuYV), which is transmitted by the aphid and can cause considerable yield losses to susceptible OSR varieties (Hackenberg et al., 2020). TuYV tolerance is well established in commercially grown OSR varieties, and so management of aphids infestation is no longer required (AHDB, 2023). Although host resistance to insects has been well characterised in a range of plant and insect families, there are currently no CSFB-resistant WOSR varieties on the market (Smith and Clement, 2012; White et al., 2020). In fact, there are currently no commercial varieties of OSR that possess resistance directly against any insect pest (Hervé, 2018). Identification of insect resistance genes within *B. napus* has been challenging due to the limited genetic diversity of this species. Strong selection for traits such as low erucic acid, and low seed glucosinolate content have led to a genetic bottleneck which may also have reduced the presence of resistance mechanisms against insect herbivory in elite cultivars (Chen et al., 2015; Mason and Snowdon, 2016).

Despite considerable efforts over the past few decades, no flea beetle resistant varieties of *B. napus* have been identified (Soroka and Grenkow, 2013). *B. napus* "DNK.89.218" may be an exception, which saw a yield reduction of only 1 % from *P. cruciferae* infestation in the field despite feeding damage comparable to other lines. Although the authors of this study stated that follow up research into the resistance of this variety was being conducted, no subsequent studies have been published on this genotype in the two decades since the study took place, which may indicate that the finding was not repeatable (Brown et al., 2004).

B. napus is the most preferable host for CSFB, but little gain has been made in

understanding the mechanisms behind this inclination. Other species of Brassicaceae which are resistant to flea beetle have been identified from numerous studies, demonstrating that genetic resistance to CSFB is present (Bodnaryk and Lamb, 1991; Chenwang Peng et al., 1992; Döring and Ulber, 2020; Palaniswamy and Lamb, 1992a,b). However, specific resistance genes or mechanisms are yet to be identified. The general consensus is that resistance is likely controlled by many genes, each contributing small additive effects.

1.2 Introgression of resistance from related Brassicaceae

Due to an apparent lack of resistance genes within *B. napus*, breeding strategies are focused on introgression of insect resistance through alternative means. Transgenic approaches have been investigated previously to produce OSR with significantly increased trichome densities in Canada, which reduced flea beetle damage (Soroka et al., 2007). Utilisation of genetically modified oilseed rape possessing well-established insecticidal transgenes could also be assessed, such as the *Cry* protein toxins from *Bacillus thuringiensis* (Halfhill et al., 2002). However, transgenic material is currently under strict regulation in the UK and Europe. Therefore, genetic resistance must come from germplasm that can be introduced to WOSR through traditional breeding mechanisms. The generation of transgenic varieties also necessitates a greater understanding of CSFB physiology, and identification of specific resistance mechanisms, which are currently limited.

In Europe, strategies to introduce genetic variability into *B. napus* often utilise inter-specific transfer from relative *Brassicas*. For example, *B. napus* can be "resynthesised" by recreating the historic hybridisation between the cultivated progenitor species, *B. oleracea* and *B. rapa* (Katche and Mason, 2023). These progenitor species are more genetically diverse than *B. napus*, and so possess a greater variability in traits such as resistance which can be utilised for breeding (Mei et al., 2011). Resynthesised *B. napus* lines have been demonstrated to show increased resistance to both disease (Ding et al., 2013; Rygulla et al., 2007) and other insect specialists of *Brassicas* such as the cabbage stem weevil (*Ceutorhynchus pallidactylus*). However, in the case of CSFB, many accessions of these two progenitor species have been previously screened for flea beetle resistance, and no resistance has been identified (Soroka and Grenkow, 2013). In fact, of the 100 accessions representing five *Brassica* species (*B. carinata*, *B. juncea*, *B. napus*, or *B. rapa*) tested in this study, all were as susceptible as *B. napus*. As the progenitor species do not appear to possess resistance to CSFB, it does not appear to be possible to introduce CSFB resistance into *B. napus* through resynthesis from these two species (Eickermann and Ulber, 2011). Instead, inter-specific hybridisation may be a more promising route for introgression of resistance (Siemens, 2002). For example, in the same study by Soroka and Grenkow (2013), a close relative within the Brassicaceae family was found to be the most resistant to flea beetles: white mustard (*Sinapis alba*).

S. alba is a commercially grown species used to produce condiment mustard, but wild accessions with substantial genetic diversity are found in abundance across Europe and Central Asia (Granot et al., 1996). The resistance of *S. alba* to both adult and larvae stages of cabbage stem and crucifer flea beetles has been substantiated in multiple studies, and can clearly be seen in the field (Fig. 1.3) (Bodnaryk and Lamb, 1991; Brown et al., 2004; Döring and Ulber, 2020; Gavloski et al., 2000; Hiiesaar et al., 2006; Palaniswamy et al., 1997). Hybrids between *S. alba* and *B. napus* recieve lower levels of *P. cruciferae* feeding, demonstrating that this is a heritable trait (Gavloski et al., 2000). Although interspecific breeding can be challenging, *in vitro* techniques such as ovary culture and embryo rescue make hybridisation feasible (Brown et al., 1997). For example, the species barrier was overcome between *S. alba* and *B. napus* to introgress resistance to the cabbage seedpod weevil into OSR (Shaw et al., 2009). In addition, *S. alba* is the only species where intra-specific variation to flea beetle resistance has been observed (Soroka and Grenkow, 2013). Identification of resistance genes and mechanisms through comparisons between species is extremely challenging, as the vast majority of differences are confounding. Comparing individuals of the same species reduces these confounding characteristics. Therefore, intra-specific comparison of *S. alba* varieties can be used to illuminate CSFB resistance mechanisms. A published genome of *S. alba* is not yet available, and so the full genetic diversity is yet to be understood. Despite this, *S. alba* is clearly a desirable source of resistance genes for developing CSFB resistant OSR. Therefore, further study of *S. alba* resistance to the CSFB is warranted.

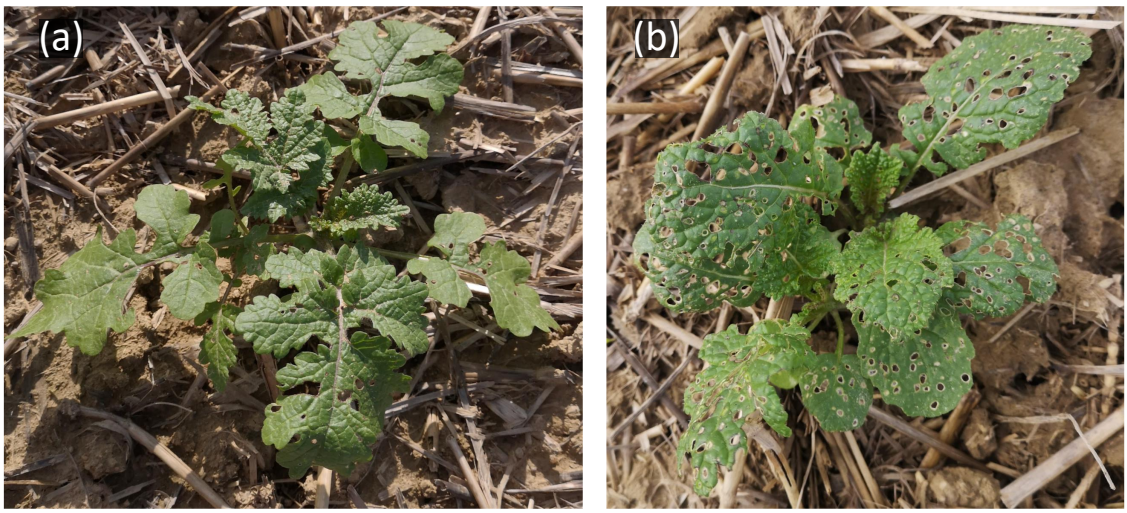

Figure 1.3: Comparison of *Sinapis alba* (a) and *Brassica napus* (b) in a field trial. Characteristic shot-holing damage is present on *B. napus* from cabbage stem flea beetle feeding, but is almost completely absent on *S. alba* due to resistance. These two plants were grown in adjacent plots in a field trial located in Mondonville, France.

1.3 Types of insect resistance

Plants have evolved the ability to resist herbivore damage by insects using different mechanisms which were first categorised by Painter (1951). A framework consisting of three main mechanisms was described: reducing feeding (antixenosis), reducing suitability as a host (antibiosis), and limiting symptom development due to damage (tolerance). It should be noted that these categories are not always clearly delimited, though they form a useful framework for clarifying resistance mechanisms to aid in their understanding. More recently, this framework was revised to also consider defences which may be induced upon detection of feeding damage or constituent to the plant at all times, as well as separate those that are direct or indirect (Stout, 2013). Often, multiple forms are used to repel as many pests as possible. In the Brassicaceae, antixenosis, antibiosis and tolerance traits have all been shown to impart resistance to a range of insect pests. For *S. alba*, there has been little focus on the type of resistance or the underlying physiological and genetic mechanisms which controls flea beetle resistance. However, a few candidate mechanisms have been identified which are discussed here.

1.3.1 Antibiosis

Antibiosis is the ability to limit insect populations by reducing their survival or reproduction through toxic or anti-nutritive compounds. A brilliant case study demonstrating incorporation of an antibiosis trait through traditional breeding is the gene *Sm1*, used to control the major spring wheat pest, orange wheat blossom midge (*Sitodiplosis mosellana*). It was noted that some winter wheat varieties were more tolerant to wheat midge, as newly hatched larvae feeding on winter wheat failed to grow and eventually died (McKenzie et al., 2002). Molecular markers for the gene were subsequently developed (Thomas et al., 2005), and *Sm1* has since been bred into most recommended list varieties (AHDB, 2023). Due to the specific and highly effective action of *Sm1* resistance to the wheat midge may also rapidly develop. Preferably then, resistance traits should be numerous (and therefore less vulnerable), or imbue partial resistance to increase durability (Chen et al., 2014; Liu et al., 2014; Zhu et al., 2012).

In Brassicaceae, an indirect antibiosis trait is the ability to attract parasitoids using volatile emissions. Volatiles from *S. alba* are attractive to a parasitoid wasp (*Diadegma semiclausum*) of the diamond back moth (*Plutella xylostella*), and volatile production is increased by moth infestation (Bukovinszky et al., 2005). However, no volatiles have been identified which attract flea beetle parasitoids. There may be potential for this, as there numerous parasitoid wasps of CSFBs such as *Tersilochus microgaster*, an endo-parasitic wasp predating upon CSFB larvae (Bonnemaison, 1965), or *Microctonus brassicae* which parsatises the adults (Jordan et al., 2020). However, infection rates of CSFB by parasitoids are estimated to be low, which is likely exacerbated through use of pyrethroids. Parasitoids

may also be unable to reduce flea beetle numbers sufficiently before extensive damage to the crop occurs, as herbivory can be rapid. Therefore, parasitoids are unlikely to effectively control CSFB alone, and assessing volatile attractants to parasitoids is not a priority.

Direct antibiosis has also been observed in *S. alba* against insect larvae. For example, larvae of the cabbage seedpod weevil (*Ceutorhynchus obstrictus*) had reduced mass and prolonged development when feeding on *S. alba*, though it is not clear if this is actually due to reduced feeding (antixenosis) rather than antibiosis (Tansey et al., 2010). Interestingly, *S. alba* has been found to reduce survival and mass of CSFB larvae (Döring and Ulber, 2020). The mechanisms behind this antibiosis are not currently known, though the authors speculate that plant secondary metabolties or nutrient profile may play a role in altered larvae survival. In addition, before further work into mechanisms proceeds there is a need to replicate these results in field experiments to evaluate CSFB larvae performance under natural conditions and ensure the result was not confounded by laboratory rearing of this non-model insect.

Antibiosis to adult flea beetles has not been identified, despite screening accessions of both *B. napus* and *S. alba* (Palaniswamy et al., 1997). Antibiosis also does not appear to be as widespread as antixenosis among the *Brassica* species tested (*B. juncea*, *B. napus*, *B. rapa* or *B. carinata*), as there were no significant differences in the mass gained by crucifer flea beetles when feeding on these species, though there were differences in foliage consumed (antixenosis) (Palaniswamy et al., 1997). Antixenosis may therefore present a more promising type of resistance for use against CSFB in OSR.

1.3.2 Antixenosis

Antixenosis is the best-studied form of resistance against the crucifer flea beetle and CSFB. Antixenotic traits can repel herbivory through various methods, including production of antifeedants and physical defences such as trichomes. WOSR deploys both of these strategies against pests, and the most characteristic defence trait of the Brassicaceae are a class of metabolites called glucosinolates. Alone, glucosinolates are not toxic to herbivores or to the plants tissues. However, WOSR also possess stored enzymes, myrosinases, which hydrolyse glucosinolates to toxic products: isothiocyanates. Producing these compounds deters feeding by slugs, pigeons and a wide range of insect pests (Giamoustaris and Mithen, 1995). Conversely, glucosinolates appear to be attractive to specialist insect herbivores of the Brassicaceae. For example, the *Brassica* specialist butterflies *Pieris rapae* and *Pieris brassicae* can detect glucosinolates on the surface of the leaves which stimulates them to oviposit on these plants (Chew and Renwick, 1995). Likewise, crucifer and cabbage stem flea beetles are attracted to isothiocyanates, and are able to detoxify these to overcome the hosts defences (Beran et al., 2018; Pivnick et al., 1992).

The potential of glucosinolates for flea beetle antixenosis is well studied, but results are often contradictory. Although glucosinolates stimulate CSFB adult feeding (Bartlet

et al., 1994) and are vital for CSFB acceptance (Bartlet and Williams, 1991; Tóth et al., 2007), no differences in herbivory by CSFB were observed for seedlings with different glucosinolate profiles and concentrations (Bartlet et al., 1996). Furthermore, transgenic *Arabidopsis thaliana* containing a 4-fold range of glucosinolates also saw no differences in CSFB feeding (Nielsen et al., 2001). However in a study by Soroka and Grenkow (2013), *S. alba* lines which were lower in total glucosinolates were more susceptible to *Phyllotreta cruciferae* and *Phyllotreta striolata* flea beetles. The variability of these results may be due to the challenge of studying flea beetles, as their preferences are not always clear or easily replicable. These results may also indicate that flea beetle preference may rely in part on specific quantities of particular glucosinolates, opposed to high or low profiles as a whole. The CSFB responds more strongly to glucobrassicin than some other glucosinolates for example (Isidoro et al., 1998). Glucosinolates may even act in synergy with each other to form the phenotype; this would not have been easily observed in these studies assessing changes to many types of glucosinolate at once. Such synergistic effects have been found for other metabolites in other species, such as the combination of furanocoumarin xanthotoxin and myristicin, which significantly increased toxicity to *Heliothis zea* caterpillars in Apiaceae (Berenbaum and Neal, 1985). Synergy between glucosinolate-derived compounds has been found for antimicrobial activity, though does not appear to have been studied for insect resistance (Wu et al., 2011).

Specifically for *S. alba*, this species possesses a relatively unique glucosinolate called sinalbin (*p*-hydroxybenzyl glucosinolate). It was suggested that sinalbin may be the key defence trait which causes CSFB resistance in *S. alba* (Bodnaryk, 1991). However, *S. alba* varieties significantly reduced for sinalbin had no difference in *P. cruciferae* or *Phyllotreta undulata* (small striped flea beetle) feeding preference (Bodnaryk, 1997; Hopkins et al., 1998). Furthermore, transgenic *Arabidopsis* which produced sinalbin was no more resistant to *P. cruciferae* or *Phyllotreta nemorum* (yellow striped flea beetle)(Nielsen et al., 2001). Therefore, glucosinolates alone do not appear to be sufficient to explain the variation seen in feeding preferences between *S. alba* and susceptible Brassicaceae. It is also important to consider that manipulation of the glucosinolate profile could increase susceptibility to other pests. For example, while low-glucosinolate lines of *S. alba* saw no change in *P. cruciferae* feeding, these lines were significantly more susceptible to the generalist *Mamestra configurata* (bertha armyworm) (Bodnaryk, 1997). Thus for the farmed crop it may be preferable to investigate resistance mechanisms other than glucosinolates for controlling the CSFB in addition to other pests.

An alternative antixenosis mechanism for *S. alba* is the presence of a high density of trichomes (Lamb, 1980). These hair-like protrusions can act as physical barriers, and also cause antibiotic reactions. *Brassica* species with an extremely high density of trichomes, such as *B. villosa*, show resistance to *P. cruciferae* feeding (Palaniswamy and Bodnaryk, 1994). Resistance of *Brassicas* to flea beetle feeding due to trichomes has also been well studied in Canada. Soroka et al. (2007) developed a transgenic hairy *B. napus* line that

showed reduced feeding by *P. cruciferae* feeding. These trichomes appeared to make feeding more physically challenging for the flea beetles (Soroka et al., 2011). However, glabrous (hairless) cotyledons of the transgenic lines were still significantly more resistant. Other metabolic pathways were also altered by the transgene due to shared regulatory components, including anthocyanins, glucosinolates, waxes and lignin (Gruber et al., 2018; Onyilagha et al., 2004). As metabolites such as anthocyanins have previously been linked to insect resistance (Wang et al., 2014), and increased lignin may make the host tougher to chew through and slow the rate of feeding, it may be that trichomes were not the primary cause of resistance in these studies but instead a linked pathway was. Although increasing trichome density of OSR tissues could increase resistance, there are also other mechanisms for investigation implicated by these results.

The composition of the cuticle may also play a role in flea beetle antixenosis within Brassicaceae. The cuticle is composed of cuticular waxes, which consist predominantly of very long-chain fatty acids (Koch and Ensikat, 2008). The specific chemical structure is dependent on the plant species, and is also influenced by the environment (Baker, 1974). These derivatives include alkanes, wax esters, aldehydes, ketones, and primary and secondary alcohols. Waxes can either be intercalated within the polymers that make up the cuticle to form "intracuticular waxes", or waxes can form the most external surface of the plant as "epicuticular waxes". Epicuticular waxes self-assemble into crystalline protrusions of structures dependent on the major components, and so their chemical composition can be inferred from observations with microscopy (Koch and Ensikat, 2008).

It is not yet certain if the composition of these wax components can influence insect resistance, though some work has found correlations between specific wax components. For example, resistance to the autumn gum moth (*Mnesempala privata*) in *Eucalyptus globulus* was correlated with levels of aliphatic phenylethyl and benzyl wax esters, but not to the quantity of wax alone (Jones et al., 2002). An increased quantity of epicuticular wax has also been found to reduce feeding by *P. cruciferae* and numerous other *Phyllotreta* spp. (Bodnaryk, 1992b; Bohinc et al., 2014). However, these prior studies of flea beetle resistance in response to wax have not assessed wax composition. In addition, the waxes themselves may not be the metabolite involved in defence; other metabolites are also deposited at the surface of the cuticle and can be embedded within the wax film. For example, sugars on the cuticle surface can influence insect attraction (Derridj et al., 2012). Many insects gather information about the quality of the host by probing metabolites at the surface of the leaf with antennae or tarsi. This is also likely to occur in CSFB, which have specialised hairs on their antennae (sensilla chaetica) which respond to chemicals present at the leaf surface when the insect probes the leaf (Isidoro et al., 1998). It would be interesting to further investigate specific wax profiles through chemical analysis techniques such as mass spectrometry (MS), and exposure of these specific metabolite to CSFB to assess changes in resistance.

There is still much potential for investigation of antixenotic traits for CSFB resistance,

especially within *S. alba*. The implicated traits discussed here would be worth investigating further by comparing accessions which contrast for CSFB resistance, and characterising the differences between them.

1.3.3 Tolerance

Tolerance traits limit the consequences to fitness without impacting the insects behaviour. In field trials, breeders are currently investigating early vigour as a form of tolerance within *B. napus* germplasm (DEKALB, 2016). Enhancing early vigour of WOSR can aid seedlings in rapidly growing past the two-leaf stage, when seedlings are most vulnerable to CSFB feeding. Genes relating to early vigour have been identified within *B. napus*, and selecting for these could reduce CSFB impact (Hatzig et al., 2015). However, although tolerance is of interest, few studies have assessed Brassicaceae tolerance in response to flea beetle feeding. This is likely due to the resources required to bring plants through to seed-set for measuring the impact of herbivory to yields in a controlled environment.

In addition to antixenosis, tolerance is considered to be an important resistance mechanism for flea beetle feeding in *S. alba* (Brown et al., 2004). To date, one study has assessed plant performance after flea beetle feeding to understand tolerance (Bodnaryk and Lamb, 1991). In this study, seedlings of *S. alba* show some tolerance to low levels of flea beetle feeding damage as their cotyledons continue to grow the same rate as undamaged seedlings. Conversely, *B. napus* seedlings exhibit a reduction in growth proportional to cotyledon damage. In the field, *S. alba* is more tolerant to reductions of seed yield than *B. napus* when damaged by flea beetles (only a 7.3 % loss compared to 17.4 % in *B. napus*); however, in field trials it is difficult to separate antixenosis and tolerance due to imprecise scoring (Bodnaryk and Lamb, 1991).

Although tolerance traits against flea beetles are understudied, the challenge of scaling up these experiments severely limits the throughput of phenotyping many accessions. Potentially, it would be preferable to first identify accessions of *S. alba* which show antixenosis to CSFB, and subsequently investigate these specific varieties for tolerance as well as antibiosis. These accessions may have evolved many forms of CSFB resistance due to selection pressure, so if antixenosis is identified other forms may also be present.

1.3.4 Induced Plant Defences

Plants possess some phenotypic plasticity in their ability to express resistance. Constitutive expression of resistance traits can be energetically expensive as they can require the use of fitness limiting resources (e.g. nitrogen) (Baldwin, 2001). Therefore, constitutive expression where not required can incur a fitness penalty to the plant. In addition, inducing resistance can allow the plant to tailor the response deployed to best target an attacker. This is important for traits which can increase susceptibility to

some attackers despite increasing resistance to others. For example, accumulation of cucurbitacins in *Cucurbita moschata* (winter squash) can repel some insect herbivores while attracting others, and the CSFB is attracted to the isothiocyanates of Brassicaceae which are typically repellent to generalist species such as the grey field slug (Carroll and Hoffman, 1980; Pivnick et al., 1992).

Induction of defences is common across plant families in response to a broad range of stimuli. This includes not only insects, but bacterial and fungal pathogens, viruses, nematodes, arachnids, and parasitic plants (He et al., 2004; Li et al., 2023; Miyazaki et al., 2013; Salcedo et al., 2017; Whitham et al., 1994; Xie et al., 2022). The pathways underpinning pest detection and the subsequent change in phenotype have been characterised especially well for defence against pathogens, and this knowledge has been utilised to identify hundreds of resistance genes and successfully introgress them into elite crop varieties around the globe. Detailed reviews on breeding inducible pathogen resistance have previously been covered for crops such as tomato (*Solanum lycopersicum*), wheat (*Triticum aestivum*) and rice (*Oryza sativa*), to name a few (Bakala et al., 2021; Chitwood-Brown et al., 2021; Jiang et al., 2020).

During their contact with the plant, pests and microbes shed conserved molecular fragments which are detected by the plant using trans-membrane proteins, termed pattern recognition receptors (PRRs) (Couto and Zipfel, 2016). PRRs are typically receptor-like kinases (RLKs) or receptor-like proteins (RLPs) lacking a protein kinase domain (Dodds and Rathjen, 2010). The molecular patterns they bind to are named after their source, either pathogen-/damage-/microbe- or herbivore- associated molecular patterns (PAMPs/DAMPs/MAMPs/HAMPs). Detection of the pattern by the PRR triggers pattern triggered immunity (PTI), a cascading response of signalling networks which ultimately results in expression of a resistance phenotype (Boller, 1995). Plants are also able to respond to differences in spatio-temporal patterns of damage, as well as damage caused to the leaf surface from insects walking (Bricchi et al., 2010; Tretner et al., 2008).

An example family of PRRs are proteins consisting of nucleotide-binding and leucine-rich repeat domains (NB-LRR proteins), which are encoded by R genes on a gene-for-gene basis between the pest and plant (Dangl, 1995). As NLRs are intracellular, R genes have been studied primarily in insect species that interact directly with the intracellular components by pierce-feeding, such as aphids, whitefly and planthoppers (see: Table 1 of Douglas and Tooker (2016)). However, very few plant NLR proteins that respond to insects have been characterized. To date, only one intrinsic PRR of herbivore elicitors has been identified. Lepidopteran larvae secrete "inceptins" in their oral secretions during feeding (Schmelz et al., 2006). Inceptins may technically be termed as DAMPs, as they are proteolytic fragments of the plant chloroplastic ATP synthase, activated by insect digestive enzymes. An inceptin receptor (INR) was identified through forward genetic screening. The mapped INR demonstrated inceptin-inducible binding,

immune signalling though ROS, and provided resistance to beet armyworm (*Spodoptera exigua*) when expressed in tobacco (Steinbrenner et al., 2019). A further HAMP receptor may be OsLRR-RLK1, a recently discovered plasma membrane-localized, early responsive leucine-rich repeat receptor-like kinase (Hu et al., 2018). OsLRR-RLK1 is essential for perception of feeding of the the striped stemborer, *Chilo suppressalis*, and for initiating defence.

Following perception of herbivory, a complex network of signalling is triggered. Early signalling includes depolarization of the plasma transmembrane to initiate a rise in cytosolic Ca^{2+}, leading to production of reactive oxygen species (ROS) and elevated mitogen-activated protein kinase (MAPK) activity in distal tissues. These networks result in phytohormone-driven signalling and activation of transcription factors driving defence-response genes (Maffei et al., 2006; Mithöfer et al., 2001).

The hormone signalling pathways involve mainly jasmonates (JAs, the precursors and derivatives of jasmonic acid) and salicylic acid (SA), though there is significant cross-talk with other pathways (e.g. ethylene, abscisic acid and auxin) (Yang et al., 2015). The specific downstream effects are dependent upon cross-talk between the interconnected networks of phytohormones triggered by the particular insect herbivore. Generally, the SA pathway is associated with response to feeding on living tissues and causing limited tissue damage, especially by pathogens and non-chewing insects i.e. those which feed upon the cell contents/phloem such as aphids, leaf-hoppers, and mites (Erb et al., 2012). Conversely, JAs generally up-regulate defence to necrotrophic pathogens and chewing arthropod herbivores. For example, JAs have been linked to modulation of defensive secondary metabolites and volatiles, as well as proteinase inhibitors (Balbi and Devoto, 2008). Mutants defective in the synthesis or perception of JAs are also more vulnerable to attack (Li et al., 2004). JAs have therefore previously been hypothesised for importance in regulation of CSFB resistance. Expression of major classes of secondary metabolites involved in Brassicaceae defence, such as glucosinolates in *B. napus*, are upregulated by JA application (Bodnaryk, 1994). This response is very similar to that elicited by CSFB feeding, and JA application in wholes plants significantly decreased feeding by CSFB (Bartlet et al., 1999a). JAs function by interacting with JAZ proteins, which repress JAs-dependent genes; JAs/JAZ interaction leads to targeted degradation of the JAZ proteins and allows transcription by the MYC2 transcription factor (Staswick, 2008). The defence responses induced downstream of the JA or SA pathways in response appear to be specific to defend against the respective chewing or phloem feeding insects; when cabbage plants (*Brassica oleracea*) were previously exposed to leaf herbivory by diamondback moth caterpillars (*Plutella xylostella*), performance of the root-feeding cabbage root fly larvae (*Delia radicum*) was strongly attenuated, but performance was not impacted by previous exposure to cabbage aphids (*Brevicoryne brassicae*) (Soler et al., 2012).

Plant-herbivore interactions do not stop with this one layer of molecular arsenal. Some insects possess the ability to suppress plant immune responses by releasing "effector"

molecules into the wound site (Felton and Tumlinson, 2008; Hogenhout et al., 2009; Musser et al., 2002). Some plants may possess intracellular receptors known as disease resistance (R) proteins which are able to detect effectors, leading to the activation of Effector Triggered Immunity (ETI). While PTI is considered to be a generalist response to attack, ETI is more specific to the particular herbivore (Flor, 1956). This "zigzag" of defence can have multiple layers of attack and response between the host and attacker (Jones and Dangl, 2006). In chewing insects the use of effectors is less well characterised, though some evidence suggests that the bacteria present within chewing herbivore secretions can disguise the insect and manipulate the plant into inducing the SA defence pathway. For example, oral secretions of the African cotton leafworm (*Spodoptera littoralis*) and *P. brassicae* detected by *Arabidopsis* suppressed plant genes associated with insect defence, and increased larval weights after feeding (Consales et al., 2012). Effector molecules may also be present in Coleoptera, but is as of yet unstudied.

1.4 Summary

The CSFB is a problematic pest of oilseed rape, and there is a need to develop resistant varieties to reduce economic losses due to this insect. Of the three major forms of resistance, antixenosis is the simplest to study in a controlled environment. The experimental throughput of both antibiosis and tolerance is dictated by the life-span of the insect and the plant respectively, which can be up to 12 months in this case. Conversely, antixenosis can be tested in the time it takes to observe flea beetle feeding; just a few days.

A plethora of resistance mechanisms have been hypothesised for flea beetle resistance, such as glucosinolates, waxes, and trichomes. However, the importance of these resistance mechanisms has not been well characterised. Previous studies have been unable to confirm that these traits are controlling the observed resistance. In part, this is due to variability introduced from using wild (and therefore poorly controlled) populations of CSFBs in experiments. This challenge has also been exacerbated because genetic differences between resistant and susceptible individuals have not been investigated, which could link resistance to specific pathways. Resistance traits that are inducible are of particular interest as they allow for a nuanced, durable response to herbivory. Complex molecular signalling pathways are required for induced resistance, which are poorly understood for chewing insects such as CSFB. Greater understanding of underlying regulatory elements may also play an essential role for breeding WOSR against CSFB.

Previous studies have generally compared resistance between species, but this makes identification of resistance mechanisms particularly challenging due to many other confounding differences. Ideally, varieties of the same species with contrasting levels of resistance should be compared. Resistant and susceptible varieties could be characterised and compared to understand the physiological characteristics defining resistance, and the

genetic pathways which may drive these. To date, the only Brassicaceae with contrasting intra-specific flea beetle resistance is *Sinapis alba*, and the specific flea beetle resistance mechanisms of this generally resistant species have not been identified.

There is also potential to develop a more robust phenotyping assay by better controlling the cabbage stem flea beetles that are used in experimentation.

1.5 Project Aims

This project aimed to identify and characterise intra-specific differences in cabbage stem flea beetle antixenosis within two species of Brassicaceae: *Brassica napus* and *Sinapis alba*. The aim of each chapter is as follows:

- Characterise the life cycle of the CSFB in the laboratory to obtain a controlled population for use in experiments.
- Identify CSFB resistant and susceptible varieties of *B. napus* and *S. alba*.
- Associate differences in morphology and/or chemical composition to the differences in CSFB resistance.
- Associate genetic regions and differences in gene expression to CSFB resistance.

1.6 General Materials and Methods

1.6.1 Rearing methodology of the CSFB

The rearing protocol developed in Chapter 1 can be seen in figure 1.4. This protocol was used to rear and maintain all insects used in experimentation throughout this project. All insects which originated from this colony were under 21 days post-eclosion when used in experiments unless otherwise stated.

The rearing protocol was successfully transferred to a collaborator (Innolea; Mondonville, France), and an independent colony was established using locally caught CSFB. This colony was established during a placement at the site, and the subsequent rearing and experimental work using this colony was performed by staff at Innolea. The Innolea CSFB culture was established using a combination of larvae-infested plants, and also using eggs produced by fertile autumn-collected adults which were taken from the field and reared in boxes for several weeks while eggs were laid, and the eggs were removed and infested onto potted plants. The same protocol was used at both sites to maintain all CSFB unless otherwise stated.

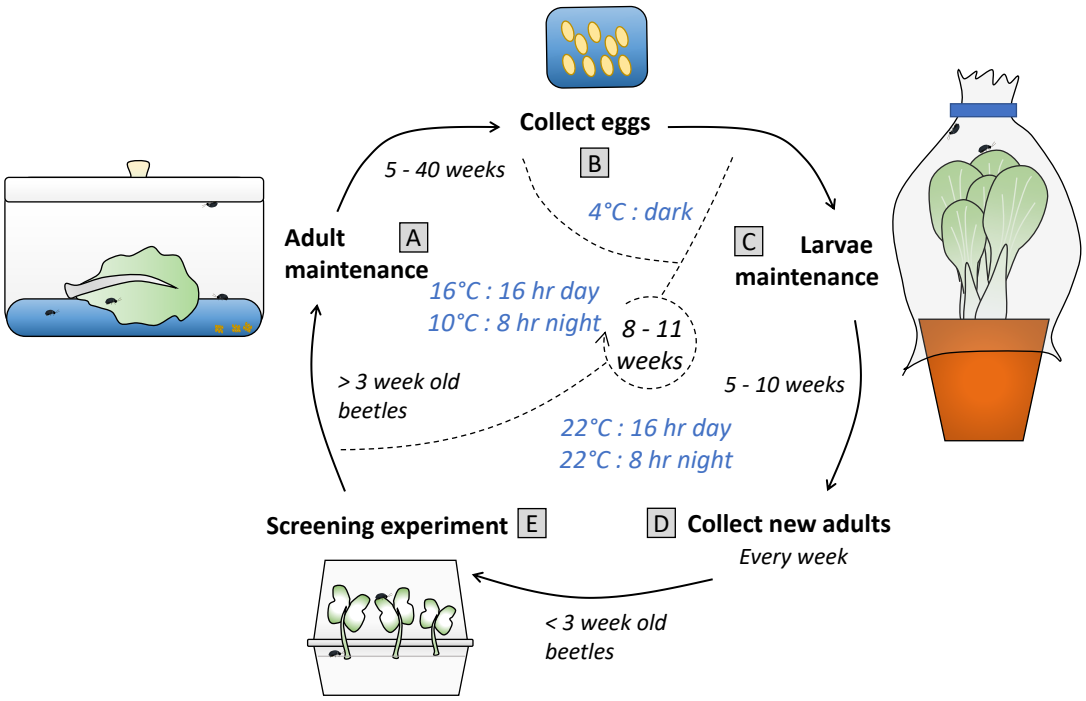

Figure 1.4: Population cycle of cabbage stem flea beetles in the captive colony, detailing maintenance stages and period for use in experiments. (A) Up to 50 adult CSFB are maintained in plastic boxes consisting of a 174 x 115 x 60 mm clear plastic container with a ventilation hole in the lid plugged with a bung, lined with a single sheet of blue roll moistened with water, and freshly cut *Brassica rapa* Pekinensis Group, var: F1 Hilton (Chinese cabbage), changed weekly. Beetles lay eggs onto blue roll beginning approximately 63 days (9 weeks) post-pupation, which are collected using a paintbrush onto dampened blue roll. (B) Eggs are maintained in a dark refrigerator for up for 4 weeks at 4 °C. (C) Eggs are reared through to adults by infesting two potted 6-8 week old *Brassica rapa* subsp. chinensis (L.) Hanelt (cv. China Choi) (pak choi), enclosed in a micro-perforated plastic bag (38 x 90 cm; Focus Packaging). Plants are watered approximately once per week, allowing soil to dry between waterings. (D) Adults are collected weekly between 35-70 days (5-10 weeks) post-infestation, with the majority collected at 40 days. (E) Beetles up to 3 weeks in age are used in experiments screening germplasm for feeding preference. Beetles over 3 weeks in age are retained for egg collection, starting around 10 weeks post-pupation and ending at death at approximately 280 days (40 weeks). All plant material is grown in pesticide free F2 compost. Beetles are maintained in a growth cabinet at 16 °C day, 10 °C night, and moved to a controlled environment room at 22 °C, for a period of 21 days starting at 8 weeks post eclosion; both 16 hr day length. Humidity was not controlled.

1.6.2 Plant growth conditions

All experimental plant material was grown in a controlled environment room (CER) at L16:D8 photoperiod at 22 °C constant. All experiments for screening germplasm against the CSFB also took place in this CER unless otherwise stated. Experimental seed was

sown in excess to allow selection of the most uniform seedlings. Twenty seed were arranged uniformly in a 0.98 L polypropylene square pot (Poppelmann Teku) containing pesticide free F2 compost (Levington), which was moistened with tap water and covered with 1 cm additional compost. Pots were placed into trays inside custom-made PVC cages, with the front covered by a magnetic thrip-proof panel, and placed into the CER for approximately 7 days. For the growth stage selected the cotyledons were fully expanded and the growing point of the first pair of true leaves was just visible. The cotyledon stage is particularly vulnerable to adult feeding, and the uniformity of the shape of cotyledons allows for increased ease of scoring compared to the true leaves. Plant material was transplanted into the experimental containers 24 hours prior to the start of any experiments to prevent the potential induction of stress responses confounding results.

Food plants for the CSFB were grown in a heated glasshouse with an approximate photo-thermoperiod of L16 (23 °C):D8 (20 °C), though temperatures did fluctuate beyond this due to the partial exposure to the external environment. *Brassica rapa* Pekinensis Group, var: F1 Hilton, (Chinese cabbage) was sown into black polypropylene pots as above, and once the first true leaves were visible (approximately 7 days later) were transplanted into individual pots of the same size. Once more than 4 pairs of true leaves had expanded (approximately 4 weeks after sowing), mature leaves were cut at the base of the petiole and placed into rearing boxes until all mature leaf tissue was harvested, usually after 2-3 weeks of use. *Brassica rapa* subsp. chinensis (L.) Hanelt (cv. China Choi) (pak choi) were maintained in the same way as Chinese cabbage, but once 4 pairs of true leaves had expanded (approximately 4 weeks later) two plants were transplanted into 5 L polypropylene pots (Soparco). Plants were maintained in these pots for a further 2 weeks before they were used for infestation with CSFB larvae.

1.6.3 Statistical analysis

All statistical analysis was performed in Genstat 20th edition version 20.1.0.23823 (VSN International Ltd.). Where transformations were used to normalise residuals, the data was back-transformed for plotting and reporting. Data filtering, processing, and plotting of graphical figures was performed with R version 4.2.1.

2. Optimising rearing of the cabbage stem flea beetle in a controlled environment

2.1 The need to establish a captive colony

Previous work testing feeding preferences of CSFB generally utilised field-caught specimens; either the captured adults (Bartlet et al., 1999a, 1994; Mathiasen et al., 2015b), adults obtained by rearing field-collected larvae (Willis et al., 2020), or allowing field-captured adults to lay eggs and rearing these through to a new generation of adult beetles (Döring and Ulber, 2020; Højland et al., 2015; Michael Koritsas, 1990). However, as the CSFB is univoltine, the adult stage is only available for approximately 6 months of the year (Ortega-Ramos et al., 2022). In addition, adult CSFB behaviour changes significantly over these 6 months. Approximately 2 months are spent in aestivation during the summer, where very little feeding occurs (Såringer, 1984). Conversely, extensive feeding is seen in the autumn on the newly sown winter oilseed rape crop in preparation for egg laying. Previous experiments have therefore preferentially used adults caught from the wild just prior to this autumn period. Although this is a good way to obtain large numbers of adults which are feeding (i.e. not aestivating), it is severely limiting to experimental throughput. Ideally, adults should be available for experimentation year-round. Using wild-caught insects also poses the risk of introducing insects with diseases or parasitoids into experiments, which may alter their behaviour. It has also previously been observed that the quantity of CSFB herbivory can vary among flea beetle populations and their stage in the reproductive cycle (Gavloski et al., 2000; Henderson et al., 2004).

It is therefore desirable to maintain a colony of CSFB in controlled conditions whereby populations can be staggered for use in experiments all year. Furthermore, insect phenology can be controlled to reduce variance introduced by using wild-caught insects. The CSFB is not widely reared in a laboratory environment, and so protocols for rearing are yet to be published. No literature has been published for rearing flea beetles for a sustained period of time (i.e. for more than one generation). Typically the adult stage is maintained in the laboratory after capture from the field only long enough to complete experimentation. The Beran group are an exception and have established CSFB colonies, though they have not published on the methodology (Ahn et al., 2019; Beran et al., 2018).

Upon starting the project, a small colony had been established at the John Innes Centre (JIC; Norwich, UK) by Jessica Hughes and Anna Jordan. This rearing protocol was modified from Beran et al. (2018). However, this colony did not lay a sufficient number of eggs to maintain the population, and insect mortality was high. Approximately 200 eggs per female are expected, with up to 1000 eggs per female reported (Mathiasen et al., 2015b; Vig, 2003). However, far fewer than this were observed in the insectary colony. This was severely limiting to experimental throughput, and it was not possible to phenotype a large panel of accessions for resistance. The environmental conditions selected for rearing

were chosen for practicality opposed to maximising fecundity. Therefore, there was scope to optimise the rearing technique, which was necessary to obtain a large, consistent, and controlled population of CSFBs. In addition, the dynamics of the colony had not been studied. It was clear that the lifecycle was altered, as egg laying occurred at a much earlier age in the insectary colony. However, when aestivation occurred, when eclosion from pupae could be expected, and when egg laying peaked had not been described. This information was also vitally important for establishing a robust rearing protocol.

2.2 Optimisation of rearing temperature

The CSFB encounters a wide range of temperature conditions of the northern hemisphere during it's roughly year-long life. The adult insect experiences a hot summer and mild autumn, while the immature stages experience the cold winter and mild spring. Although the interaction between environmental and endogenous stimuli are not understood in the development of the CSFB, high temperatures appear to negatively impact survival of the adult (Bonnemaison and Jourdheuil, 1954; Mathiasen et al., 2015b; Såringer, 1984; Schrödter and Nolte, 1954). High temperatures also decrease fecundity, potentially due to triggering re-absorption of the oocytes (Bonnemaison, 1965; Mathiasen et al., 2015b). The optimal rearing temperature for both fecundity and survival appears to be 16 °C (Mathiasen et al., 2015b). Egg laying per female at this temperature was 665 eggs/female, and decreased above this to 371 eggs/female at 20 °C. 50 % survival was 186 days at 16 °C, but just 78 days at 20 °C. This suggests that the rearing temperature of 22 °C used to maintain CSFB at the John Innes Centre was a limiting factor to fecundity and survival of the colony.

The previous experiments testing temperature on CSFB survival and fecundity used CSFB populations from Denmark (Mathiasen et al., 2015a,b). A population of CSFB initiated from a UK population may possess similar environmental requirements, as the climates of Denmark and the UK are alike. However, there may be important genetic differences between populations influencing environmental preference, as flea beetles are not believed to migrate long distances (Bonnemaison, 1965). It is important to verify if there are optimal rearing temperatures for CSFB and if these align with those previously reported using a CSFB population.

Furthermore, these rearing experiments have only tested adults collected at the end of the summer after they have lived 3-4 months of their adults lives (Mathiasen et al., 2015a,b). This is because CSFB can be found in abundance during the late summer (July/August) and are easiest to collect from the crop at this time. These autumn-collected adults have already experienced hot temperatures of summer. In Norfolk, UK, the maximum daytime temperature for the last 30 years in July and August has averaged 22 °C (Met Office, 2020). Although previous studies have found that these temperatures negatively impact fecundity and survival of older adults, the fact that these insects do

experience these temperatures in the wild raises the question of whether they are better adapted to these high temperatures when at a younger age. Only Såringer (1984) tried testing the impact of different temperatures on survival of newly eclosed adults. However, only temperatures from 18 °C to 28 °C were assessed and so these conditions were not compared to the potentially more optimal 16 °C. In addition, these temperatures were applied throughout the life cycle, and were not varied to simulate the natural seasons. The number of eggs per CSFB at all temperatures was significantly lower than the control insects kept outdoors due to high mortality. The need to test variable temperatures on survival and fecundity has been previously highlighted by Mathiasen et al. (2015b), as all previous experiments have only tested fecundity and survival at constant temperatures (Bonnemaison and Jourdheuil, 1954; Mathiasen et al., 2015b; Såringer, 1984).

High summer temperatures appear to accelerate the developmental rate of the ovaries in the cabbage stem flea beetle in wild populations (Bonnemaison, 1965). In addition, the development of the ovaries peaks in September after the diapause is finished (Bonnemaison and Jourdheuil, 1954). The period of CSFB oviposition is also earlier if post-aestivated CSFB are maintained at warmer temperatures (Mathiasen et al., 2015b). Increased temperatures have been found to increase the rate of gonad development and increase fecundity in insects such as butterflies, fruit flies, and mealybugs (Barker and Herman, 1976; Hodin and Riddiford, 2000; Zhang et al., 2021). Potentially then, a higher temperature during gonad development may be a requirement to increase fecundity in captive CSFB populations. As temperature can clearly impact CSFB sexual maturity and egg laying, it is clearly of interest to investigate if temperature can be exploited to optimise fecundity.

It was hypothesised that rearing at a cool temperature could significantly prolong CSFB lifespan to maintain a captive colony. In addition, constant rearing at cool conditions from the moment of pupation was thought to be insufficient to initiate egg laying, as high aestivation temperatures may be necessary to initiate ovary development.

To test if a cooler temperature was preferential for survival of a UK-sourced population of CSFB, wild-caught CSFB adults were reared at a cooler temperature, alongside CSFB maintained at 22 °C as previously at the JIC insectary. An additional experiment was performed to test if a period of heat would increase fecundity and survival. A short period of increased temperature was given to simulate summer during aestivation at different time points in the CSFB adult life cycle. It was believed this would better reflect the cool/hot/cool, spring/summer/autumn conditions experienced by adult CSFB in the wild.

2.3 Dynamics of the captive colony

In addition to optimisation of the rearing technique, it was vital to understand the dynamics of the captive colony to develop a robust rearing protocol. The specifics of

how the life cycle was altered by captive rearing were not understood. For example, how many eggs could be expected and when, how long adults survived, if egg viability changed with adult age, how many adults could be expected and when, or when the aestivation period may take place. There was also concern that adult feeding may change with the age of the adult, but at what time feeding may be highest was also unknown. Understanding these dynamics was necessary for planning large-scale phenotyping screens using CSFB performed in later chapters.

It is important to know when new adults will be available for experimentation. Therefore, adult emergence from plants infested with eggs was tracked. This also allowed for identification of the optimal number of eggs to infest plants with, as well as a basic understanding of how long eggs can be stored in cool conditions before viability of these eggs decreases, reflected in the reduction of adults produced. Previous research found that larval and pupal development of the CSFB are linked to temperature, and higher temperatures decrease the time for development (Kaufmann, 1941). It was hypothesised that rearing the immature CSFB at 22 °C would decrease the time taken to obtain adults for use in experimentation.

The aestivation period of the CSFB is believed to be genetically fixed, called "prospective diapause", as it cannot be prevent by changes in temperature, day length, or food availability (Såringer, 1984). During aestivation feeding almost completely ceases, and so at this stage the CSFB cannot be utilised for assessing resistance of plants to CSFB herbivory. In the UK the aestivation period lasts for approximately 4 weeks, beginning after the harvest of mature OSR in July and ceasing in August after sowing of the new crop (Alford, 1979; Såringer, 1984). However, as the CSFB life-cycle appeared shortened in the laboratory culture established previously, the exact time of aestivation was unknown. It was therefore necessary to determine when the aestivation period occurs by measuring when feeding ceased.

2.4 Aim and objectives

The aim of this study was to characterise the life cycle of the cabbage stem flea beetle (*Psylliodes chrysochepala*) in the laboratory to obtain controlled population for use in experimentation. The objectives were to record how insects responded to different environmental conditions to optimise rearing to obtain a culture of CSFB which could be maintained in perpetuity. This chapter also aimed to understand the key life stages of the CSFB in a reared colony to identify when adults would be available, and when adults feed to allow for planning of experiments using CSFB in future work. The different aspects of the flea beetle life cycle were recorded; the numbers of eggs laid and when egg laying occurred, and whether the number was influenced by adult age. Also, the egg hatch and adult eclosion success rates, and the magnitude of feeding with adult age, were recorded.

2.5 Materials and Methods

2.5.1 Initiation of the Colony

A laboratory culture of CSFB was established from two collections in the east of England. Approximately 2,000 adult cabbage stem flea beetles were gathered from a heavily infested WOSR crop near Peterborough, UK (July 2019). The mature crop was shaken into a high-sided bucket to dislodge the adults, and they were removed with an insect aspirator into 50 mL centrifuge tubes plugged with a foam bung.

Adults were maintained in 174 x 115 x 60 mm clear polythene boxes with four 1 cm diameter ventilation holes drilled into the lid covered in 0.3 mm fine mesh, and an access hole plugged with a bung in the center of the lid. The base of the boxes was lined with a sheet of blue roll moistened with water, and freshly cut mature leaves of *Brassica rapa* Pekinensis Group, var: F1 Hilton, (Chinese cabbage) was provided as food in excess and changed weekly. Adults were quarantined for 4 weeks at ambient conditions in the laboratory to allow for larvae of the parasitoid wasp *Microctonus brassicae* to emerge and be removed from the remaining population (Jordan et al., 2020). Insects were then moved into a controlled environment room at 22 °C, with 16 hr day length. Humidity was not controlled.

The second collection was of larvae-infested material. 40 whole WOSR plants infested with CSFB larvae were removed from a field in Cambridgeshire, UK (November 2019). To remove other insects from the field, all above-ground plant tissue was submerged upside down for approximately 10 seconds into a large bucket filled with SB Plant Invigorator (Stan Bourard Limited), prepared according to package instructions. Plants were potted and enclosed in a micro-perforated plastic bag (38 x 90 cm; Focus Packaging), and placed into trays in an unheated glasshouse. Plants were bottom-watered as required, approximately once weekly. In May 2020 adults began emergence. All adults were removed from each plant at least every 7 days, and were stored in boxes as above.

2.5.2 Adult survival at two rearing conditions

The CSFB collected at the adult stage were used to test if temperatures cooler than 22 °C was preferable to increase CSFB adult survival. At the end of the four weeks quarantine, for each treatment (n=3) boxes of 51 beetles each were placed into either a growth cabinet at 16 °C day, 10 °C night, or a 22 °C CER, both with 16 hr day lengths (for a total of n=6 boxes). Approximately once every 7 days the beetles were moved to a fresh box, and surviving adults were recorded. The experiment was halted after 67 days to rescue the individuals in the treatment with high mortality, and allow them to lay eggs to continue the colony. The difference in the dynamics of survival over time was analysed using a repeated measures ANOVA. Due to low repetition, sphericity could not be calculated

as the variances between time points were not similar, and so any potential violation of sphericity was corrected by using a Greenhouse-Geisser correction.

$$Survival = T \times S \times W$$

Where T is the temperature treatment considered as a factor, S is the subject/individual box of beetles as a factor, and W is the week/timepoint of recording.

2.5.3 Survival and fecundity at variable temperatures from eclosion

The impact of temperature on the total number of eggs laid per beetle has not previously been assessed for adults from the point of eclosion, only on older adults. The F1 generation of adults, obtained by rearing the wild-caught CSFB adults, were used to compare two temperatures for rearing, in addition to a variable temperature treatment to more closely simulate natural environmental conditions. The wild-caught adults laid eggs onto the blue roll in boxes, and these were transferred to clean moistened blue roll using a fine-tipped paintbrush. The eggs were reared through to adults by placing them at the base of two 6-8 week old *Brassica rapa* subsp. chinensis (L.) Hanelt (cv. China Choi) (pak choi) in a 27 cm diameter pot of pesticide free F2 compost (Levington), enclosed in a micro-perforated plastic bag (38 x 90 cm; Focus Packaging). The bag was taped up to the rim of the pot with PVC tape to prevent beetles falling under the pots. Infested plants were then maintained in the 22 °C CER. Every 7 days all adults were removed, and adults of the same age (± 7 days) were combined into boxes as above and allocated to either two constant conditions, or one of 7 treatments where the temperature was varied in the same way at different ages. For the treatments with variable temperatures, boxes initially started at 10 / 16 °C, then were moved into 22 °C for a 21 day "heat treatment" at different ages: 4, 5, 6, 7, 8, 9, or 11 weeks post-eclosion. It was hypothesised that coinciding the temperature change with a physiological readiness for sexual maturity was necessary, but at what age this occurred was unknown. Hence testing this temperature increase at different ages was needed.

In this preliminary screen, the treatments were replicated at most twice. The controls were maintained at a constant condition of either 10 / 16 °C, or 22 °C. Approximately 55 beetles were used per replicate, however this number varied with adult availability. Approximately every 7 days, fresh food and blue roll was provided and the number of eggs was counted for each box until all insects died at 293 days (42 weeks) post-eclosion. As the number of adults in each box was variable, all egg count data is normalised per beetle. Note that the sex of the insects was not controlled, though the sex ratio is approximately 1:1 for CSFB (personal observations, unpublished). For this preliminary experiment the results were not analysed statistically due to low repetition. Instead, the results were used to indicate the most promising conditions for further repetition.

This heat treatment experiment was repeated at the condition that appeared most favourable to fecundity, controlling for insect sex and number. Adults were collected from

the larvae-infested material harvested from the field, and maintained as described above. Every 7 days the bags were emptied completely. CSFB were sexed using the first tarsal segment of the front pair of legs (Bonnemaison and Jourdheuil, 1954), and 5 females and 5 males collected of the same age (± 7 days) were combined into boxes as above. (n=3) Boxes were allocated to either the constant 10 / 16 °C growth cabinet, the 22 °C CER, or maintained at 10 / 16 °C with a 21 day heat treatment at 22 °C at 9 weeks post-eclosion. Every 7 days the number of eggs laid and adults remaining were counted. A one-way ANOVA was used to compare the total number of eggs laid per beetle between the different rearing conditions. A one-way ANOVA was also used to compare the time-point when 50 % mortality occured for the three treatments.

This heat treatment was also replicated by collaborators at a partner site (Innolea). Between 8 and 150 beetles of the same age (± 7 days) were collected and allocated to a box (n=7). The insects were reared at 10 / 16 °C with a 21 day heat treatment at 22 °C constant at 8 weeks of age. From the date of eclosion until beetles death, beetles and eggs were recorded every 7 days (for between 232 - 289 days). The data collected from this independent colony aimed to replicate the heat treatment experiment with an independent colony. The number of eggs produced at the collaborators site was recorded and compared. A two-way ANOVA was performed to compare the number of eggs per adult between the two sites. A square root transformation was used for the total eggs per beetle to stabilise variances.

2.5.4 Dynamics and viability of egg laying in captive populations

The colony at Innolea was also used to assess the dynamics of egg laying over time in a captive population by the collaborators. It was hypothesised that the number of beetles maintained together may influence egg laying, as lower egg laying was observed here than had previously been reported and a major difference was the number of insects (Mathiasen et al., 2015a,b). Therefore, the number of eggs laid per insect were compared against the number of beetles within the box at for every reading. 16 boxes in total were measured, and the number of insects placed in each box varied from 18 to 122. All boxes were reared at 10 / 16 °C with a 21 day heat treatment at 22 °C constant at 8 weeks of age. The first data point was when the insects first laid eggs. Recordings before this period were excluded to prevent superfluous readings at 0 when the adults weren't yet old enough to lay eggs. The number of insects and the number of eggs were counted approximately every 7 days. The impact of the insects age was also considered here, as there was potential that fecundity would vary with beetle age. Measuring how insect age impacted fecundity would both help to explain how beetle density impacts fecundity, and be useful for colony maintenance.

A generalized linear model was performed, which used poisson regression with a log link function, to predict the number of eggs laid per adult compared against the number

of adults in the box, and the age of those adults. This model was selected because the relationship between density and egg laying was exponential, the variance of Y (eggs laid per adult) was not constant in regard to X (adults per box), and because Y would not be a negative number.

$$Y = \beta + D \times A + \epsilon$$

Where β is the random intercept, D is the density of beetles, A is the age of the beetles, and ϵ is the random error term.

The viability of eggs was also measured by collaborators at Innolea. Eggs were collected using a paintbrush and transferred to damp blue roll in petri dishes, and maintained at 4 °C in a fridge. The number of eggs that hatched per batch was recorded by viewing them under a microscope. Batches of between 31 and 680 eggs were tested (n=33). The mean hatch success was found by calculating the mean across all replicates. The impact of the age of the adults that laid the eggs was also assessed (n=31). Two batches were excluded as the age of the adults was not known. A simple linear regression was used to calculate if adult age significantly altered egg viability, and the impact of this. The model included adult age and egg number per batch to account for potential egg-density effects. A square transformation was applied to the percent of eggs hatched data to normalise the residuals.

$$Y = \beta + A + \epsilon$$

Where β is the random intercept A is the age of the beetles, and ϵ is the random error term.

2.5.5 Dynamics of adult eclosion in captive populations

Once plants were infested with eggs, the next step was to measure the number of adults, and when they eclosed. The number of adults emerging from infested plants was assessed at two sites, JIC and by collaborators at Innolea. At JIC, between 47 and 380 eggs were placed onto potted plants (maintained as above) consisting of two 6-week-old *B. rapa* subsp. *chinensis* (*n=29*). Plants were sampled approximately every 7 days, though the sampling rate was increased when emergence was at its peak. All replicates had successful collections at atleast three time-points. Bags were confirmed empty of adults for a period of at least 14 days before being discarded. At the Innolea site, between 270 and 990 eggs were eggs were placed onto *B. rapa* subsp. *chinensis* (*n=13*), and collection of adults was halted at 47 days post-infestation. More eggs were available at Innolea as a team of three researchers were involved in rearing at this site, whereas only one undertook the work at JIC.

When measuring the dynamics of eclosion over time, only replicates where the first time point of adult collection occurred at most 42 days post-infestation were included (*n=26*

at JIC, and *n=13* at Innolea). At the Innolea site, bags were sampled with a higher frequency around the date of eclosion, approximately every 1-3 days. A LOESS-smoothed line of best fit was fitted with an alpha value of 0.5, combining data from both sites. The maximum peak of the moving average demonstrated the peak in eclosion.

A t-test was used to compare the mean percent of adults that eclosed from added eggs between the two sites. It was hypothesised that there would be no difference between the two sites if the protocol was robust. This comparison also gave additional confirmation of the expected percent of adults that should be collected from a given number of eggs.

The number of larvae (or "larvae load") that the two 6-week-old *B. rapa* subsp. *chinensis* could support was assessed by comparing the number of adults collected to the number of eggs added. The maximum larvae load was determined by fitting a linear correlation, and observing deviation in the number of adults emerging from this linear fit as the number of eggs increased. A decline in the number of adults emerging suggests that the maximum larvae load has been reached.

2.5.6 Feeding variability with insect age

An F3 population of adult CSFB 0 to 7 days post-eclosion were obtained through rearing adults captured in July 2019 from near Peterborough, UK using the methodology outlined in the general materials and methods. These third-generation adults were screened for area eaten against *B. napus* variety "Arrow" every 7 to 14 days and scored quantitatively using the method developed in Chapter 2. Briefly, for each replicate six 7-day-old whole seedlings were transplanted into water agar, and four adult CSFB were added for 48 hours. Damage was scored destructively by quantifying the percent area of leaf area missing at the end of the 48 hours using macros developed in ImageJ. Initially (*n=6*) replicates were used, but as beetles died during the experiment this declined to (*n=4*) at 21 days old, and (*n=3*) at 115 days. The experiment was halted at 115 days (16 weeks) when too few beetles remained to continue. The change in feeding over time was analysed using a one-way ANOVA, comparing percent area eaten to the age of the insects (as a factor). A log transformation was applied to the percentage eaten to ensure normal residuals. A Bonferroni post-hoc test was used to compare the area eaten between weeks, and determine at what time CSFB feeding was at is highest and lowest.

2.6 Results

2.6.1 Adult survival at two rearing conditions

When rearing adults collected from the wild post-aestivation, a significant reduction in survival was seen for adults reared at 22 °C compared to 10 °C / 16 °C [Fig. 2.1]. When CSFB were reared at 22 °C, 50 % survival at 22 °C was 28 days, whereas 50 % mortality was

not reached within the experiment for the 10 °C / 16 °C treatment, though it was close at 46 % mortality when the experiment ended at day 67. Survival in the population maintained at 22 °C decreased faster, and this decline was seen after just the first measurement at 7 days. For both treatments, the rate of mortality was consistent over time, though the rate of mortality in the 22 °C condition was immediately and consistently higher; when a linear regression was fitted, the hot (22 °C) treatment has a gradient of -4.7622 adults per week, which was more than double the rate of mortality in the cool (10 °C / 16 °C) treatment where the gradient was -2.0924 adults per week. From the repeated measures ANOVA ($\epsilon = 0.2136$), there was a significant decrease in the number of insects over time in both groups, as expected ($F = 24.554$, $P < 0.001$). There was also a significant difference between the gradients, showing that there was a difference in the rate of mortality between the two rearing temperatures ($F = 7.16$, $P = 0.017$).

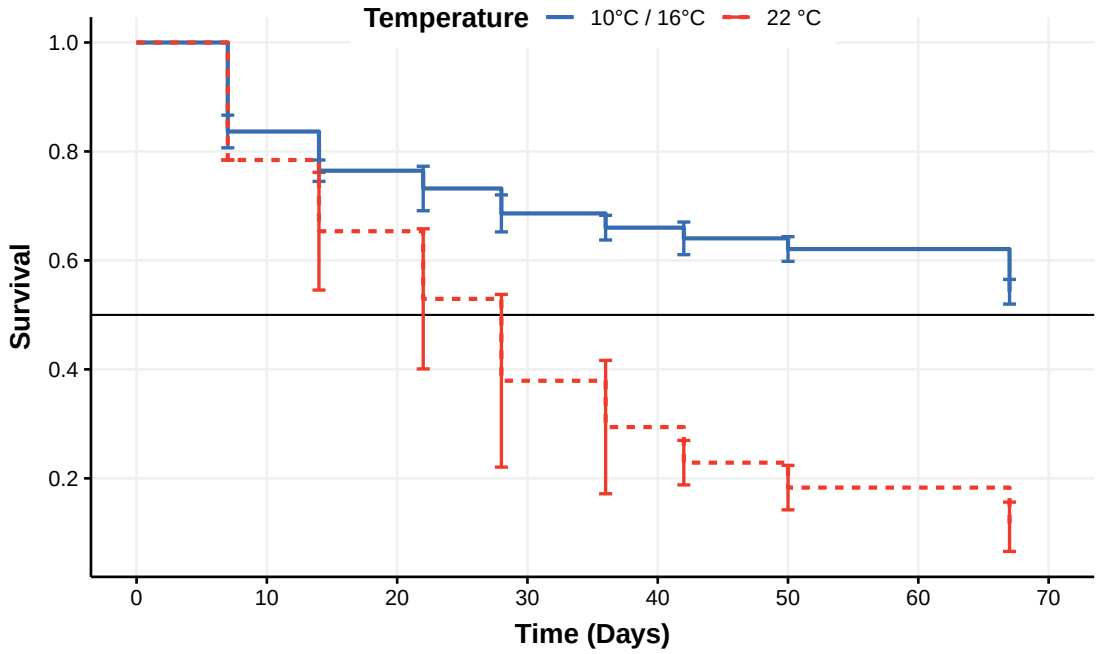

Figure 2.1: Mean survival for adult CSFB collected post-aestivation and reared using two conditions, either at 22 °C constant or 10 °C day / 16 °C night, with 16 hr day length for both treatments (n=3, 51 individuals each). The horizontal line shows 50 % survival. Error bars show the standard deviation. Insects were collected from the wild in July at several months old (post-aestivation).

2.6.2 Survival and fecundity at variable temperatures from eclosion

In the preliminary experiment testing fecundity during variable temperature treatments, egg laying increased greatly for beetles given a heat treatment at 8 or 9 weeks post-eclosion [Fig. 2.2]. 350 eggs per adult were obtained from the 9-week heat treatment, and only 26 from the control maintained at 10 / 16 °C. A heat treatment at 8 weeks appeared to have

a similar impact as 9 weeks, increasing egg laying to 280 eggs per adult. A smaller increase in egg laying was seen when heat treatments occurred at 4 or 5 weeks post-eclosion, to 61-105 eggs per adult. A modest level of egg laying was also seen at 11 weeks, where 61 eggs per adult was obtained. However, at 6 or 7 weeks, egg laying was similar to the controls, as only 14-27 eggs per adult were laid. For insects reared at 22 °C constant, egg laying appeared to be higher than when reared at 10 °C / 16 °C, with 97 eggs laid opposed to 26 ± 22 (SD). These preliminary results suggest a high treatment at 9 weeks may increase fecundity, and were used to select conditions for testing further.

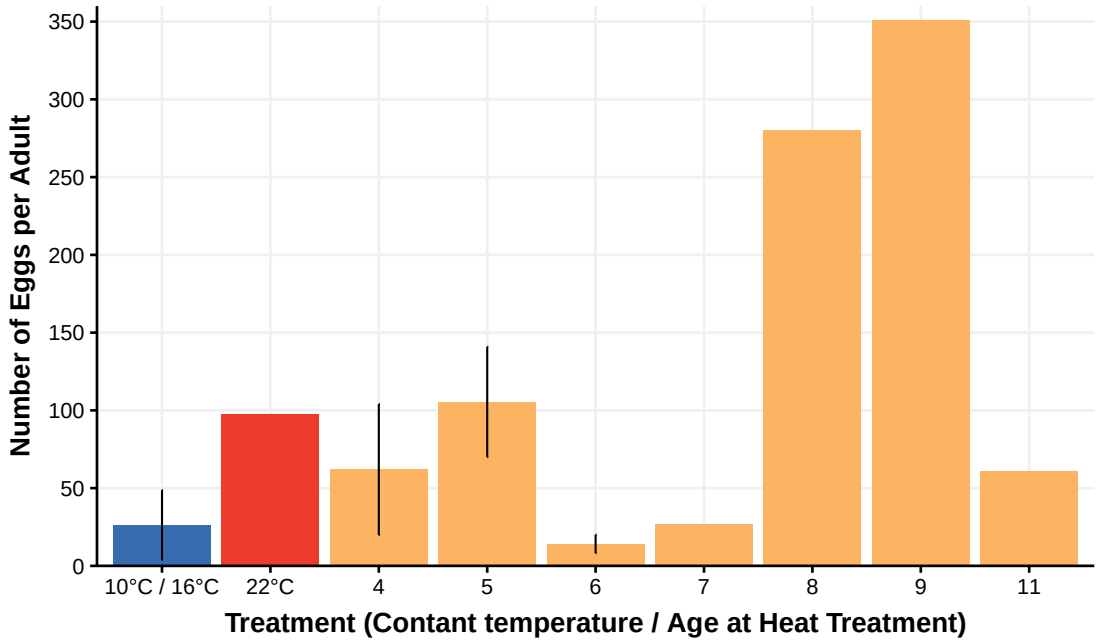

Figure 2.2: Mean number of eggs laid per CSFB by CSFB adults during their lifetime, when given heat treatments at different times post-eclosion. (n = 1 For the 22 °C control, 7, 8, 9, 11 weeks. n = 6 for 10 / 16 °C control. n = 2 for 4, 5, 6 weeks). Where more than 1 replicate was available, error bars show the standard deviation.

A repeated measures ANOVA was performed to test for differences in survival over time for the three groups. As expected, there was a significant difference in beetle number over time, demonstrating that beetles died during the 300 day experiment ($F = 69.88$, $P < 0.001$). There was also a significant difference in the number of beetle over time between groups, demonstrating that mortality was different between some of the treatments ($F = 3.03$, $P = 0.044$). This difference is small, and the large amount of variance between replicates of the same treatment can be clearly seen [Fig. 2.3]. The rate of beetle mortality is not different before, during, or after aestivation. Instead a constant decline in survival is seen from 21 days. Observation of the data suggests that this difference in groups is due to an increased rate of mortality in the insects reared at 22 °C constant, compared to the two other groups (a heat treatment at 9 weeks post-eclosion, and 10 / 16 °C), which

had very similar survival dynamics.

A one-way ANOVA was used to compare the age at which 50 % mortality occurred between the three treatments. There was no significant difference in when 50 % mortality occurred ($F = 1.92$, $P = 0.227$) [Fig. 2.3]. The mean number of days until 50 % mortality was 88 days for the 22 °C treatment, but 141 days for the 10 / 16 °C treatment, and 164 days for the insects that received at heat treatment at 9 weeks post-eclosion. It appears that 50 % survival occurs much sooner when reared at 22 °C than in the cool condition and condition with a "heat treatment". This difference is 1.60x to 1.86x faster mortality in the 22 °C treatment. However, the variance between replicates was very high and so this was not significant. However, as the rate of mortality was significantly different between groups, assessed using the repeated measures ANOVA above, this data does suggest that there may be a difference in the 22 °C treatment which was not captured here due to low replication. This would support the data collected in figure 2.2, and also in previous experiments (Mathiasen et al., 2015b), that rearing CSFB at 22 °C causes increased mortality compared to moderately cooler temperatures. Variability may have been greater for this experiment than that performed used wild insects (figure 2.1) as fewer insects were used here, so each beetle contributed a greater weight to the mean survival rate.

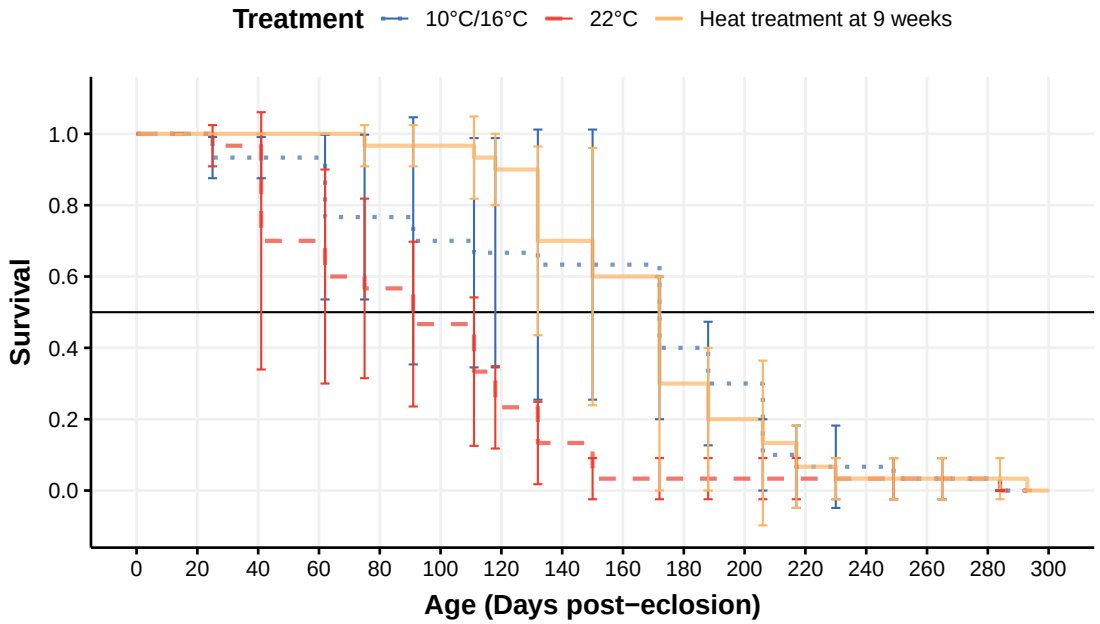

Figure 2.3: Mean survival for adult CSFB collected post-aestivation and reared at three conditions $n = 3$, 10 individuals per replicate). Either 10 / 16 °C, 22 °C, or a heat treatment at 9 weeks post-eclosion where adults are moved from 10 / 16 °C to 22 °C for 21 days. The horizontal line shows 50 % survival. Error bars show the standard deviation. Age is ± 7 days.

There were no significant differences observed in fecundity for the three rearing treatments (when eliminating site as a variable) ($F = 1.43$, $P = 0.276$) [Fig. 2.4]. Although it appeared that egg laying was highest for beetles maintained at 10 / 16 °C (42.60 ± 35.65 eggs), then egg laying was reduced slightly for adults given a 3-week long heat treatment at 9-weeks of age (34.75 ± 24.10), and egg laying was lowest for adults maintained constantly at 22 °C (4.77 ± 11.93 eggs), this was not significant. The only occurrences of no egg laying was within two replicates of the 22 °C. These insects never laid eggs despite reaching egg laying age. However, the variation between replicate boxes was very high despite controlling for beetle age and sex. This variance also led to the mean number of eggs laid per adult to be much lower than in Fig. 2.2, where for the most part only 1 to 2 replicates were collected.

The two sites were compared for just the rearing condition of a heat treatment at 9 weeks of age. These results were similar, as there was no significant difference in the number of eggs laid between the two sites ($F = 0.07$, $P = 0.798$). As only one treatment was tested at Innolea, the interaction between treatment and site could not be calculated. Despite increased replication at Innolea (n= 7, compared to n=3 at JIC), the variance around the mean was similar at the two sites. For the Innolea population, the number of beetles placed in each box at the start of the measurements was a range from 8 to 150, unlike at the JIC site which used 10 per replicate. It was therefore hypothesised that beetle density may have influenced the number of eggs laid and contributed to this high level of variance. Therefore, the number of beetles and the eggs laid per beetle were compared.

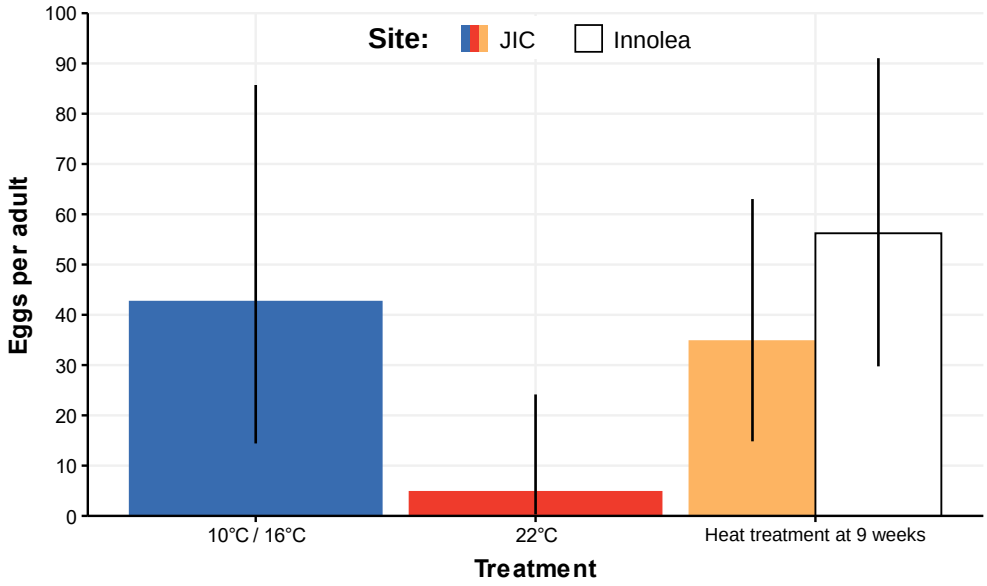

Figure 2.4: Total eggs laid per adult for three treatments. Either 10 / 16 °C, 22 °C, or a heat treatment at 9 weeks post-eclosion where adults are moved from 10 / 16 °C to 22 °C for 21 days. The 9 week treatment was replicated at two sites, either JIC (n=3) or Innolea (n=7). 10 individual insects composed each replicate at JIC, but the number of individuals at Innolea varied from 8 to 150. Error bars are the predicted standard error.

2.6.3 Dynamics and viability of egg laying in captive populations

The number of adults and the number of eggs laid every 7 days was compared for 16 replicate boxes at Innolea. The density of adults maintained together significantly impacted fecundity of the adults [Fig. 2.5]. There was negative, exponential correlation between the number of eggs laid per adult and the density of adults within a box (R^2 = 0.45). The most successful adults were maintained in boxes of fewer than 10 adults. The log linear regression analysis demonstrated that the age of the adults did not impact the number of eggs laid per adult ($P = 0.288$), and also did not significantly interact with the number of adults (t^* = -0.62, $P = 0.537$). Therefore, age was removed as a parameter and the model was re-run to compare the number of eggs laid per adult to the number of adults in the box only. From this log linear regression analysis, the number of adults in a box (i.e. beetle density) had a large and significant impact on the number of eggs each adult lays (predicted y-intercept = 7.374 ± s.e. 1.161, $P < 0.001$).

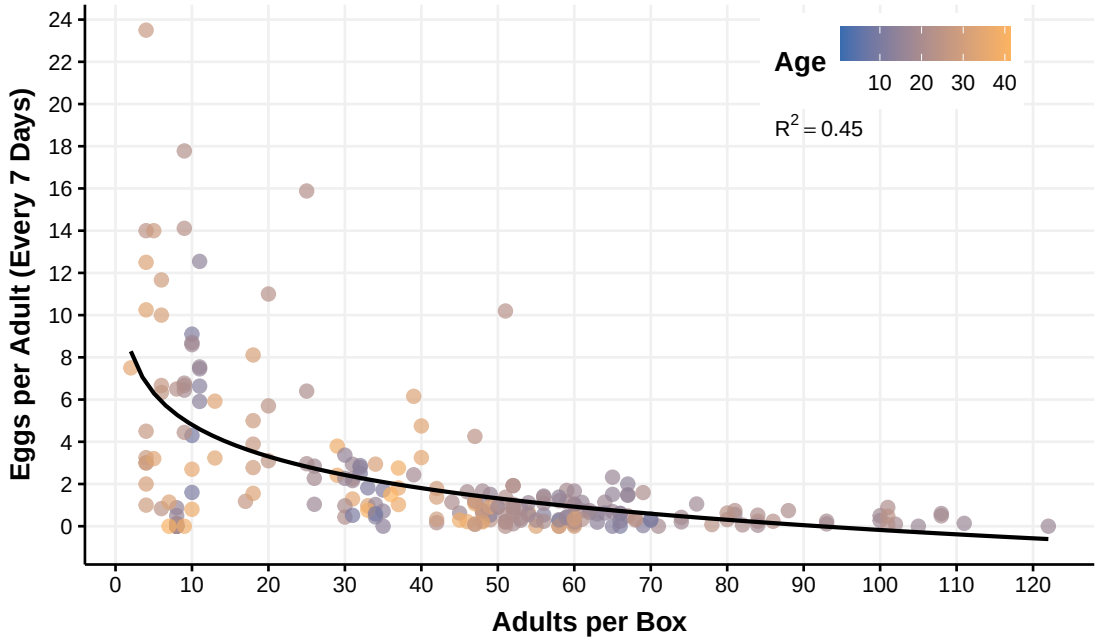

Figure 2.5: Adults per box (beetle density) compared to the number of eggs laid per adult within that box in the seven days since the last recording, for (n=16) boxes of adults. An exponential line of best fit is fitted to the data, and the R^2 represents the fit of the values around this exponential line. The age of the adults (weeks post-eclosion) at the time of egg collection is also shown as a gradient from 0 weeks (blue) to 40 weeks (yellow).

Once adults started laying eggs, they were laid consistently until they died [Fig. 2.6]. Of the 16 boxes tested here, CSFB first started laying eggs at 63 days, and continued up to 290 days. The number of eggs laid appears to be dependent on age, but this was not found to be significant in the previous analysis. Eggs laid per adult starts at around 1 egg per adult at 63 days, increasing to 2 eggs per adult by 120 days. There is a broad peak of more than 4 eggs per adult laid for adult between 160 and 220 days of age. Past 220 days, eggs laid per adult declines rapidly until egg laying stops at approximately 280 post-eclosion. Although egg laying appears to peak in the middle of the adult life, this peak is due to differences in beetle density.

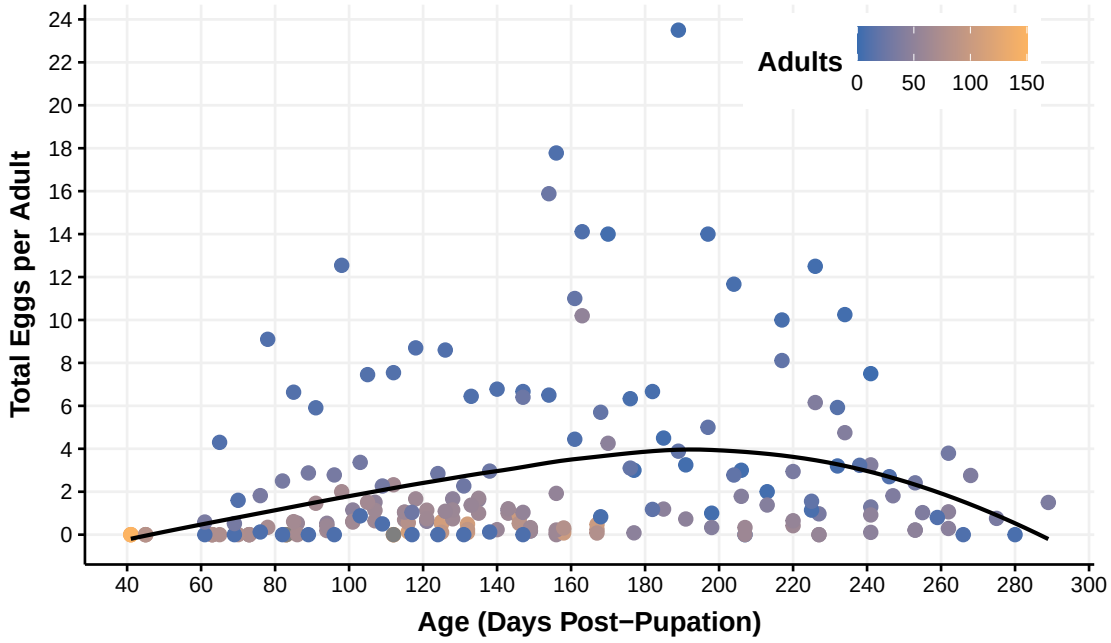

Figure 2.6: Eggs laid per adult cabbage stem flea beetle over the course of the adult life, for a laboratory-maintained colony. (n=16) replicate boxes were tested, and the number of adults varied over time shown as a gradient from 1 beetle (blue) to 150 beetles (yellow). Boxes initially started with between 8 to 150 beetles. A LOESS-smoothed line of best fit is plotted with an alpha value of 1.

The viability of eggs compared to the age of the adult that laid them was analysed using a linear regression model, where the predictors were the age of the adults that laid the eggs, and the number of eggs per petri dish. A mean of 38.3 % (SD: ± 15.6 %) of eggs hatched into larvae. The number of eggs stored together on a petri dish did not significantly impact the % of eggs that hatched ($t(26) = 1.32$, $P = 0.197$). Therefore it does not appear that eggs are able to influence the hatching success of their neighbours when stored in this way. The linear model was re-run with the number of eggs per dish removed. Egg viability is significantly decreased in older adults [Fig. 2.7] (t(27)-5.57, s.e. 0.0175, $P = 0.026$). Viability of the eggs from the youngest adults was approximately 46 %, but just 28 % for the oldest adults. For every day increase in age, the number of eggs hatched decreased by 0.19 % ± 0.03 %. Therefore, the viability of eggs from the oldest adults was 43.13 % lower than eggs laid by the youngest. However this is not a strong correlation ($R^2 = 0.14$). Therefore, there were clearly other variables not measured here that impact hatch rate.

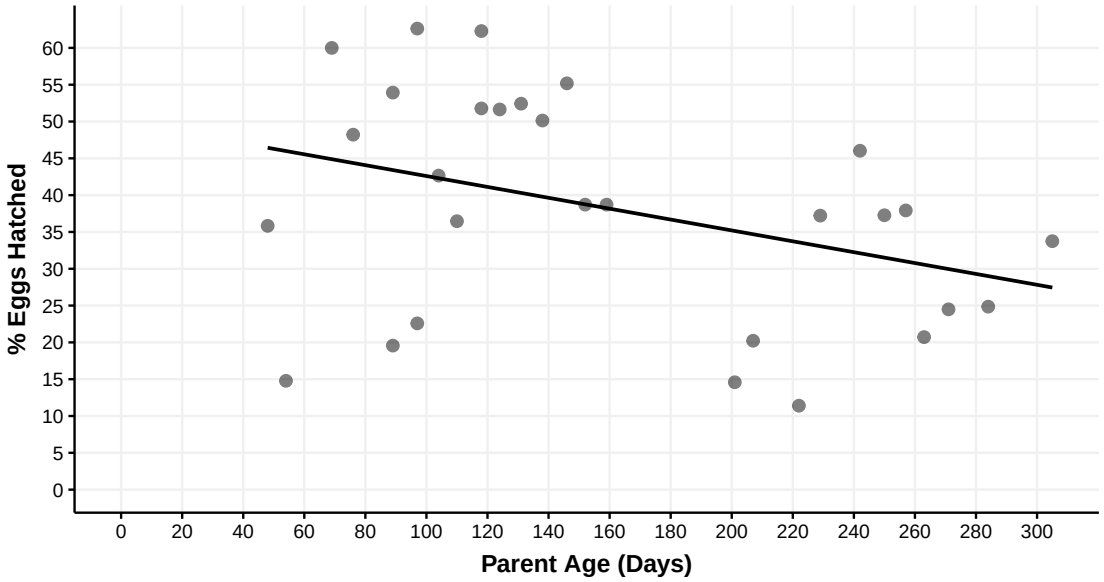

Figure 2.7: Percent of eggs that hatched from adult cabbage stem flea beetles of different ages in the captive population (*n=33*). Age is days post-eclosion.

2.6.4 Adult emergence in captive populations

The number of adults that eclosed after infesting plants with eggs was counted and compared between the two sites. The number of adults that eclosed from plants infested with eggs was 24 % ± 16 % (SD) of eggs in the JIC population, and 31 % ± 8 % (SD) in Innolea's population. The mean number of adults eclosing was not significantly different across sites ($F = 3.49$, $P = 0.066$) [Fig. 2.8].

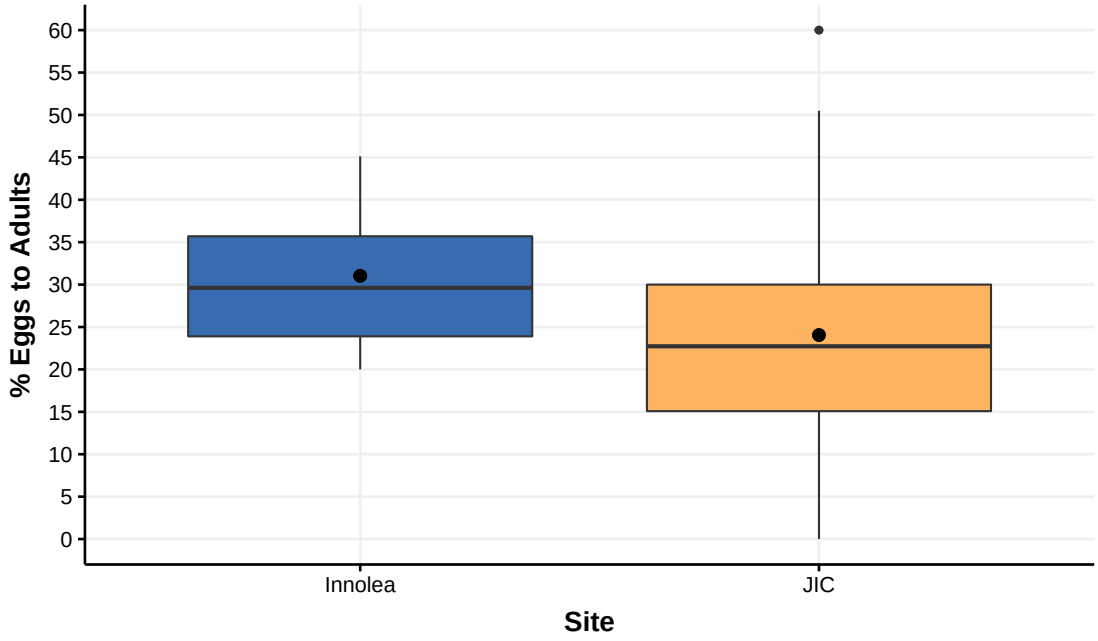

Figure 2.8: The percent of eggs that successfully eclosed as adults at two sites, the John Innes Centre (JIC; *n=29*) and Innolea (*n=13*).

When assessing the dynamics of adult eclosion over time, there is a clear peak in adult emergence at 42 days (6 weeks) post-infestation with eggs [Fig. 2.9]. This peak was the same across both sites, though the sampling rate at Innolea was slightly higher and so the peak is more precise at this site, at 40 days. The lower peak in the data at Innolea is due to this higher sampling rate. The broader peak at JIC site due to different sampling frequencies, and the peak emergence is spread across 40-50 days post-infestation. JIC data also sampled up to 100 days, though only stragglers were collected past around 60 days which may have been insects that were missed during earlier collections, as beetles are easily hidden among the plants or soil.

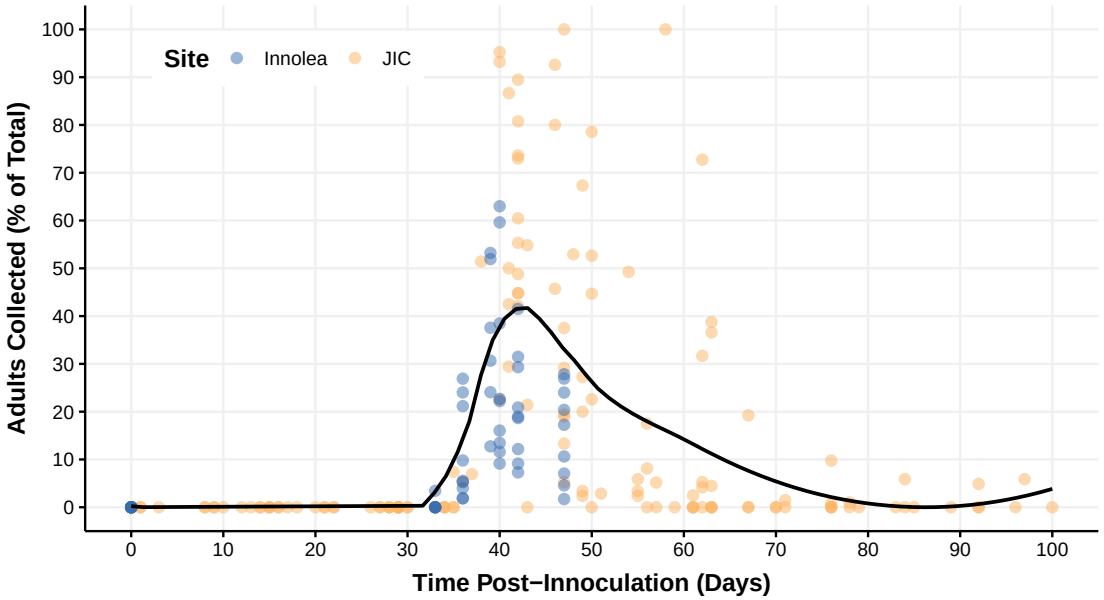

Figure 2.9: Adult eclosion over time from plants infested with CSFB eggs, measured at two sites, the John Innes Centre (JIC; yellow; *n=26*) and Innolea (*n=13*; blue). A LOESS-smoothed line of best fit is plotted with an alpha value of 0.5.

The plants used here to rear the immature stages of the CSFB through from eggs to adults were sufficient to support up to 990 eggs without a decline in the success rate. A simple linear regression model was used to compare the number of adults that eclosed to the number of eggs added onto plants for the two sites. The regression was not significantly different across the two sites, demonstrating that the maximum larvae load was not different between the two sites ($P = 0.758$). Site was removed from the model, and a new model was fitted using only egg numbers as a predictor of the number of adults collected. There was a significant linear regression, demonstrating that the number of adults collected was significantly impacted by the number of eggs added ($F = 178.91$, $P < 0.001$). The R^2 of the linear regression was 0.81, which suggests that a linear correlation is a good fit and that there was no decline in the rate of adult collection for the numbers of eggs used for infestation here. Therefore the maximum larvae load did not appear to be reached using 980 eggs. Although the number of adults produced was not impacted, at such a high density the increased competition over resources may impact insect development. The potential effects to adult mass or body size were not recorded here, though smaller insects could be expected when more eggs are introduced and the competition is higher.

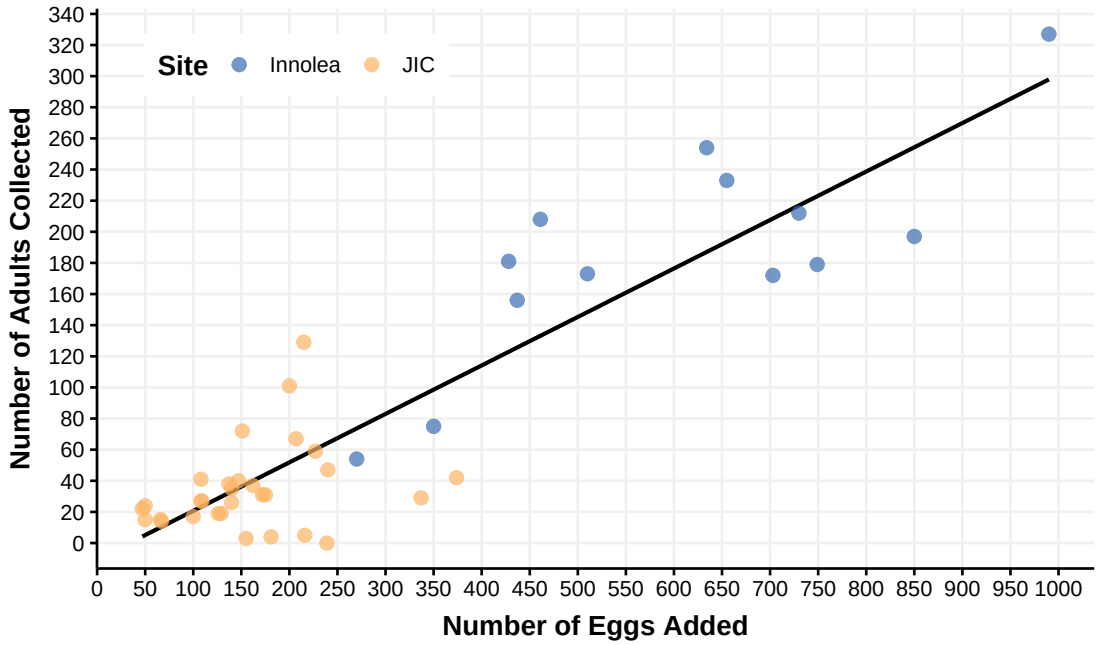

Figure 2.10: The number of adult cabbage stem flea beetles collected from the number of eggs used to infest two mature pak choi plants at two sites, the John Innes Centre (JIC; yellow; *n=29*) and Innolea (*n=13*; blue)

2.6.5 Feeding variability with insect age

The variation in adult feeding with age was quantified by giving adults the same variety of *Brassica napus* each week and measuring percent area damage. Initial feeding of newly pupated adults at 7 days post-eclosion is very high [Fig. 2.11]. Feeding steeply declined from 7 days of age, until no feeding was observed at 21 days. From this time, almost no feeding occurred until 42 days, at which point feeding picked up to around 3 % of leaf area eaten at 56 days. This level of feeding was maintained approximately 21 days, then decreased again to around 1 % at 85 days until the experiment was halted at 115 days post-eclosion.

The percentage leaf area eaten varied significantly with beetle age ($F = 33.23$, $P < 0.001$). The Bonferroni post-hoc test showed that the feeding at 7 days of age was significantly higher than at any other time point, with 18.98 % (± 2.64 % SD). Feeding at 27 days was significantly lower than at any other time point, with a mean of 0.05 % (± 0.01 % SD) eaten. Between 55 days and 69 days, feeding is significantly higher than between 21 and 42 days. Feeding then decreases again at 85 days, which is not significantly different from the level of feeding from 21 to 69 days.

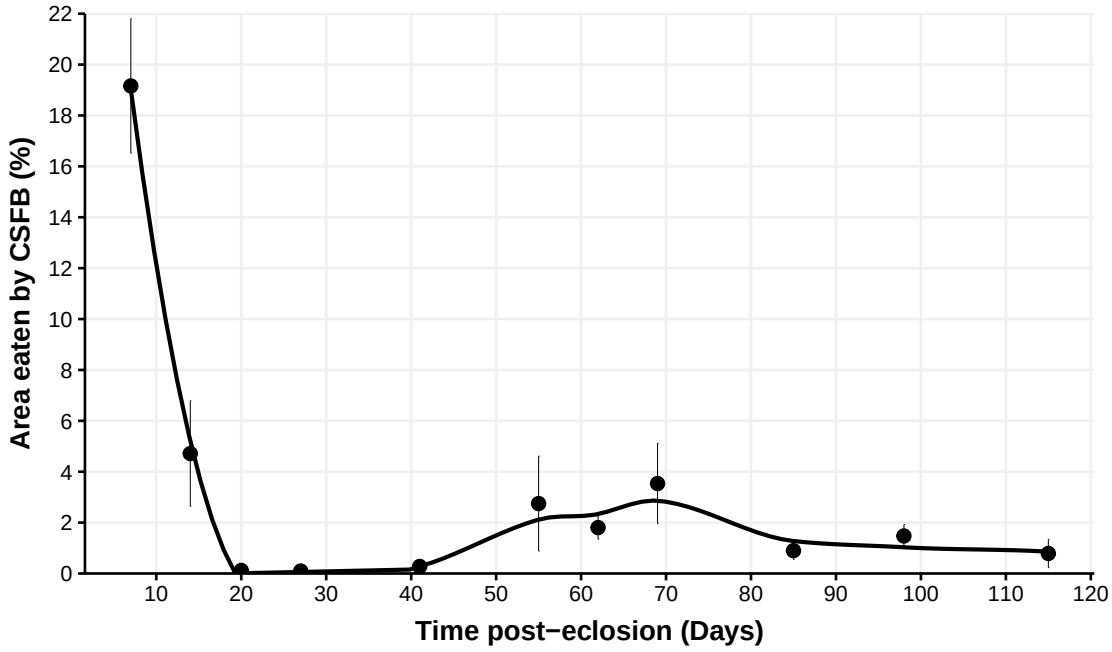

Figure 2.11: Change in the quantity of feeding by cabbage stem flea beetle (CSFB) adults over time post-eclosion. Error bars are the standard deviation. (n=6) replicates for the first data-points at 7 and 14 days, (n=4) from 21 days, and (n=3) for 115 days only. A LOESS-smoothed line of best fit is plotted with an alpha value of 0.5.

2.7 Discussion

2.7.1 Adult survival at two rearing conditions

The day/night temperatures used here reflect the average high day/night temperatures in Norfolk, UK, during September and October (Met Office, 2020), which is what the these wild-caught insects would have experienced *in situ*. In addition, mortality was previously demonstrated to rapidly increase above 16 °C, so this experiment preferentially used a cooler condition to exercise caution. In previous studies, estimated survival between 8 °C and 16 °C was not significantly different (Mathiasen et al., 2015b). As the variable temperature used here fell within this range, it was not thought that using this variable temperature would significantly alter mortality.

Mortality over time was compared for adult CSFB collected from the field, reared in two conditions. Temperature had a significant effect on the survival of CSFB. Survival was immediately and significantly reduced when rearing at 22 °C compared to 10 / 16 °C, and the rate of mortality was significantly higher. The significant interaction between the rearing temperature and the time indicated that CSFB survival was dependent on the time spent in the different temperature conditions. The rate of mortality in the hot condition was approximately 2.3x higher in the hot treatment, so the difference between

the treatments increased as time went on. It has previously been reported that rearing post-aestivated adult CSFB at 16 °C can prolong life expectancy (Mathiasen et al., 2015b). This holds true for the CSFB tested here from a population caught in the UK, opposed to Denmark, and reared using a different method.

The 50 % mortality for both conditions was reached faster here than has previously been observed. For the results here, 50 % mortality for the 22 °C treatment was reached in just 28 days, whereas for (Mathiasen et al., 2015b) this was reached in 78 days for a similar temperature (20 °C). However, although this is a similar temperature, the two temperatures were not exactly the same. Just a few degrees can significantly alter survival, for example a 4 °C difference (between 16 and 20 °C) decreased 50 % survival time by 42 % (Mathiasen et al., 2015b). However, this does not explain the difference in time to 50 % survival at the cool condition. In the 10 / 16 °C used here, survival was expected to be at least 150 days as in (Mathiasen et al., 2015b). 46 % mortality was reached at day 67, and extrapolating this suggests that 50 % mortality would have been reached shortly after 70 days (10 weeks). Potentially, this is due to differences in rearing conditions; here, the insects here were maintained in higher densities of 51 insects in an area of 1200 cm^2 at a density of 24 cm^2 / beetle. Conversely, (Mathiasen et al., 2015b) maintained just a single male and female in a container of 209 cm^2, or a density of 105 cm^2 / beetle. The increased density used here may have been high enough to induce a crowding stress response due continued contact between individuals, and a lack of sufficient room for free movement. The impact of crowding stress has not been studied in adult leaf beetles, but crowding can induce a range of physiological responses in other insects, including increased mortality (Hodjat, 2016). Decreasing adult CSFB density by using larger containers with more food, or fewer insects in the containers used here, may therefore increase survival. Due to this potential stress, the impact of beetle density on fecundity was assessed in later experiments.

During this experiment, egg laying started at 67 days (9.6 weeks) in both conditions, with a total of 14 eggs in the 10 / 16 °C and 6 in the 22 °C condition, at which point the 50 % mortality for the 22 °C treatment had been reached. The experiment was halted at this time to rescue the remaining adults and maximise their eggs laid to continue the colony. Although the experiment was not continued to assess egg laying, these results suggest that fecundity could be increased in the cool condition as the majority of the insects were still alive when egg laying began, whereas nearly all insects had died in the hot condition. This result also suggests that the life cycle of the CSFBs maintained at 22 °C was not accelerated, which may have explained the higher mortality rate. These results also suggest that for the crop in the wild, a hot summer may limit egg laying in the following autumn/winter period due to increased mortality of the adults.

To establish a colony of CSFB it was important to test the impacts of these temperatures on adults from the date of eclosion. As adult CSFB live for many months, their suitability towards particular environmental conditions may change over time.

2.7.2 Survival and fecundity at variable temperatures from eclosion

Fecundity

For the preliminary experiment testing the impact of rearing temperature on fecundity, exposure to a high temperature appeared to increase CSFB fecundity. The insects reared constantly at 22 °C had increased egg laying compared to insects reared at 10 / 16 °C, despite high temperatures decreasing survival in this study and in previous research (Mathiasen et al., 2015b). This suggested that a high temperature exposure is required to increase the number of eggs laid. This may not have been identified in previous experiments testing the impact of temperature on CSFB fecundity, as these experiments used adults which had already experienced high temperatures in the wild (Mathiasen et al., 2015a,b). The greatest increase in eggs laid was seen with a heat treatment at 8 to 9 weeks post-eclosion. At this age, captive insects appear to have already experienced aestivation [Fig. 2.11]. This result reflects previous observations of CSFB ovary development occurring only after exposure to high temperatures (Bonnemaison, 1965), and also that ovary development peaks after aestivation is finished (Bonnemaison and Jourdheuil, 1954). The fecundity of CSFB given a heat treatment earlier than 8 weeks in age was comparable to the controls. This suggest that these insects may not have been old enough for a high temperature to influence their ovary development. Similarly, the beetles treated at 11 weeks saw fewer eggs laid than at 8/9 weeks, potentially because these beetles were too old for the temperature change to have effect. The change in fecundity seen here appears to align with the conditions experienced *in situ*, and imply that a heat treatment timed to coincide with physiological milestones can alter fecundity. Potentially, fecundity may be maximised by using a short period of high temperature to induce sexual maturation, and then rearing at a cooler temperature to prolong survival, which ultimately allows for beetles to lay more eggs. Why temperature can impact gonad development in other insects is not well understood, but may be due to increased efficiency of cell development and hormonal signalling in warmer temperatures Mirth et al. (2021).

It is important to note the results seen in this preliminary experiment were not replicated. There is a considerable difference between the fecundity of insects given a treatment at 6/7 weeks, and 8/9 weeks post-eclosion. The 6/7 week insects had fecundity lower than any other treatment, whereas at 8/9 weeks fecundity was highest. The age of the insects used here was not exact and varied by ± 1 week, so it is unexpected that the results vary so considerably. Instead, the variance indicates that there are confounding factors, highlighting the need for replication.

Replication controlled for the date of eclosion, the number of insects per replicate, and the number of individuals of each sex. All insects eclosed within 7 days of each other, and just 10 insects were used per box. Under these conditions, there were no significant differences between treatments. As the density of insects was shown in later experiments to

influence fecundity, this may be one factor that significantly impacted the results here and introduced variance which could not resolve differences with the low replication (n=3). Although density was initially controlled in the repeat, beetles died at different rates between treatments, and so density was no longer controlled, and fecundity would have been impacted by this.

Due to the time taken to complete this experiment (approximately one year), the results at the early stages were used to decide upon a rearing protocol. As the best evidence available at the time indicated that a heat treatment increased fecundity, this was chosen as the rearing method. However, the final results indicate that a heat treatment does not significantly alter fecundity. Regardless, as the established protocol was not detrimental it was maintained going forward for consistency and uniformity in beetle rearing between experiments. Ideally, this temperature experiment would be repeated with greater replication and a constant density of insects (e.g. one male and one female per replicate at all times). This would be much more labour intensive, as each pair would require their own food, substrate, and cage, rather than 10 individuals having shared resources. Repeating this experiment in this way could draw more robust conclusion about how variable temperatures impacts CSFB fecundity in a reared colony. However, the aim here was to develop a protocol which could maintain a colony, which was achieved. Although the optional conditions were not established, the protocol provided a sufficient number of CSFB for future experimentation, and so no further time or resources were used on CSFB rearing optimisation.

The rearing protocol was successfully transferred to the collaborator Innolea (France). There was no significant difference in fecundity at this site, which suggests that rearing protocol is robust as it gives the same results for both French and English CSFBs, in two different laboratories. These results also suggest that the ideal rearing conditions between geographically distant CSFB populations are similar, as the two populations respond in the same way to the same rearing conditions.

Survival

The mortality over time was also assessed in the repeat. The 50 % mortality was significantly different for insects reared in a constant cool condition or those insects given a heat treatment at 9 weeks of age, and the insects reared at 22 °C constantly. As has been observed with insects collected post-eclosion, mortality rate was much higher for insects reared at 22 °C constant (Mathiasen et al., 2015a,b). As CSFB experience a period of heat early in their life, they may have been adapted to this high temperature when younger, and so time to 50 % mortality may have been the same between all treatments. However, this does not appear to be the case.

For a captive culture of CSFB, 16 / 10 °C is a preferable temperature for rearing compared to the 22 °C conditions used previously at the JIC. This work supports previous

results that the optimal rearing temperature is close to 16 °C (Mathiasen et al., 2015b). This data would also indicate that for the only published literature that mentions use of a reared colony of CSFB, the temperature was likely higher than the optimum for the insects, as the authors used a temperature of 24 °C (Ahn et al., 2019).

Hotter temperatures accelerate ovary development and oviposition of CSFB (Bonnemaison, 1965; Mathiasen et al., 2015b), and increases fecundity and ovary development of other insects (Barker and Herman, 1976; Hodin and Riddiford, 2000; Zhang et al., 2021), and did not significantly decrease mortality. Although it was not conclusive that rearing with a heat treatment would increase CSFB fecundity due to limitations created by the experimental design, it did not appear to have a negative impact. With the potential to increase fecundity and better reflect the conditions experienced in the wild, rearing of CSFB adults going forward used a heat treatment.

2.7.3 Dynamics and viability of egg laying in captive populations

For this rearing protocol, CSFB appear to lay more eggs when maintained at a lower density, as eggs laid per adult shows as exponential trend [Fig. 2.5]. Egg laying is consistently low where more than 50 adults were within a box. When maintained at low densities of 10 or fewer adults (< 120 cm^2 per beetle), adults yielded at least 5 eggs per adult per week, or around 50 eggs per week per box. However, the number of eggs laid per adult below 50 adults is very variable. This variation is reflected in the fit of the exponential model ($R^2 = 0.45$). This suggests that heterogeneity in host response to crowding stress is important. An exponential decrease in fecundity in response to stresses has previously been observed in other insects, such as an exponential decrease in *Drosophila melanogaster* fecundity due to age, and in confused flour beetles (*Tribolium confusum*) due to high density or infection with *Hymenolepis dimin* (Boyce, 1946; Maema, 1986; Novoseltsev et al., 2003). Cannibalism by adults has not been reported for flea beetles, and no evidence of cannibalism (e.g. discarded elytra) was observed here. Therefore, crowding appeared to directly affect the adults, though the mechanism was not determined. It is important to note however that the number of eggs laid in each 7 day period are linked, as the same beetles were recorded over time. Potentially, a female which lays a large number of eggs in one week may lay fewer the next.

Crowding may impact fecundity by reducing the opportunity for copulation, resulting in the reduced fertilisation of eggs in the ovaries. Alternatively, host-plant deterioration may alter fecundity. Density-dependent oviposition control in response to host-plant deterioration has previously been found in other beetle species, such as the lady beetle *Epilachna niponica* (Ohgushi, 1996). Although CSFB were always provided with food in excess, the damage that occurred to the leaves was greater when density was higher as the amount of leaf area per beetle appeared to be consistent regardless of beetle density. Potentially, females may reduce egg laying in response to the heavy damage of the provided

leaves, which may be perceived as an inadequate host for their offspring. This may be a response so females do not lay eggs that compete, as if a host plant dies due to an excess of larvae, larvae must migrate to a new host during which period they are vulnerable to predation and parasitisation. As (Boyce, 1946) found that fecundity is also reduced if the number of flour beetles in a container is higher, despite the number of adults per gram of food remaining constant, fewer adults of CSFB should be maintained together to potentially increase CSFB fecundity.

The age of the adult did not significantly alter the number of eggs laid. This can be seen more clearly in Fig. 2.6. It initially appeared that adult age influenced the number of eggs laid, as a peak around 2/3rds of the way into the adult life span was seen in Fig. 2.6. However, this data does not account for beetle density. Beetle densities were always highest when beetles were young, and as beetles died over time there were fewer adults remaining when adults were oldest. Therefore the "peak" in egg laying seen in this figure does not accurately represent how egg laying varies over time. However, it is useful for demonstrating that CSFB lay eggs for the majority of their adult life in the laboratory colony, from around 60 days to 300 days. This is vital to know in order to understand when to expect eggs for preparing experiments. The mean egg laying period is 227 days (32.4 weeks) long.

Viability was hypothesised to decline with age, as eggs collected from older adults were sometimes pale and flattened opposed to bright yellow. Potentially, the eggs of these older females were not fertilised, as observation under a dissecting microscope did not see the presence of an embryo. The results collected here also found that the age of the adult significantly influences successful hatching of the eggs, with older adults producing fewer offspring. It was not tested here whether the effect comes from the female or male dependent. This could be tested by mating females and males of known ages, and varying the age of one or the other. Importantly though, the older adults also have more than a quarter of their eggs successfully hatch which is a sufficient number to perpetually maintain a colony.

2.7.4 Dynamics of adult eclosion in captive populations

Eclosion of adults from plants infested with eggs is lower than the number of eggs that actually hatch. The mean hatch rate for eggs was 38 % ± 16 % (SD), with eggs from some younger adults having a success rate of up to 63 %. The mean rate of eclosion between JIC and Innolea sites was 28 % of the eggs which were laid reaching adulthood. Therefore, around 10 % of CSFB mortality occurs at the immature stages in the colony, either from death of larvae or pupae. This was not studied here in detail, but there is potential to increase adult numbers further by modifying the rearing conditions of larvae and pupae, especially the temperature. Here, immature CSFB (larvae/pupae) are maintained at 22 °C constant, which is much higher than the winter and spring temperatures these stages would

experience in the wild (Bonnemaison and Jourdheuil, 1954). These high-temperature conditions may contribute to mortality in larvae and pupae in the adult stages, so lower rearing temperatures could be tested for increasing the success of adult eclosion. However, these rearing conditions also decreased the time taken to eclosion, compared with wild populations *in situ* (Mathiasen et al., 2015b). Here, the time from egg infestation to adult eclosion had a clear peak at just 40 days, but *in situ* this can take approximately 8 months (September to May) (Bonnemaison and Jourdheuil, 1954). It was also observed that CSFB adult size decreased compared to the size of field-collected adults, potentially because there is a reduced amount of time to feed and accumulate mass. Cooler rearing conditions may extend the time taken to eclose and also prevent a reduction in size. However, accelerating immature CSFB development increases the ease of experimental planning, as plants can be infested with eggs just 6 weeks prior to experimentation with CSFB adults to coincide with peak eclosion. As the egg-to-adult success rate is sufficient to obtain the required number of adult CSFB and maintain a robust colony, it was considered unnecessary to optimise rearing of immature CSFB further.

A linear correlation was observed for the number of eggs added to plants, and the number of insects received [Fig. 2.10]. There was no decline in adult emergence for up to 990 eggs, (500 eggs per plant) without viability being impacted. Higher egg loads were only tested at the Innolea site, but suggest that the two 6-week-old pak choi plants can support large numbers of larvae for rearing a CSFB colony. These results demonstrate that this rearing method is suitable to obtain a sufficient number of CSFB adults to maintain a colony, and use in experimentation. Although egg loads beyond 990 were not tested, it was very rare to have many hundreds of CSFB eggs available at any one time, and so it is unlikely that there would be an opportunity to exceed this.

Although there was no significant difference between sites for eclosion success, there was higher variance and a trend towards lower eclosion success at the JIC site. This is likely due to sporadic issues with thrip infestations impacting the health of the host plants at JIC. Although this was not serious enough to warrant exclusion of the data, it probably did impact performance compared to the Innolea site, which did not face this issue. Variation in egg success between replicates would also have been influenced by other factors, for example the age of the adults laying the eggs, as discussed above. The results across sites were consistent for time to eclosion. The data at Innolea was more precise, as sampling was more frequent (once every 1-2 days, opposed to once every 7 days at JIC). Sampling was more frequent at Innolea as a team of three researchers were working on rearing, whereas at JIC only one person undertook the work alongside experimentation. Although the peak was broader at JIC because of this, the timing was the same across sites. These results confirm that the protocol is robust, as the results were replicated using two colonies with distinct origins.

These data give an understanding of how many adult CSFB can be expected from a specific number of eggs, and when. These results also demonstrate that immature

CSFB development were accelerated in a laboratory colony. Finally, results demonstrate how many eggs to infest onto a given number of plants. These results are useful for understanding when to prepare plant material for egg infestation, and also when to prepare plant material for experimentation to coincide with peak adult eclosion. As the seedling stage (7 days old) is of interest for phenotyping CSFB antixenosis, experimental material can be sown 32 days post-infestation of eggs.

2.7.5 Feeding variability with insect age

The highest quantity of feeding was observed in the youngest beetles, from 1 to 21 days post-eclosion. These results were substantiated using a different batch of CSFB up to 63 days (5 weeks; data not shown). Using CSFB at the age where they feed most heavily will make any differences in antixenosis more apparent, as the quantity of feeding can vary more substantially. Therefore, only beetles less than 21 days in age were selected for experiments screening feeding preferences. It is also important to try and use insects of exactly the same age, as the feeding quantity is quite variable within the first 21 days. However, there is a significant decline in the area eaten between 0 to 21 days and the exact point where this decline occurs was not captured here; sampling more frequently to fill in the days between what was recorded here would be beneficial to pin-point the exact age where insect feeding significantly decreases. For this reason, all insects selected for subsequent experimentation differed in age by 7 days at most. Although there is still variability in feeding for insects 7 days apart in age, it was not feasible to obtain enough insects that were closer in age than this. Use of multiple insects per replicate during feeding experiments could account for slight differences in feeding quantity due to age.

It is important to note that in the field this is not the age at which CSFB adults would be exposed to seedlings of OSR (Bonnemaison and Jourdheuil, 1954). Potentially, their preferences could differ at different ages, so contrasts in antixenosis should be confirmed using insects of more relevance; either those of an appropriate age, or by testing plants *in situ* (i.e. in a field trial).

Aestivation is a period of metabolic reduction, including depression of digestion, movement, and growth (Cowan et al., 2000). Aestivation in the colony it appears to take place between 21 days and 42 days post-eclosion, as during this time only almost no feeding occurs. As CSFB aestivation is genetically fixed it was expected to occur in the colony (Såringer, 1984). Therefore, it is highly likely that aestivation is occurring during this period, as this was the point of lowest feeding. Furthermore, aestivation in wild populations occurs before egg laying. Egg laying in the colony started at 63 days, which coincided with an increase in feeding. To validate if aestivation was occurring, the metabolic rate of the CSFB could be measured directly by recording gaseous exchange through a technique such as closed-box respirometry (Lownds et al., 2022).

The dynamics of feeding in this captive colony can also be extrapolated to the feeding

behaviour observed in the field. The higher magnitude of feeding between 0 to 21 days post-eclosion correlate with what is seen for adults feeding on the newly sown crop, and when adults can be collected from the field. The period of lower feeding centred around 63 days corresponds to when adults are found on the mature crop and can be collected from the grain store.

2.8 Chapter summary

The results in this chapter describe the dynamics of a captive colony of CSFB. Different rearing temperatures were also tested to optimise adult CSFB survival and fecundity, though the impact on fecundity was inconclusive. Flea beetle survival was increased when rearing at cooler temperatures from eclosion, which has only previously been found for older CSFB adults collected from the field. The dynamics of egg laying and adult emergence were also described, which allowed planning of experiments. A peak in adult emergence after egg infestation was seen at 40 days (6 weeks) post-infestation. There was no significant effect on adult age on the quantity of egg laying so long as adults were at least 63 days old. Therefore, the generation time for this colony is approximately 103 days. The rearing protocol developed from the results of this chapter is outlined in the general materials and methods.

For a box of 10 CSFB reared at 10 / 16 °C with a 21-day 22 °C period at 9 weeks post-eclosion, the expected dynamics are described: A mean of 9 adults will reach egg laying age at 63 days, then 50 % mortality is reached at 164 days, and all beetles will die by 293 days. For a density of between 5 to 10 adults, approximately 5 eggs per adult are laid per week. For 3 to 5 adults, 6 eggs are laid. 2 adults lay 7 eggs per adult per week, and one adult will lay 8 eggs per week. Therefore, from a box of 10 adults, 750 eggs can be expected during the life span in a 203 egg-laying period from 63 to 266 days. Of these eggs, 285 should hatch (mean success of egg hatch is 38 %), and 180 adults should eclose from plants infested with eggs (24 % mean success of eclosion from eggs). A mean of 18 adults are produced for each CSFB when reared in this way, which far exceeds the minimum necessary replacement rate.

There appears to be no increased benefit to increasing the population size by keeping insects at densities higher than 10 per box. For example, densities of 50 adults per box yields 1 egg per adult per week, which is the same total egg yield as when keeping 5x fewer insects. This suggests that workload could be reduced significantly if lower insects numbers are maintained. All adults could be retained for 21 days during the period they feed heavily, and can be used in phenotyping experiments testing antixenosis. Then, adults can be culled to increase the ease of maintaining the colony. As female CSFB need mate only once (Mathiasen et al., 2015b), egg laying could be further increased by increasing the female:male ratio. Sex was not controlled here, and there was approximately an equal number of males and females. However, maintaining fewer insects will more rapidly

reduce genetic diversity, and increases the risk of unintentionally selecting the population for traits divergent from wild insects. Genetic diversity could be monitored by sequencing individuals and comparing how similar micro-satellite loci are over time, such as in (Azrag et al., 2016). However, to date no genome sequence for CSFB is available, so monitoring of genetic diversity is not possible. Therefore, maintaining slightly larger colonies and introducing wild populations periodically (e.g. once per year) is advisable to mitigate a genetic bottleneck.

As these results allow maintenance of a CSFB colony, this technique also allows study of CSFB antibiosis.

3. Measuring production of reactive oxygen species in response to extracts from cabbage stem flea beetle

3.1 Introduction

3.1.1 Induced defences from insects

Induction of defence responses upon perception of a herbivore can allow a tailored defence response to resist the attacker (Carroll and Hoffman, 1980; Pivnick et al., 1992). Plants are able to recognise insect herbivores through detection of a range of behaviours, such as feeding (Lin et al., 1990), crawling (Peiffer and Felton, 2009), oviposition (Bassetti et al., 2022), and defecation (Ray et al., 2016b). Secretions left behind by the insect such as saliva, regurgitant, frass, or oviposition fluid can trigger these defence responses when they come into contact with the plant tissues (Felton and Tumlinson, 2008). The specific HAMPs within these secretions with characterised effects on the plant immune response have previously been discussed, and they can be found in Figure 1 of Snoeck et al. (2022).

In 1997 the first herbivore elicitor was isolated from the oral secretions (OS) of *Spodoptera exigua* (beet armyworm) (Alborn et al., 1997). Oral secretions compose not just saliva from the glands, but also secretions from the insect gut (Schmelz, 2015). Application of the elicitor to maize (*Zea mays*) was able to induce volatile emission, implicated in attraction of natural enemies, and so was termed "volicitin". Volicitin is a fatty amino-acid conjugate (FAC), and FACs have since been the best characterised class of HAMPs. FACs have been found in the OS of Lepidopteran larvae (Yoshinaga et al., 2008), as well as Diptera and Orthoptera (Yoshinaga, 2016). As FACs are vital for nitrogen metabolism in insects they are a necessary component of their biology (Yoshinaga et al., 2008). Therefore, it is logical that plants have evolved a mechanism to detect a constituent of insect secretions which cannot be easily hidden. Since this initial discovery, many other types of elicitors from OS have been discovered. These include caeliferins, bruchins, large molecules such as glucose oxidase and β-Glucosidase, as well as molecules released from the plants by cellular damage (DAMPs; which are particularly relevant for chewing herbivores which cause significant destruction to tissue), such as oligouronides and inceptins (Stahl et al., 2018).

HAMPs from saliva have been particularly well studied in phloem-feeding insects. For example, a protein component of the salivary sheath used for feeding by the brown planthopper (*Nilaparvata lugens*), *N. lugens* Salivary Protein 1 (NlSP1), is detected by rice (*Oryza sativa*) (Ji et al., 2017). NlSP1 triggers accumulation of ROS, expression of defence-related genes, and reduces planthopper performance. HAMPs termed "tetranins" from the saliva of another phloem-feeder, the two-spotted spider mite (*Tetranychus urticae*), also increases ROS accumulation and the expression of defence genes, as well as biosynthesis of JA, SA, ABA, and volatiles to attract predatory mites (Iida et al., 2019). In

addition to HAMPs, effectors which promote susceptibility have been characterised from phloem-feeders, such as Sm9723 from the salivary glands of the grain aphid (*Sitobion miscanthi*) (Zhang et al., 2022b). Sm9723 was found to suppress the defence response of tobacco and is necessary for aphid survival and fecundity.

For Coleopteran species specifically, induced defence induction is not as well studied compared to other insect groups such as Lepidoptera and Aphididae, and no elicitors from Coleoptera have been reported to date. However, Coleoptera are believed to deposit large volumes of OS onto the plant during herbivory (Wang et al., 2016). This can be in the form of saliva, or as regurgitant originating in the gut. Some Coleoptera do not possess true salivary glands, and for the Chrysomelisdae family to which the CSFB belongs, the gnathal glands (e.g. mandibular and maxillary glands) are numerous yet unstudied in relation to their role feeding (Gergerich, 2002; Srivastava, 1959). It is currently unknown if regurgitate is involved during feeding of the CSFB (Srivastava, 1959).

The oral secretions of beetles do appear to be capable of eliciting defence responses. For example the larval regurgitant of the Colorado potato beetle (*Leptinotarsa decemlineata*) increased production of ethylene and enzymes involved in defence (Kruzmane et al., 2002). For the Mexican bean beetle (*Epilachna varivestis*), the "regurgitome" contains many unique proteins, and 13 % of these encoded extracellular proteins previously implicated in plant defence responses such as defensins and attacins (Gedling et al., 2018). Future analysis of these specialised proteins in the context of Coleopteran herbivory may aid in understanding the pathways involved in defence induction in response to Coleopteran feeding. For the Colorado potato beetle and the false potato beetle (*L. juncta*), oral secretions have also been demonstrated to release microbes into the feeding site (Wang et al., 2016). This can induce SA defences and disguise the herbivore as a microbial pathogen, likely by releasing microbial elicitors into the wound. This also repressed JA defence pathways, and led to increased growth of the beetles (Chung et al., 2013; Sorokan et al., 2020). Therefore for those Coleopteran species where oral secretions come from the gut, the microbiome is also important to consider. Potentially, saliva or the regurgitate of CSFB may contain elicitors which could be detected by the host plant during feeding, and as with other herbivores these may modulate defence pathways.

It is not only secretions from the mouth of insects that can trigger defence, but also elicitors from frass (faeces). Two plant chitanases from the frass of the fall armyworm larvae (*Spodoptera frugiperda*) induced an accumulation of SA, and increased expression of pathogen defence genes in maize. This also suppressed herbivore defences and increased susceptibility to the insect while reducing growth of the fungal pathogen *Cochlioblus Heterostrophus* (Ray et al., 2016a, 2015). The response of the host to frass appears to be highly dependent upon the host species and the particular herbivore. For example, further work by Ray et al. (2016a) found that in rice herbivore defences are induced in response to fall armyworm frass, while pathogen defences are suppressed. This same study also included testing of a member of the Brassicaceae, white cabbage (*Brassica*

oleracea var. oleracea Platinum dynasty) against the larvae of the cabbage looper (*Trichoplusia ni*). An oscillating response was seen that resembles the zigzag scheme of effector-triggered immunity, where JA precursor transcripts accumulated in the initial 4 hours then significantly decreased past 48 hours, at which point pathogen associated transcript levels significantly increased. The frass of chewing insects outside of Lepidoptera have not been studied to date, though beetle frass can also come into prolonged contact with the plant. For example, the frass of CSFB adults sticks to the leaf surface leaving the plants visibly covered as a result (Fig. 3.1). Potentially then, it may be of interest to assess if host Brassicaceae can respond to CSFB frass, which could be studied for breeding resistance.

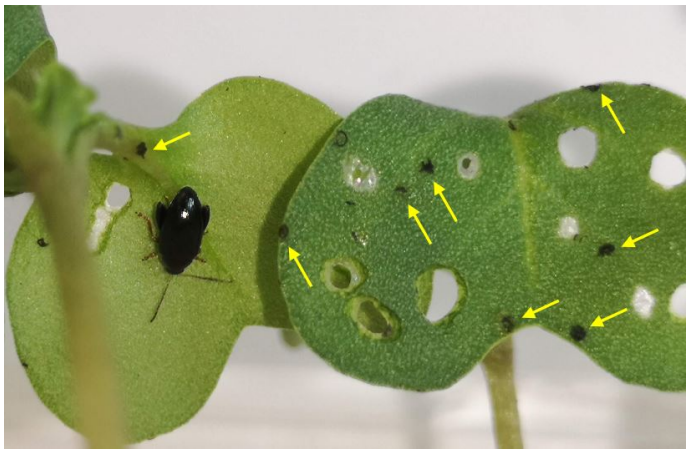

Figure 3.1: Frass from cabbage stem flea beetle adults is found across both surfaces of the cotyledons after feeding. The frass (dark fibres) is highlighted with yellow arrows. The damage to the two seedlings here was the result of 48 hours of feeding by the one beetle in the laboratory, pictured.

Components related to egg-laying such as oviposition fluid can also elicit a defence response. When eggs comes into contact with the leaf surface, they can induce a response similar to the "hypersensitive response" triggered by pathogens, leading to localised necrosis (Hilker and Fatouros, 2016). The death of the leaf tissue to which the insects eggs are attached can kill the eggs through desiccation, or detachment from the plant. The ability of a host plant to respond to insect eggs has been extensively studied for Lepidopteran insects, especially for *Brassica* species in addition to *Arabidopsis*. *Brassica nigra* is able to respond to eggs of *P. brassicae* and *P. rapae*, leading to lower hatching rates and higher parasitism by *Trichogramma* wasps, and the magnitude of the response varies with plant genotype (Fatouros et al., 2014; Griese et al., 2017; Shapiro and DeVay, 1987). Specific Lepidopteran egg elicitors have also recently been identified, such as phosphatidylcholines from *P. brassicae* eggs (Stahl et al., 2020).

For the Colorado potato beetle, eggs laid onto the plant surface caused necrosis of the leaf tissue and subsequent egg drop from a hybrid variety of wild potato (*Solanum berthaultii*), leading to greater larvae mortality in the field (Balbyshev and Lorenzen,

1997). Although egg-related defences have been identified in *Brassica* species, and in other plants in response to Coleoptera, egg laying is less likely to be relevant to defence response induction in the case of CSFB. CSFB adults lay eggs in the soil near the host plant, but not necessarily in contact with the plant surface (Såringer, 1984; Vig, 2003). Therefore, potential signalling molecules from the eggs are unlikely to come into contact with the plant.

The majority of the work studying the ability of Brassicaceae species to detect HAMPs and effectors has focused on the response of *Arabidopsis*, as it a convenient laboratory model for studying complex networks such as those involved in defence (Koornneef and Meinke, 2010). Understanding the defence pathways of *Arabidopsis* has particular relevance for *B. napus* as both species are part of the Brassicaceae family; therefore homology between the defence pathways of these species is greater than for some other crops, such as rice (David De Vleesschauwer, 2014). Studies with *Arabidopsis* and insect defence induction have typically assessed aphids; for example, multiple salivary proteins (Mp56, Mp57, Mp58) from the green peach aphid *Myzus persicae* decrease aphid reproduction on *Arabidopsis thaliana* when expressed transgenically (Elzinga et al., 2014). To date, only one elicitor specific to chewing insects has been demonstrated to have action against Brassicaceae: β-glucosidase. Artificially damaged cabbage leaves treated with gut regurgitant of cabbage butterfly (*Pieris brassicae*) caterpillars released a volatile mix similar to that of herbivore-damaged plants. β-glucosidase was identified as a key elicitor, as β-glucosidase treatment releases a similar volatile blend, which is highly attractive to the parasitic wasp *Cotesia glomerata* (Mattiacci et al., 1995).

Despite the strong effects of FACs in tobacco and maize, *A. thaliana* does not appear to respond (Schmelz et al., 2009) and other *Brassica* species remain unstudied. However, there is evidence to suggest other effectors are able to alter resistance pathways within plants. The trichome density of *Brassica nigra* (black mustard) has been observed to change in response to the oral secretions of different chewing insect herbivores such as *P. rapae* and *T. ni* (Traw and Dawson, 2002). Trichome density increased 76 % and 113 % respectively for *P. rapae* and *T. ni* in response to feeding upon a set leaf area. No trichome density changes were observed from feeding by *P. cruciferae*, which may suggest something unique about flea beetle feeding compared to the other two chewing herbivores.

Induced responses require genetic reprogramming. To date, a single gene has been implicated in damage response of *B. napus* after flea beetle herbivory. *Brassica napus Auxin Re-pressed Protein 1 (BnARP1)* was highly expressed in a germplasm library after *(P. cruciferae)* feeding, and acts to repress auxin transport, increasing the interaction between JAs and auxin signalling pathways (Wu et al., 2017). Due to JAs core role in defence response, this gene is an interesting candidate for further study. Although over-expression of *BnARP1* in *Arabidopsis* failed to reduce flea beetle feeding, this does not rule out a role for *BnARP1* in defence, as only antixenosis (deterrence) was measured. Therefore, it would be interesting to consider the role of *BnARP1* for CSFB antibiosis. In

addition, *Arabidopsis* may lack the downstream genes induced in *B. napus* by *BnARP1* against flea beetles. Interestingly, *BnARP1* over-expression did significantly improve survival after *Sclerotinia sclerotiorum* infection, suggesting *BnARP1* could be a generalist defence-response factor. This is supported by a study by de Souza et al. (2019), who found that expression of an ortholog of *ARP1* was increased in soybean in response to nematode infestation. *BnARP1* may therefore act as a regulator of other genes specific to stress type. Assessment of genes regulated by *BnARP1* may therefore also be of interest.

3.1.2 Measurements of defence induction

Induction and measurement of a defence response is not just useful to understand if a CSFB-inducible defence response is present in Brassicaceae, but it is also desirable as a means for development of a high-throughput screening assay. Use of live insects in feeding assays can be particularly time consuming. For the CSFB this is further confounded as beetle feeding is variable between flea beetle populations and their stage in the reproductive cycle. This makes quantification of resistance challenging, as the magnitude of feeding is variable between experiments (Gavloski et al., 2000; Henderson et al., 2004). Therefore, use of beetle "extracts" in a 96-well plate assay system could facilitate screening resistance to large populations much more rapidly than feeding trials using living insects. This system could be used to identify lines with contrasting ROS induction responses, which could then be screened against live CSFB in future tests to verify if defence induction correlates positively with reduced CSFB feeding and understand any potential mechanisms of resistance to the CSFB.

Induction of ROS is characteristic of defence response signalling, and is an important class of molecules for long distance stress signalling (Choi et al., 2017; Miller et al., 2009). ROS production in response to chewing herbivores has been identified; for example ROS accumulates after herbivory by Egyptian cotton leafworm larvae (*Spodoptera littoralis*) and *T. urticae*, which was not observed due to mechanical wounding alone (Leitner et al., 2005). ROS levels also increase upon addition of the a Lepidopteran FAC elicitor to *Arabidopsis* (Block et al., 2018), and herbivory is required for a ROS burst in *Medicago truncatula*, while mechanical damage does not elicit any ROS (Leitner et al., 2005). Recent work has demonstrated that ROS production is likely nuanced on the herbivore species, perhaps to elicit a specific defence response. For example, in *Arabidopsis*, knocking out the ROS-inducing gene *RBOHD* can reduce defence to *M. persicae* but increases resistance to *S. exigua* and *T. ni* (Miller et al., 2009).

The production of ROS has been used as a method to assay defence responses to insects by utilising its oxidative capacity. For example, oxidation by ROS of iodide to iodine in iodine-starch staining causes a visible colour change (Leitner et al., 2005), and photons can be produced and quantified from oxidation of luminol by ROS when catalysed by a peroxidase (Bisceglia et al., 2015; Zhu et al., 2016). Using this luminol assay, the dynamics

of the response to well characterised PAMPs have been demonstrated. For example, the response to flg22 from flagellin, a bacterial protein, elicited a SA response which is both rapid and transient (Felix et al., 1999; Hickman et al., 2019). In contrast, the JA defence response is extended and less intense (Zhang et al., 2020). This prolonged but significant induction of ROS has been demonstrated for insect-derived extracts too, and was used to identify genes involved defence such as *BAK1* (Prince et al., 2014).

3.1.3 Aims

The aim of this chapter was to assess if a stress response was induced by treatment of Brassicaceae germplasm with potential elicitors from CSFB. To do this, an attempt was made to measure ROS production using a luminol-based assay system previously used for green peach aphid extracts and PAMPs. The level of ROS produced from different lines of *S. alba* and *B. napus* was measured after exposure to crude extracts of whole CSFB or CSFB frass.

3.2 Materials and Methods

3.2.1 Measurement of reactive oxygen species from leaf discs of *Brassica napus* and *Sinapis alba*

The production of ROS was quantified using a luminescence-based assay (Lloyd et al., 2017). Seedlings of *B. napus* or *S. alba* were grown as previously described in the general materials and methods to the cotyledon stage, and three seedlings were used for each treatment. A total of 24 discs were used per line for test extracts (8 technical replicates per seedling). For the control extracts, 8 discs were used to reduce space on the plate to one column. Discs were cut from the cotyledons of each seedling, avoiding the mid-vein, using a 4 mm cork borer with ejector (Fisher Scientific, product number 12863952). Discs were ejected into each well of a white 96-well plate (Greiner Bio-One Ltd, product number 655075) containing 200 μL sterile H_2O per well. Plates were then wrapped in kitchen tin-foil and left overnight in the dark at ambient conditions, and remained in the dark until < 5 minutes prior to placing in the plate reader. 24 hours after cutting the discs the H_2O was replaced with 200 μL of the luminol-based assay system diluted in sterile H_2O, consisting of luminol (34 μg mL^{-1} final concentration; Wako Chemicals) and horseradish peroxidase (20 μg mL^{-1} final concentration; Sigma).

3.2.2 Preparation of extracts

An extract of whole CSFB was prepared using adults collected from the colony. Beetles were placed into a 2 mL Eppendorf and snap frozen in liquid nitrogen. The beetles were ground to a fine powder using a pre-chilled mortar and pestle, then the powder was

removed with a cooled spoon to a 50 mL Corning tube on ice. The powder was suspended in sterile H_2O to a stock concentration of 20 mg mL^{-1}, and the extract was tested at a final concentration 100 μg mL^{-1} or 1000 μg mL^{-1}. The extract was tested against a single *S. alba* line 97, and two *B. napus* lines previous observed by Jessica Hughes of the John Innes Centre to show a significant difference in CSFB adult feeding in laboratory choice assays; these were named "resistant" (R) and "susceptible" (S).

Frass extracts were prepared similarly to (Ray et al., 2015), without fractionation or sterilisation. Adult beetles from the colony were moved to an empty rearing box 24 hours prior to adding the plate to the plate reader, 50 adults per box, and maintained in the CER as previously. After 24 hours a sterile fine-tipped paintbrush was used to remove frass into a 15 mL Corning tube. Sterile H_2O was added to the tubes, and the frass was homogenized for 30 seconds using a vortex mixer to form a slurry. A 200 μL pipette was used to separate the liquid from the solids into a fresh tube.

To test if any elicited response could be due to DAMPs originating in the feed, an extract of the feed was also prepared as a control. A freshly cut leaf of *Brassica rapa* Pekinensis Group, var: F1 Hilton, (Chinese cabbage) grown as previously was harvested 24 hours prior to the experiment, and added to a 2 mL Eppendorf containing a 4 mm diameter stainless steel ball (Bearing Supplies). The tube was shaken using a mixer mill (Retsch) at 25 rpm for 1 minute to macerate the sample, then sterile H_2O was added at a ratio of 200 mg wet weight leaf to 1 mL sterile H_2O. The tissue was re-suspended by using a vortex mixer for 30 seconds. A 200 μL pipette was used to separate the liquid from the solids into a fresh tube, and stored in the refrigerator overnight at 4 °C. Both the frass and cabbage leaf extracts were used at a final concentration of 100 μg mL^{-1} (wet weight) as not enough frass was obtained to test higher concentrations. Lines tested were *B. napus* lines R and S.

Immediately prior to placing in the plate reader, extracts were added to the appropriate wells using a 12-tip multichannel pipette. Replicates for each extract were arranged in columns so that test extracts were added at the same time across each row. Test extracts and controls were prepared on the day the discs were placed into the plate reader. The negative control used an equivalent volume of sterile H_2O (blank). For the frass/cabbage leaf experiment only, the positive control used Flagelin 22 (flg-22, 20 μg/ml final concentration, EZ BIOLAB). Plates were placed into a Varioskan LUX multimode plate reader (Thermo Scientific, Waltham, MA) with SkanIt RE Software (version 6.0.1.6) and photon counts were recorded every 30 seconds for at least 300 minutes. Data was imported into Python Version 3.8 (Van Rossum and Drake Jr, 1995) and plotted using the MatPlotLib library Version 3.1 (Hunter, 2007).

An average of the technical replicates was calculated for each time point, and the relative light units (RLU) at each time point were plotted over time to visualise the dynamics of the ROS production/immune response. The total RLU emitted across the 5 hours was also calculated for each treatment, and a two-way ANOVA was used for each

experiment to compare the blank to the corresponding treatment to assess if more ROS was produced in those individuals receiving a treatment.

3.3 Results

3.3.1 ROS induction in response to whole cabbage stem flea beetle extract

B. napus and *S. alba* were tested for ROS production in response to whole ground CSFB (Figure 3.2). At a concentration of 100 μg mL^{-1} CSFB in water, no significant production of ROS was observed in the samples exposed to the test extract compared to the controls ($F = 1.022$, $P = 0.447$).

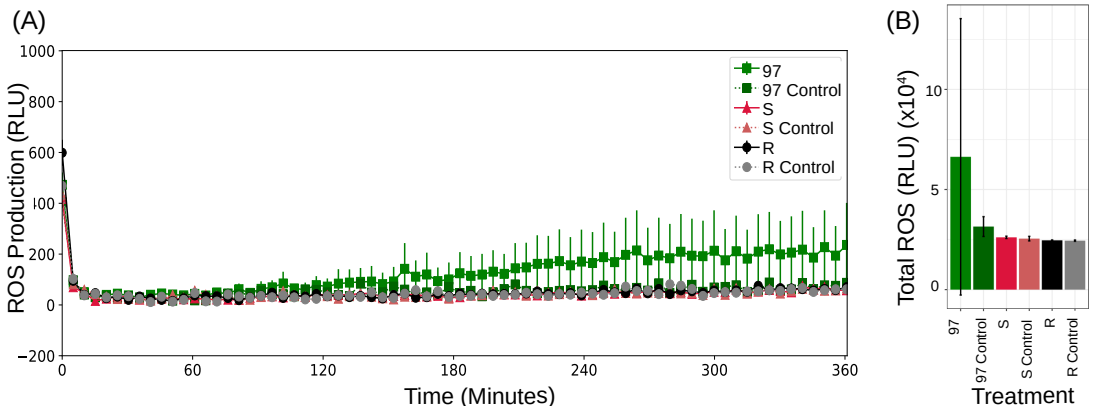

Figure 3.2: Leaf discs of *B. napus* lines R and S, and *S. alba* line 97 were elicited with water (Control), or whole CSFB-derived extract in water at a final concentration of 100 μg mL^{-1}. ROS bursts were measured using a luminol-based assay for 300 minutes. (A) Mean ± SE of ROS produced with points plotted every 5 minutes. (B) Total ROS ± SE produced over 300 minutes. Experiments used 3 replicate seedlings per treatment. RLU, relative light units.

B. napus and *S. alba* were also tested for ROS production in response to whole ground CSFB at a higher concentration of 1000 μg mL^{-1} (Figure 3.3). This concentration was also unable to elicit a significant production of ROS in the test extract compared to the controls ($F = 1.211$, $P = 0.362$).

B. napus was tested for ROS production in response to frass obtained from CSFB adults at concentration of 100 μg mL^{-1} (Figure 3.4). An extract of the CSFB feed was also tested, in addition to flg-22 as a positive control. As expected, the flg-22 treatment elicited responses in both lines after several minutes, peaked at around 15 minutes, and had halted by 30 minutes. However, both the frass and leaf extracts did not elicit a significant production of ROS in the test extract compared to the controls ($F = 0.723$, $P = 0.619$).

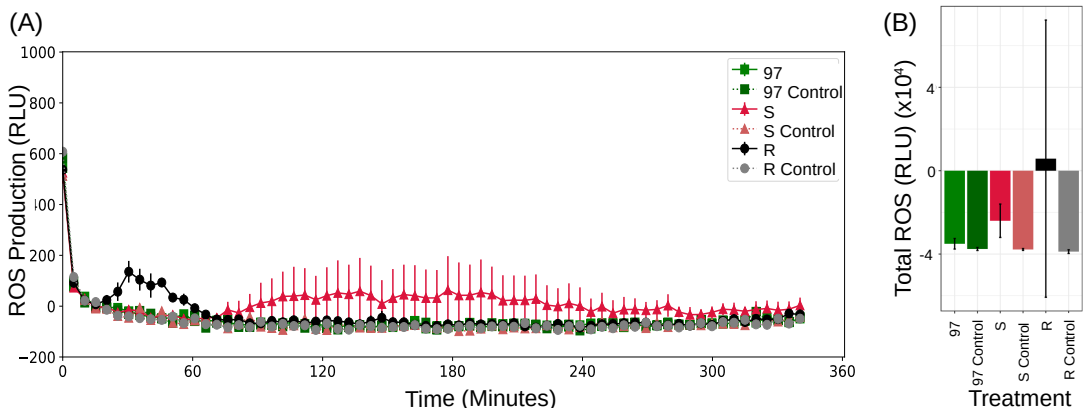

Figure 3.3: Leaf discs of *B. napus* lines R and S, and *S. alba* line 97 were elicited with water (Control), or whole CSFB-derived extract in water at a final concentration of 1000 μg mL^{-1}. ROS bursts were measured using a luminol-based assay for 300 minutes. (A) Mean ± SE of ROS produced with points plotted every 5 minutes. (B) Total ROS ± SE produced over 300 minutes. Experiments used 3 replicate seedlings per treatment. RLU, relative light units.

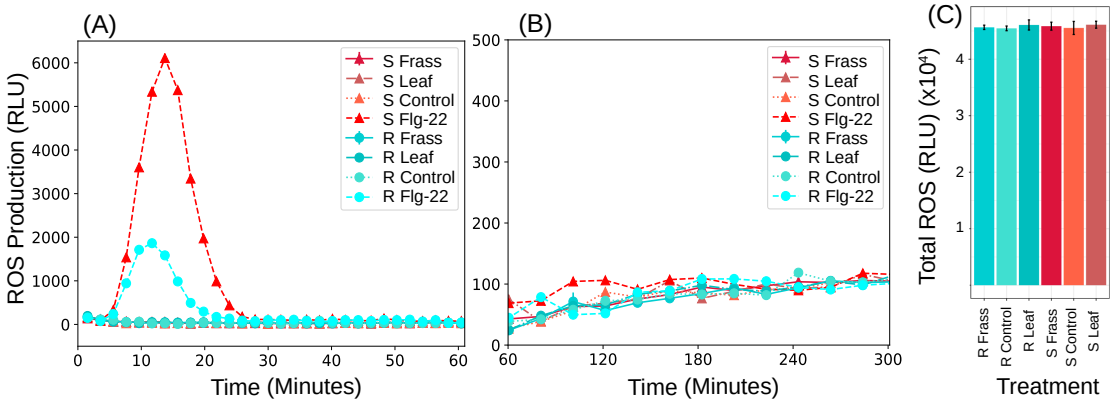

Figure 3.4: Leaf discs of *B. napus* lines R and S were elicited with water (Control), Chinese cabbage leaf (Leaf), CSFB frass at a final concentration of 100 μg mL^{-1}, or flg-22. ROS bursts were measured using a luminol-based assay for 300 minutes. (A) ROS production is plotted every 2 minutes for 0 to 60 minutes and (B) every 15 minutes for 60 to 300 minutes. (C) Total ROS detected over 5 hours for the two varieties and three treatments. RLU, relative light units.

3.4 Discussion

The study assessed the ability of CSFB extracts to elicit an accumulation of ROS in three Brassicaceae accessions. For extracts derived from whole CSFB and from CSFB frass, no significant elicitation of ROS was generated. One individual from *S. alba* line 97 deviated from the negative control in response to the 100 μg mL^{-1} whole-CSFB treatment. The dynamics of the ROS curve in figure 3.2A resemble what has previously observed with green peach aphid extracts, whereby ROS production increases from approximately 80 minutes and peaks around 280 minutes (Prince et al., 2014). However, the ROS produced

for this line was not significantly higher than the control. In addition, the same response was not observed at the higher concentration. Therefore, this does not constitute sufficient evidence to demonstrate that the whole CSFB extracts were able to elicit an accumulation of ROS in this line. Previously, *Brassica nigra* appeared to be unresponsive to *P. cruciferae* flea beetles, whereas feeding by *P. rapae* and *T. ni* was detected and led to increased trichome density which is associated with increased defence against herbivores (Traw and Dawson, 2002). The Brassicaceae tested here may similarly be unable to respond to CSFB feeding.

Aphid extracts used by (Prince et al., 2014) were at a final concentration of 5000 μg mL^{-1}, which is higher than those tested here at 100 μg mL^{-1} and 1000 μg mL^{-1}. The peak of RLU observed here was around 100 to 200 RLU from whole beetles tested against the 100 μg mL^{-1} extract, whereas from aphids the peak was over 1000 RLU. Potentially, the extracts tested here were not concentrated enough to elicit a sufficient production of ROS to be detected. However, this is unlikely as this is still a highly concentrated extract and so it is unlikely that a response was missed, but instead elicitation did not occur.

It should be noted that the negative RLU recorded in the experiment with whole CSFB extracts is due to the baseline of ROS being set too high automatically by the Varioskan plate reader; it is normal for some photon emission to be triggered by the light in the laboratory and decrease once the plate reader closes. For this particular experiment the ROS production upon entering the plate reader was slightly higher than usual, however this should not alter the conclusions of the experiment. If a ROS burst was triggered by the extracts, it was expected that this would still have been observed as a significant increase above the negative control.

In the frass extract, the positive flg-22 control elicited a strong response which resembled those seen previously when used at this concentration (Bisceglia et al., 2015; Fabro et al., 2011). However, no significant induction of ROS was observed for any of the test extracts (cabbage leaf or CSFB frass). A limitation of this experiment is that due to insufficient room on the 96-well plate *S. alba* line 97 was excluded. This species may have responded differently to *B. napus*, as *B. napus* is generally a susceptible species to CSFB and so the selected varieties may not be capable of responding to elicitors even in an optimised assay.

It is perhaps unusual that no response at all was generated from whole-CSFB extracts; it may be expected that the high concentrations of chitin derived from the exoskeleton could induce some response in the tested varieties. Chitin is a well studied elicitor in the context of fungal pathogens, where it is a structural component of the cell wall (Wan et al., 2008). Although fungal chitin may be structurally different to chitin derived from insects, arthropod chitin also elicits immune responses in plants, and chitin used in most chitin immunology assays is sourced from shellfish (Poncini et al., 2017).

Previous studies assessing the ability of insect extracts to elicit plant immune responses

used fractionated extracts. De Vos and Jander (2009) tested green peach aphid saliva fractionated to contain compounds < 10 kD or > 10 kD, and only the < 10 kD fraction was an effective inducer in *Arabidopsis*. Subsequently, this was narrowed to a fraction containing the 3- to 10-kD fraction (De Vos and Jander, 2009; Prince et al., 2014). For the generalist grasshopper herbivore *Schistocerca gregaria*, the oral secretions were also fractionated "to narrow the spectrum of possible effectors" (Schafer et al., 2011). Conversely to the response in aphids however, the larger > 10 kD fraction induced a greater response in *Arabidopsis* than the < 10 kD fraction, and in this instance both fractions induced a response. As both small and large compounds can induce defence responses, the CSFB extracts were not fractionated as it has not been reported what size proteins may elicit a response from this insect. However, by nature of being a whole insect, the CSFB extracts used here may contain an abundance of potential inhibitory metabolites or proteins which may have been removed by fractionation in previous studies. As the composition of a whole CSFB is not known, inhibitory metabolites can only be speculated upon. Regardless of their potential identity, future work with whole CSFB extracts would benefit from fractionation to remove potential inhibitors, though there is the potential that many inhibitors are present and cannot be easily removed through fractionation alone. Extracts of the CSFB gut, which will likely contain elicitors where regurgitate is relevant, may also be possible by dissection (Ahn et al., 2019).

The frass extracts used here were also unable to elicit a production of ROS in the tested varieties. As with whole CSFB extracts, the assay was not tested sufficiently to determine the cause of this. Potentially, CSFB frass may be unable to elicit a defence response unlike what has been seen in some other insects. Alternatively, there may be no elicitation due to the presence of inhibiting metabolites preventing the assay reagents from activity. Another possibility is that the frass used here may not elicit a response as the composition may not contain the appropriate elicitors, as it is unlikely the frass is representative to what is found in the field as a reared colony of CSFB were utilised. The frass was obtained from insects reared exclusively on *Brassica rapa* subsp. chinensis (L.) Hanelt (cv. China Choi) as larvae, and Chinese cabbage (*Brassica rapa* Pekinensis Group, var: F1 Hilton, see general materials and methods) as adults. The composition of DAMPs, or MAMPs from CSFB gut microbes, in the frass may be significantly altered from what is found in the field where CSFB feed on many different Brassicaceae including OSR and *S. alba*, which were the species tested in the assay here (Bartlet and Williams, 1991). If DAMPs within the frass are of relevance to elicitation of a defence response, the composition of these DAMPs will be altered in the frass from the colony and may reduce the ability of *B. napus* and *S. alba* to respond. Previous research has found that applications of leaf extracts have been unable to elicit a defence responses in other plant species. For example, leaf homogenates of other legume species applied to *Phaseolus vulgaris* did not induce formation of ROS or induciton of downstream defences, whereas homogenate from other Phaseolus species did induce ROS, though production was reduced compared to the

test species (Duran-Flores and Heil, 2014). Potentially then, the cabbage leaf and frass extracts used here may not be capable of heterospecific ROS elicitation. To verify this, this experiment could be repeated using frass from CSFB reared on *B. napus* or *S. alba*, or collected from a field of OSR.

For both experiments, it is also important to note that as a specific variety of Brassicaceae with known CSFB resistance was not available for use in the development of this assay. It is challenging to optimise an assay when lacking a variety which is a known responder, as the tested lines may be incapable of detecting potential CSFB elicitors. These assays were performed to observe any clear, strong induced defence responses to CSFB, which has not previously been reported upon. If a response was observed, the assay to elicit ROS could be further optimised through fractionation of extracts, and adjusting extract concentrations. If an optimised assay could have been developed, testing of additional plant lines would have been undertaken to assess if ROS response may correlate to resistance against CSFB in laboratory screens with live insects. These results would indicate if the ROS assay could function as an effective high-throughput CSFB resistance screen. However, as there was no elicitation in any assay it suggests that the methodology needs significant adjustment, or the lines tested here were not able to respond either due to the extracts or the varieties themselves.

Further progress on the work undertaken within this chapter was halted due to UK COVID-19 lockdown measures causing the closure of the institute in 2020. As no evidence had been collected to demonstrate that the experimental design being tested would work, this work was not continued. As the aim was to identify potential contrasting lines for resistance to CSFB feeding, the method to achieve this was altered and instead future work used live CSFB for screens as this technique is better studied.

3.5 Chapter summary

This work is a very preliminary starting point for assessing induced responses in *B. napus* and *S. alba* to CSFB. *B. napus* and *S. alba* were exposed to extracts of whole CSFB in addition to CSFB frass. It was hypothesised that if a CSFB "extract" which elicits a defence response could be identified, there may be potential to use this in a high throughput screening assay to assess Brassicaceae resistance to CSFB. Many seedlings could be tested in this assay using a plate reader instead of live insects, and the ROS production could be quantified to infer resistance. In addition to increasing the rate of screening resistance, it could also increase precision by removing the variation in assays caused by using live beetles, as beetle feeding behaviour varies considerably between flea beetle populations and their stage in the reproductive cycle (Gavloski et al., 2000; Henderson et al., 2004). However, no significant elicitation of ROS occurred in the experiments undertaken, and so it does not appear that a defence response was induced to extracts of whole CSFB, CSFB frass, or the cabbage leaf tested here.

4. Identification of *Sinapis alba* genotypes with contrasting resistance to the cabbage stem flea beetle

4.1 The challenge of phenotyping flea beetle resistance

As the speed of genotyping large populations has increased, the major bottleneck for discovering resistance genes against insect herbivores is now the ability to phenotype many accessions in parallel (Goggin et al., 2015). Phenotyping for intraspecies contrasts in CSFB resistance has been particularly formidable. There are three major challenges confounding identification of CSFB antixenosis; obtaining a sufficient number of CSFB with uniform behaviour, development of a high-throughput methodology for phenotyping large numbers of accessions, and use of precise scoring mechanisms to quantify resistance. The first chapter of this thesis increased the number of available insects, and developed an understanding of the dynamics of the insects in a captive population to facilitate planning of experiments. Here, the challenge of phenotyping CSFB resistance is addressed, and a new method was utilsed to identify intraspecies contrasts.

Development of a novel phenotyping technique to score resistance to CSFB should be high-throughput, reproducible, quantifiable, and non-invasive (Bazakos et al., 2017). The majority of studies have been non-invasive in that they use whole plants. However, some previous studies assessing flea beetle resistance have used damaged tissues, such as leaf discs (Bartlet and Williams, 1991; Nielsen et al., 2001; Palaniswamy and Bodnaryk, 1994). This can be problematic, as it may alter the plant phenotype in such as manner that it is no longer representative of the true phenotype. For example, total alkaloid content was much greater in artificially damaged tobacco plants (4x controls), than when compared to real herbivory of an insect pest (2.2x controls) (Baldwin, 1988). Although use of plant parts increases the ease of phenotyping, it is important to use whole, undamaged plants for accurate phenotyping.

4.1.1 Field trials for testing hypothesise *in situ*

Previous work for phenotyping many plant accessions for flea beetle resistance have preferentially utilised naturally occurring CSFB populations in field trials. Field scoring of adult flea beetle resistance can involve estimating the visible damage to the plants, counting the number of beetles on the plants, and counting plant survival (Lamb, 1988; Lamb et al., 1993). The largest field trial to date for flea beetle resistance compared 90 OSR accessions across 2 years, but failed to identify differences in resistance (Ahman, 1993). Field trials have also been used for scoring CSFB larvae damage. Field plants can either be dissected with a scalpel to count the number of larvae (Lane et al., 1995), or through the "evacuation method", whereby larvae are allowed to naturally exit desiccating plants, and are captured for counting (Conrad et al., 2016). Again, these have failed

to identify larvae-resistant varieties. Although, field trials have successfully identified resistant *Brassicaceae* to other insects, such as resistant accessions of Brussels sprout to cabbage whitefly (*Aleyrodes proletella*) (Hondelmann et al., 2020), to date field trials have not identified intraspecies contrasts in resistance to CSFB.

The impact of the pest in field trials depends strongly on the phenology of the plant material and the environmental conditions (Fidelis et al., 2019; Lerin, 1991; Mopper and Simberloff, 1995). Plant density, plant size, and the stage of plant development are also difficult to account for in analyses (Hervé et al., 2017). Insect populations also vary in magnitude considerably between years (Emery et al., 2022), and spatially across a trial site (Warner et al., 2003). Scoring techniques in the field also fail to distinguish between the three types of resistance (antibiosis, antixenosis, and tolerance). Field-scoring for adult CSFB resistance is also limited to only a short period of the year in the autumn when adults attack the establishing seedlings. Although field trials are necessary to validate resistance *in situ*, use of a laboratory screening technique is therefore needed to find CSFB resistance.

4.1.2 Laboratory screens for controlled phenotyping of flea beetle resistance

Previously developed laboratory screens have preferentially utilised choice environments (Gavloski et al., 2000; Palaniswamy and Bodnaryk, 1994; Soroka and Grenkow, 2013). This increases the ease of phenotyping large populations concurrently by reducing labour-intensive assembly of separate chambers with separate insects. Choice environments may also improve the ease of identifying contrasts between lines, because hungry insects are not forced to feed on an unpalatable line to prevent starvation. However, previous experiments have instead found the opposite, that lines are more easily distinguished in non-choice environments. (Palaniswamy and Lamb, 1992a) performed both a choice and non-choice test for 9 species, and the differences between species were much clearer in the non-choice environment. Potentially, feeding cues are mixed in choice environments and confound the ability of the CSFB to distinguish preferential plant material. This reduction in contrast between resistant and susceptible plants in a choice environment has also been found at JIC (observations by Jessica Hughes, unpublished). Therefore, to clearly identify resistant and susceptible varieties it is important to phenotype CSFB in a non-choice environment. In addition, non-choice experiments are more representative of crop mono-cultures, so non-choice screens should be used to confirm that the resistance holds in the setting of interest.

Previous experiments have frequently relied upon visual estimation of damage, and have applied qualitative scores to phenotypes with continuous variation. In addition to being subjective to the experimenter, ordinal scales are difficult to analyse with paramertric statistics (Stevens, 1968). This hampers understanding of how the data

reflects the real resistance phenotype. In addition, qualitative scores are often imprecise. For example, the most comprehensive laboratory screen for flea beetle resistance to date was performed by Soroka and Grenkow (2013). The authors estimated damage by eye on a 0 to 10 scale to screen a large panel of *Brassicaceae* to Cruciferae flea beetle (*Phyllotreta striolata*), including 48 *S. alba* and 33 *B. napus* accessions. However, they found no difference in resistance within species. The imprecise scoring method may have limited the potential to identify small quantitative differences in resistance. For example, a similar phenotyping method for scoring by-eye could only detect differences in feeding of 18 % or greater (Palaniswamy and Lamb, 1992b). Further experiments have used scales of just 1 to 4 (Nils Conrad and Conrad, 2019).

Some researchers have opted to use the number of shot-holes as a quantitative estimator of resistance (Heath, 2017; Hiiesaar et al., 2006; Lamb, 1980). However, the size of shot-holes can vary considerably, from small "test-bites" to almost entire leaves. The feeding data obtained from chapter 1 comparing CSFB damage to the age of the insect can be used to demonstrate the wide range in actual area eaten when using shot-hole number as a predictor of damage [Fig. 4.1].

These previous screens using imprecise scoring techniques have failed to identify intra-species resistance to CSFB. Therefore, it is likely that CSFB resistance is a highly nuanced trait which cannot be identified using broad qualitative scales. Development of a more precise scoring technique could facilitate identification of intraspecies contrasts for CSFB resistance.

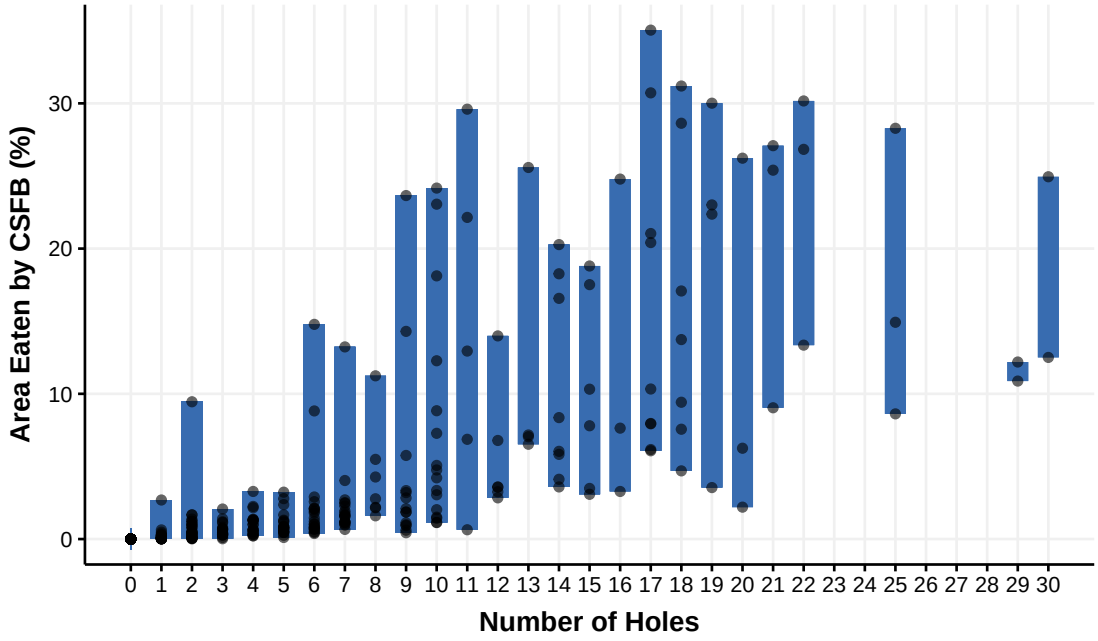

Figure 4.1: Variation in actual percent area of leaves eaten compared to counting of shot-hole number, demonstrating the imprecision of using shot-hole number as a proxy for antixenosis. This data was obtained from experiments in Chapter 1, and the precise % area eaten scoring was obtained from the ImageJ pipeline developed during this project. The ImageJ pipeline semi-automatically quantified damage to the cotyledons of 366 seedlings of *Brassica napus* var. "Arrow". Instances where there were fewer than 2 replicates for a particular number of shot-holes were excluded, leaving (*n=362*) seedlings plotted.

While quantitative scoring has been used to assess CSFB antixenosis, it has not been used on a large scale. Palaniswamy and Lamb (1992a) compared 9 species for CSFB antixenosis using photography of leaves for quantitative scoring, but noted that their method was too time-consuming to be used for further screens. The method also required that the experiment was halted with very low levels of damage, which prevents the accurate identification of small, quantitative differences in antixenosis when considering variability in feeding between seedlings.

4.2 Computational techniques for phenotyping cabbage stem flea beetle resistance

Computational advances now present an opportunity to quantify damage in a rapid, reproducible manner to identify small contrasts in antixentosis to CSFB. Software to measure herbivory from chewing insects is readily available, such as BioLeaf and LeafByte (Getman-Pickering et al., 2020; Machado et al., 2016). Although convenient for quick quantification of small numbers of samples, these software do not allow processing of large

numbers of leaves at any given time. Furthermore, they are unable to identify feeding on the leaf edges. Interpolation of the leaf boundary is vital for accurate damage scoring. Without interpolation, damaged to seedlings with more than 5 % of the leaf surface damaged are quantified as significantly underestimated [Fig. 4.2].

ImageJ is a widely used alternative for measuring herbivory (Abràmoff et al., 2005). Though ImageJ is not optimised for scoring herbivory and so can be fairly labour-intensive, the software has the benefit of scripting to process large batches of images. There is therefore potential to use scripting to increase both the throughput and the precision of scoring CSFB feeding damage on a large number of *Brassicaceae* accessions.

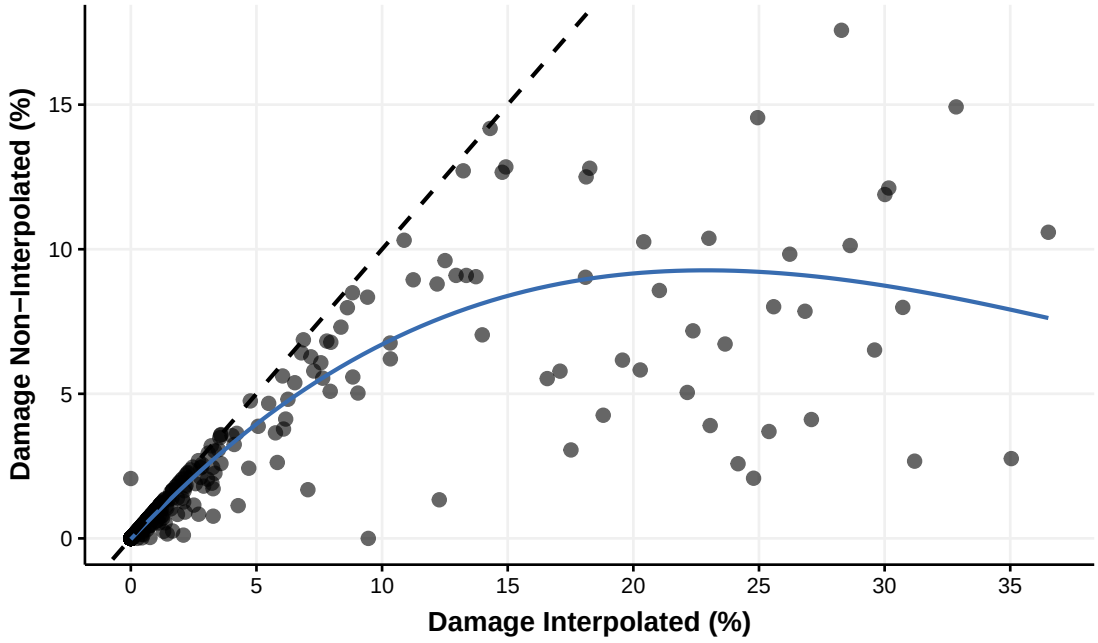

Figure 4.2: Comparison of percent area missing quantified from an ImageJ pipeline for the same samples, with and without interpolation of eaten leaf edges. The ImageJ pipeline semi-automatically quantified damage to the cotyledons of 366 seedlings of *Brassica napus* var. "Arrow". The dashed line represents the expected fit of points if interpolation of leaf boundaries was not important. The blue solid line shows a LOESS-smoothed line of best fit with an alpha value of 1.

4.3 Aim and objectives

The aims of the current study was to develop a high-throughput, robust and reliable screening method to quantify antixenosis to cabbage stem flea beetle to screen whole plants at a development stage relevant to field conditions (i.e. seedlings). Additionally, the assay would be used to identify accessions of Brassicaceae with contrasting levels of resistance to CSFB feeding. The objectives were to quantify the area of leaf eaten by

CSFB using computational methods (ImageJ). The assay would then be used to phenotype a diverse panel of 65 accessions of *Brassicaceae*, oilseed rape (*Brassica napus*) and white mustard (*Sinapis alba*). Contrasts in antixenosis between lines would be verified by testing in situations more relevant to the field, including using adults of a relevant biological stage and performing a field trial.

4.4 Materials and Methods

4.4.1 Precise quantification of area eaten using an ImageJ pipeline

Area eaten by feeding was quantified destructively. Cotyledons were cut across the lamina base and arranged onto a white board with a scale, then scanned with a desktop scanner at 1200 dpi. Images were processed using an ImageJ pipeline, as shown in Figure 4.3. The estimated undamaged leaf area was calculated automatically in ImageJ by extracting the area of the shot-holes from the interpolated images. Data from ImageJ for leaf area and area eaten was saved as a .csv file and exported to Excel. Percent leaf area eaten was calculated by dividing the total area of the eaten leaf by the estimated undamaged leaf area. (See the Appendix for the scripts used to automate the thresholding, the leaf area measurement, and the area eaten measurement.)

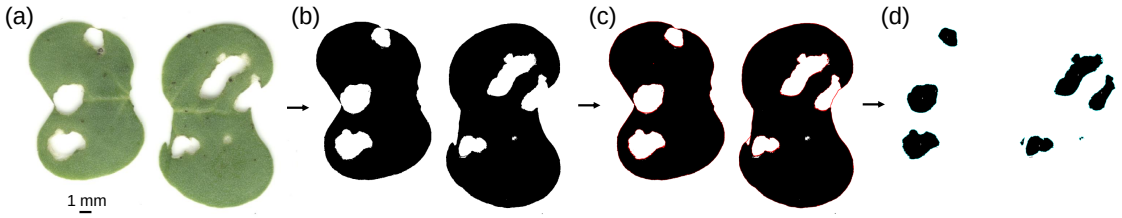

Figure 4.3: Example seedling processed using the partially automated ImageJ pipeline developed here. (a) After an experiment testing CSFB feeding was completed, seedlings were cut and scanned using a flat bed scanner, and individual seedlings were manually cropped from scanned images using ImageJ software v. 1.52a. (b) Images were automatically thresholded using Otsu's method. (c) Any eaten leaf boundaries were manually interpolated using the paintbrush tool, shown in red here. (d) The area of holes within each interpolated seedling was measured automatically using a minimum hole size of 50 pixels to exclude noise. Only steps (b) and (d) are automated here, but there is scope to fully automate this pipeline.

4.4.2 Screening a panel of *Brassicaceae* for cabbage stem flea beetle resistance

A phenotyping screen was developed to measure CSFB resistance for a large panel of whole seedlings in a controlled non-choice environment. The design was chosen to minimise space by arranging seedlings vertically. As *Sinapis alba* is an obligate out-crossing species, some lines were unfixed and so the design also aimed to reduce variance between replicates of the

same line by using multiple flea beetles and seedling per replicate. In addition, seedlings were transplanted into the experimental containers opposed to growing in them directly, as this allowed selection of the most uniform seedlings and also allowed consistency in the arrangement of plants within the container.

Six days after sowing seed (as described in the general materials and methods), the base of a plant cell culture box made of PETG (PhytatrayTM II, Sigma-Aldrich) was filled with 200 mL 1.5 % water agar for each replicate. The six most vigorous seedlings were selected for each line, and transplanted into the set agar, ensuring no damage to plant tissues to prevent induction of defences due to mechanical damage [Fig. 4.4]. In addition, un-sexed CSFB aged 1 to 20 days post-eclosion were starved for 24 hours by moving them to a fresh container with access to water but without food. 24 hours later, lids were attached and for each box four starved CSFB were added using an insect aspirator through a hole drilled into the centre of the lid. The hole was sealed with a foam bung and beetles were left to feed for 48 hours. All plant-growth and experiments occurred in a controlled environment room (CER) at 22 °C day/night, 16 hr day length, 48 % humidity. This set-up was validated by screening 20 replicates of a single genotype across 5 weeks (3 to 6 reps per week) using insects aged 8 to 16 days in age. Variance in percent area eaten between replicates within a block was low, as the mean standard deviation was just 3.5 %. This indicated the preference by the beetles in this design was able to be consistently recorded. However, the mean area eaten for each block varied from 1.6 % to 15 %. The difference between each block was due to changing the particular batch of CSFB. This variance highlighted the need for an experimental design that would resolve the differences between beetle batches (blocks).

Figure 4.4: Image of one of the chambers used for the large phenotyping screen. Each replicate consists of a single chamber containing six seedlings, transplanted from compost at 6 days old into 1.5 % water agar. The lid is attached on day 7, and 4 CSFB are added for 48 hours. After this time, CSFB are removed and damage to cotyledons is destructively quantified.

A panel of 65 accessions were screened using the assay described above for antixenotic

(non-preference) resistance, consisting of 15 accessions of *S. alba* and 50 accessions of *B. napus*. This material was provided by Innolea (Mondonville, France), apart from three benchmarking genotypes of *B. napus*. Of these, two *B. napus* varieties were previously identified by Jessica Hughes at the John Innes Centre as showing contrasting antixenotic resistance to CSFB in choice assays, named "Res." for resistant and "Sus." for susceptible. These were used to validate the results of the experiment. Line 0 (var. "Arrow") was also included sporadically when there was an excess of beetles. This genotype is a restored hybrid and was a candidate for the AHDB recommended list in 2018/19. This line was included to benchmark the rest of the material against a commercially available *B. napus* line.

An incomplete block alpha design was generated with Gendex software (module ALPHA 8.0) to assign accessions to each block. Initially, each line had 3 replicates, with 12 lines screened per block, resulting in 16 blocks. This blocked experiment was run twice, as the variance between replicates was high. Therefore, 6 replicates per line were phenotyped across 32 blocks. The blocking factor was the date the experiment was performed, with a different batch of beetles used each time to ensure young, heavily feeding beetles were utilised.

There were 22 instances where seedlings did not germinate sufficiently, and so some genotypes did not achieve 6 replicates for each genotype. Instead, these genotypes were replaced with an additional replicate of a different genotype within that block. This resulted in 22 genotypes having more than 6 replicates. Line 0 was not included in the incomplete block design, and instead was added when there was an excess of beetles. It was added into 7 blocks resulting in 20 replicates for this one genotype.

Data was imported into R version 4.2.1 (packages: ggplot2, tidyverse, dplyr) and factors influencing feeding were plotted to observe their effects. Factors considered were the number of beetles still alive after 48 hours, the number of plants per chamber, beetle age, and the block. There were no blocks or beetle ages with unusually different feeding. Data was filtered to remove instances where more than 1 beetle died during the experiment, or where fewer than 3 plants were included in the chamber. This left 2 genotypes with fewer than 3 replicates, and so these 2 genotypes were also removed from the analysis. After filtering, 14 lines of *S. alba* and 49 *B. napus* accessions remained, which included the 3 benchmarks. A Gaussian distribution of the residuals was assessed through quantile-quantile plots, skewness calculations and Schapiro-Wilk tests. Residuals were not normally distributed, as there was a characteristic positive skew as only some of the replicates were eaten particularly heavily. The data was transformed using a square-root transformation for analysis.

All lines of the two species were compared to assess if in this design *S. alba* showed higher levels of antixenosis as would be expected. An unbalanced ANOVA was utilised to account for the different number of replicates for the different genotypes. Replicate number varied from 3 up to 20 for line 0. The average (mean and median) was 6 replicates

per line. The mean number of plants per replicate was 5.9, for both *S. alba* and *B. napus*. In total, 403 chambers of 63 lines (14 *S. alba* lines and 49 *B. napus* lines) were analysed, for a total of 2387 seedlings. Percent area eaten was compared to the plant species (factor), with the week as a blocking factor to account for clustering to the experimental block, and the number of plants within a box as a covariate. The number of plants within each Phytatray was included as a covariate, as it had a linear relationship with area eaten. The age of the insects was assessed for suitability as a co-variate, but had no significant impact on the model and so was excluded from the analysis.

To compare the mean area eaten by CSFB for all of the different plant genotypes, a similar model was used. An unbalanced ANOVA compared percent area eaten to plant line (factor), with week included as the blocking factor and the number of plants per chamber as a co-variate. A Bonferroni post-hoc test was used to understand which lines were significantly different from one another.

4.4.3 Re-screening the most contrasting genotypes for validation

Non-choice experiment

Additional replication of the non-choice assays was performed for the lines of interest, controlling for insect sex and with increased replication. This was to validate the initial results seen in this experimental set-up, as a large amount of error was present. The results of the initial screen demonstrated that there were no significant differences between *B. napus* genotypes. However, there were significant differences between some *S. alba* lines. (n=4) replicate Phyatrays were screened for lines 10, 11 (*B. napus*), 86 and 91 (*S. alba*), using insects from the captive colony between 7 and 11 days old. All replicates and lines were screened at the same time in this case, unlike the previous screen which used a blocked design. Insect sex was controlled for the first time, and 2 male and 2 female CSFB, starved for 24 hours, were added to each replicate.

A one-way ANOVA was performed to compare the four lines to the percentage area eaten. A square-root transformation was used to stabilise the residuals, and a Bonferroni post-hoc test was used to identify which lines were contrasting.

Choice experiment

Some of the most contrasting genotypes were taken for further analysis. Here, an alternate chamber was used that was lower-throughput, but allowed for presenting beetles with a choice. For some of the *Brassica napus* genotypes that were not significantly different, it was hypothesised that including them in a choice experiment may help to more clearly identify antixenosis, as they would not need to feed on the resistant genotypes out of necessity. Therefore, this experiment focused on testing if contrasts between genotypes were stronger when beetles were presented with two genotypes at once. This repeated

screen also used field-caught adults to verify that the results were not limited to the laboratory-reared population. Adult beetles were captured from a WOSR field trial in Norwich, UK, at the end of October 2020. Adults were maintained separately from the reared colony to prevent potential contamination with disease, and so were kept in the laboratory at ambient conditions (approximately 24 °C with natural sunlight and no additional lighting) before experimentation commenced over the subsequent month.

A novel feeding chamber was developed by Jessica Hughes and Anna Jordan at the John Innes Centre. Twelve equally spaced slits approximately 0.5 cm in width were drilled into the rim of a 35 x 10 mm Nunc petri dish (Thermo Fisher Scientific, Inc.). The gaps were sealed with PVC tape, and the lid was filled to the brim with 1.5 % water agar. The dish was placed on a white PVC board, and after setting, an incision was made in the agar at the point of each opening. 12 seedlings were placed at each, with their cotyledons inside the dish and stem passing through the gap. Their stems were gently pressed into the cut in the agar, presenting the cotyledons inside the dish with other plant tissue left inaccessible. Foam bungs were used to fill the remaining gaps around the stems, and the lid of the dish was secured. 6 beetles starved for 24 hours were added to the dishes, and allowed to feed for 48 hours. Sex was not controlled. Half of the seedlings on one side of the dish were of one genotype, while the other half were of the other genotype. The percent area eaten was quantified destructively using the ImageJ pipeline described above.

All genotypes were not screened together at once due to a limited number of available beetles. Therefore, only two lines were screened together per block. The lines compared were 10 versus 11 (*B. napus*), 18 versus 7 (*B. napus)* and 86 versus 91 (*S. alba*). Two recombinant inbred line populations were already available for line 10 x 11, and 18 x 7, so screening these was prioritised.

Statistical analysis was performed for both percent area and unit area, as results may be skewed if cotyledon sizes are considerably different. Although leaf size did not appear to differ by eye, performing both sets of analysis verifies that leaf size does not have an impact. Leaf size was not included in the models for analysis as the true leaf size at the start of the experiment cannot be measured destructively, which was the technique used here at the end of experimentation. Although leaf size can be estimated at the end of the experiment from interpolated images, leaves were sometimes observed to shrink considerably after the experiment was finished. Therefore, scoring leaf size in this way would not be accurate. The three pairs of lines were analysed separately, as they were not directly screened against each other. Three independent samples t-tests were performed to compare the percent area eaten for the grouped lines; 10 vs 11, 7 vs 18, and 86 vs 91. Feeding damage as units area (mm^2) was also compared to these grouped lines using three additional independent samples t-tests. The date of each replicate was used as a blocking factor, which corresponded to both different batches of plants and insects used. An additional chamber was run for lines 86 and 91, giving $n = 24$ replicates, whereas lines 10, 11, 7 and 18 had $n = 18$ replicates. A square-root transformation was used to

stabilise variances of the data, excluding lines 18 and 7 when scoring area eaten (mm^2), and lines 10 and 11 when scoring percent area eaten; these were not transformed.

4.4.4 Screening the contrasting candidates at a biologically relevant age

In the wild, adult CSFB do not feed upon seedlings of the WOSR crop until they are older, and after they have aestivated. The experiments performed here used young adults as they feed more heavily to try and emphasise contrasts between lines as much as possible. To test if the preferences of adults were altered at different CSFB ages, insects were screened both pre- and post- aestivation. The adults used to screen genotypes 86 and 91 in the repeated non-choice experiment using Phytatrays were maintained until they exited aestivation and began feeding again. In Chapter 1, aestivation was estimated to occur from 21 days post-eclosion, to up to 55 days post-eclosion. Then, adults exit aestivation and a small secondary peak in feeding is seen around 70 days post-eclosion. Therefore, adults were screened again at 76 days (11 weeks) post-eclosion using the same non-choice Phytatray method. Due to a limited number of insects and because they did not differ, lines 10 and 11 were not included in this repeat at an older age.

A two-way ANOVA was used to compare the percent leaf area eaten for the two lines (factor) and age (factor), and the interaction between line and week was included. A Bonferroni post-hoc test was used to assess which lines were contrasting. No transformation was required.

4.4.5 Taking genotypes of interest to the field for comparison *in situ*

Scoring using ImageJ

A single field trial was conducted in 2021/2022 at the John Innes Field Station (Norwich, UK) to compare the two *Sinapis alba* lines 86 and 91, which were observed to be contrasting for CSFB antixenotic resistance in laboratory feeding screens. A semi-field trial was also performed in 2022 using plants grown in a controlled environment room and transplanted into trays before being placed in the field, allowing control of phenology. The 2021/2022 trial was sown adjacent to a large *Brassica napus* field trial, in a site that contained barley in the previous year. Flea beetles were from naturally occurring local populations composed mainly of *P. chrysocephala*, and neighbouring fields at the site had previously seen high CSFB infestation in at the least the two prior years. Plots were sown in a chequerboard arrangement with a density of 80 seeds/m^2 and plot sizes of 6 m x 1.2 m (7.2m^2), plus an additional two coulters (border drills), one either side of the trial plot. A border of untreated *Brassica napus* var. "Barbados" surrounded the trial. The trial, including borders, were irrigated according to best management practices, but no insecticides were applied.

The trial was sown on 24th August 2021. The first seedlings were observed 7 days after

sowing (31st August), and the first CSFB feeding was observed on day 9 (2nd September). Two destructive scorings were taken, and one score by eye. For the first score, seedlings at the cotyledon stage were sampled 7 days after emergence (14 days after sowing and 5 days after the appearance of the first CSFB feeding). For each of the 5 plots per line, 10 seedlings in a row were removed from the middle of each plot and the cotyledons were cut along the lamina base and damage was quantified using the ImageJ pipeline. The border material was also sampled from the middle of the four edges of the trial in a row, i.e. 40 plants. Although the *B. napus* var. Barbados border was not included in the trial design or subsequent analysis, it was sampled to give an indication of CSFB infestation rates in the field. A second destructive sampling occurred at the first true leaf stage, 20 days after sowing and 11 days after first CSFB damage using the same method. Border material was not sampled at this stage.

An unbalanced ANOVA was used to compare the difference between the two genotypes across the two sampling dates, as 1 seedling was missing each for lines 86 and 91 at both scoring dates, meaning one plot for each line had fewer seedlings. Ultimately ($n = 98$) seedlings per genotype were analysed, with ($n= 49$) per scoring date. A log transformation was used to normalise residuals. The original treatment structure to compare these terms and interactions compared % area eaten against line nested within each plot, with the scoring date as a blocking factor. Plot did not contribute a significance effect to the model and was removed. The final analysis compared % area eaten against line (factor), with the scoring date as a blocking factor. Although scoring date did not significantly interact with line, an additional analysis was performed where this interaction was included in the model to extract predicted means for the two lines across the two dates.

Scoring by eye

Although the seedling stage is the one most of interest economically, it was also of interest to screen the plants at an older stage. For *S. alba*, this gave CSFB more time to feed which may have made differences between the lines more prominent. A field trial conducted in the previous year found that CSFB damage peaked 20 to 28 days after sowing. This trial is not discussed here as it assessed *B. napus* lines that were not contrasting, but the data was used as a guide for estimating when damage would peak in the *S. alba* trial in 2021-2022. A third assessment at the third true leaf stage was scored by estimating damage by eye 28 days after sowing (21 days after first emergence), and the border material was also scored at this stage. Destructive scoring using the ImageJ pipeline was not possible as plants were too large to fit into a scanner, and the shape of true leaves made them difficult to accurately interpolate. As above, 10 seedlings in a row were scored. Percentage area eaten was estimated for each of the true leaves expanded beyond 3 cm in length, and mean damage per plant was calculated by totalling damage across the leaves, and dividing by the number of leaves. A one-way ANOVA was performed to compare the percent area eaten (by eye) to line (factor), with plot as a blocking factor.

For this third score, the number of plants per plot, and the number of leaves per plant were also counted. Two two-sample t-tests were performed, one to measure differences in the number of plants per line, and the second to measure the number of leaves per line. Line was considered a factor and the number of plants or leaves was considered as a variate.

In all cases, if a cotyledon or leaf was missing completely that leaf was excluded, and the damage of the remaining cotyledon or leaves was recorded only. This is because it was not possible to differentiate between heavy CSFB feeding damage from other herbivores such as pigeons, though as low levels of CSFB damage were observed the latter was more likely.

Semi-field trial using ImageJ

In the 2021 trial it was observed that phenology within the plots was not consistent. There was a particularly harsh dry-spell prior to sowing, and so germination was inconsistent across the field and approximately 50 % of seedlings within each plot were delayed by 7 days. Therefore, plants were being scored at different stages. To control for phenology, an additional semi-field trial was performed in September 2022. Seedlings were grown using the standard method in the CER for 7 days, and the most uniform seedlings were transplanted into 4 trays at day 6. Each tray contained 12 seedlings each of 86 and 91 (24 per tray), arranged in side-by-side blocks. Trays were placed directly next to a *Brassica napus* field trial which had heavy CSFB infestation on day 7 (26th September 2022), located at the John Innes Field Station (Bawburgh, Norfolk, UK). Trays remained in the field for 7 days (2nd October) before being transported back to the laboratory. Seedlings with damage were sampled and scored on day 8 using the ImageJ pipeline. True leaf tissue had started to expand so was also scored. An unbalanced ANOVA was performed to compare the level of CSFB feeding between the two lines (factor), using the tray ("plot") as a blocking factor. A square-root transformation was used to stabilise residuals.

Scoring larvae infestation

In the 2021 field trial scores for larvae infestation were also taken by sampling plants on 17th December using the larvae evacuation method, which has previously been found to be as accurate as manual dissection but more efficient (Seimandi-Corda et al., 2022). Border material was also sampled for this test to assess the CSFB infestation of the field. For each replicate, a random point in the middle of a plot was chosen and 3 plants in a row were removed. Plants were cut at the soil level and all true leaves larger than 2 cm were counted for each plant. 9 border plants of *B. napus* var. "Barbados" were also sampled, with 3 plants sampled haphazardly from 3 equidistant locations around the trial. All leaves were stripped from their stems by gently bending the petiole back towards the stem, separating the stem at the join with the petiole to minimise damage to larvae

within the petioles. Stems were then cut with secateurs into lengths of 15 cm. All plant material was placed onto chicken wire suspended above a 35 L clear plastic box containing tap water a few inches deep. Larvae would fall into the water as they could not escape over the sides of the box. Boxes were maintained inside the warehouse of the John Innes Field Station at room temperature (Bawburgh, Norfolk, UK); in these conditions plants rapidly dessicated. Larvae were collected every 7 days for 3 weeks by filtering the water through miracloth, and were transferred to ethanol in 15 mL centrifuge tubes using a fine paint-brush. Collections ceased at 3 weeks (7th January) as no additional larvae had evacuated at week 4. At the end of the experiment, plants were retained and the dry weight was recorded for each replicate. The total number of larvae and the total number of each of the three instars were counted under a dissecting microscope, and the instars were differentiated by comparing their relative sizes. Approximately, first instars were 1 mm long, second instars were 3-4 mm long, and third instars were 5-8 mm (Kaufmann, 1941).

For analysis, the number of larvae per 100 g dry weight, per plant, and per leaf was assessed. These three measurements were chosen to try and control for plant architecture which was observed to be different between the two lines; line 86 had greater stem elongation, and a greater number of smaller leaves. For each measurement, a two-way ANOVA was performed. The number of larvae was compared to line (factor) and the instar stage (either 1, 2, 3; factor). A Bonferonni post-hoc test was applied for the interaction between line and larvae stage to determine if larvae development, or total larvae, was significantly different between lines. For each measurement, a one-way ANOVA was also performed to compare line (factor) to the total number of larvae. Square-root transformations were used for all larvae data to stabilise variances.

4.5 Results

4.5.1 Identification of two *Sinapis alba* genotypes contrasting for antixenotic resistance to *Psylliodes chrysochephala*

The area eaten across the two species for all lines was compared. The species explained the majority of the difference in area eaten, with *Brassica napus* eaten significantly more than *Sinapis alba* ($F = 25.77$, $P < 0.001$). For *B. napus*, a mean of 9.7 % ($\pm$ 0.8 %) leaf area was eaten, compared to 5.6 % ($\pm$ 0.6 %) of *S. alba*; a difference of around 4 %, or 58 % the area of *S. alba* eaten compared to *B. napus* [Fig. 4.5]. Although the species are significantly different as expected, the magnitude of this difference is not as large as has previously been observed. This may be due to the effect of block, which explains a large amount of the variance ($F = 21.56$, $P < 0.001$).

As expected, the number of plants in the chamber significantly impacted the area eaten of those plants ($F = 4.86$, $P = 0.028$). When fewer plants are provided to the same

number of beetles, each plant will be eaten more than if the damage was spread over a greater number of plants. Including this in the model allows for inclusion of replicate lines that had poor germination, and therefore only have 5 plants within the chamber.

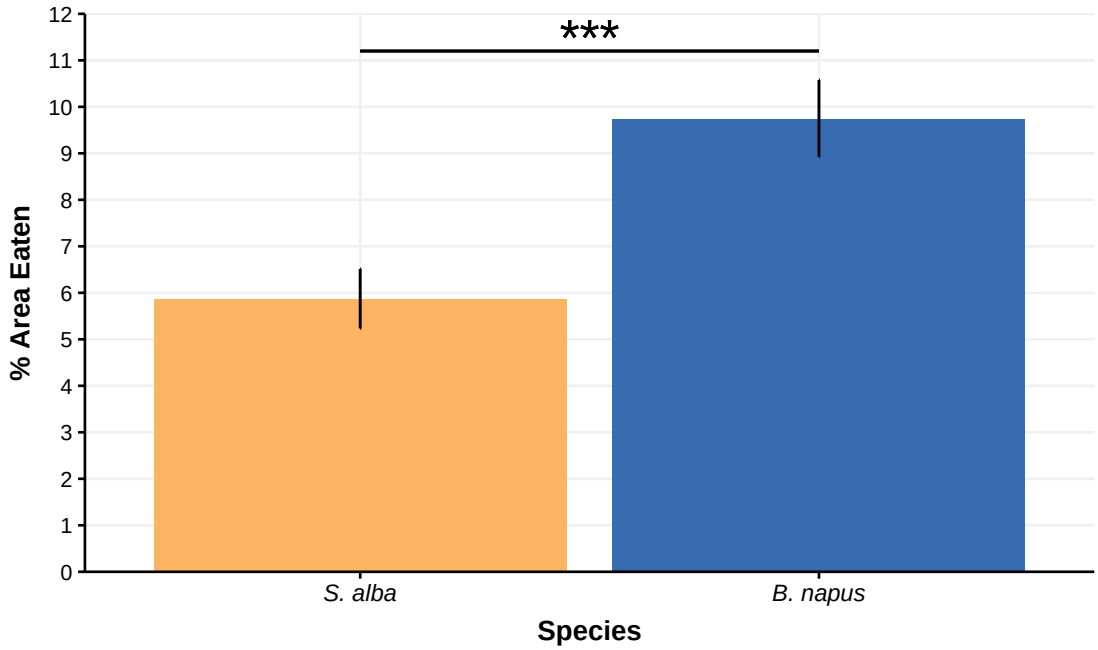

Figure 4.5: Mean area eaten as a percent of total cotyledon tissue by cabbage stem flea beetles in laboratory screens for multiple accessions of two *Brassicaceae* species. (*n=56*) for 14 lines of *Sinapis alba*, and (*n=346*) for 49 lines of *Brassica napus*. Asterisks (***) show a significance of $P < 0.001$ from analysis using an unbalanced ANOVA. Error bars represent the estimated standard error. Data is back-transformed for plotting after use of a square-root transformation.

An unbalanced ANOVA with Bonferonni post-hoc was performed to compare percent area eaten for each of the 63 lines. The biggest determinant of area eaten between all of the lines was the block, which corresponded to the use of different beetle batches ($F = 22.42$, $P < 0.001$). The differences in area eaten for each blocks was compared by re-running the model with block as the y-variate instead of plant line, and with the number of plants as a covariate. The area eaten between each block ranged from 1.57 % (± 0.03 %) to 19.34 % (± 0.08 %). This is likely due to differences in beetle behaviour between each block, due to the use of different individuals. As above, the number of plants had a significant impact on the area eaten ($F = 5.05$, $P = 0.025$).

There were lines that were significantly different from each other, showing that there were varieties with greater resistance or susceptibility to CSFB adult feeding ($F = 1.66$, $P = 0.003$). The Bonferoni post-hoc analysis shows that no *Brassica napus* lines were significantly different from each other [Fig. 4.6]. The two most contrasting lines of *Brassica napus* were line 11 (3.3 % eaten ± 1.6 %), and line 79 (20.5 % eaten ± 5.0 %). Although

not significantly different, as a large amount of variance was introduced by the block, the lines that appeared to be most contrasting were screened with additional replication to verify these results.

The *B. napus* lines that acted as checks, *B. napus* Sus. (susceptible) and *B. napus* Res. (resistant), were not significantly different from each other. Although there were no significantly different lines for antixenosis for *Brassica napus*, there were significant contrasts for *Sinapis alba*. Specifically, there was one particularly susceptible line for *S. alba*, line 86 [Fig. 4.6]. This line was eaten significantly more than lines 91, 88 and 98. Just 1.1 % (± 0.8 % ESE) of line 91 was eaten, compared to 20.5 % (± 5.0 % ESE) of line 86; a difference of 19.4 %, or 18x more of 86 eaten than 91.

S. alba line 91 was more resistant than six of the *B. napus* lines, and 88 and 98 were more resistant than three of them. It was expected that more of the *S. alba* lines would be significantly more resistant than *B. napus*, from previous studies showing that this is a much more resistant species. Potentially, the insects used within each block were not adequately controlled which introduced considerable variation (for example, CSFB age within each block was variable up to 7 days which for which the magnitude of feeding can vary greatly, see Chapter 2). The *S. alba* lines do generally cluster to the lower end of the area eaten, and there is a significant difference between the species. Repeating the analysis for the lines of highest interest, i.e. those with the greatest contrasts, with further replication and with more controlled insects, should help to better elucidate these differences.

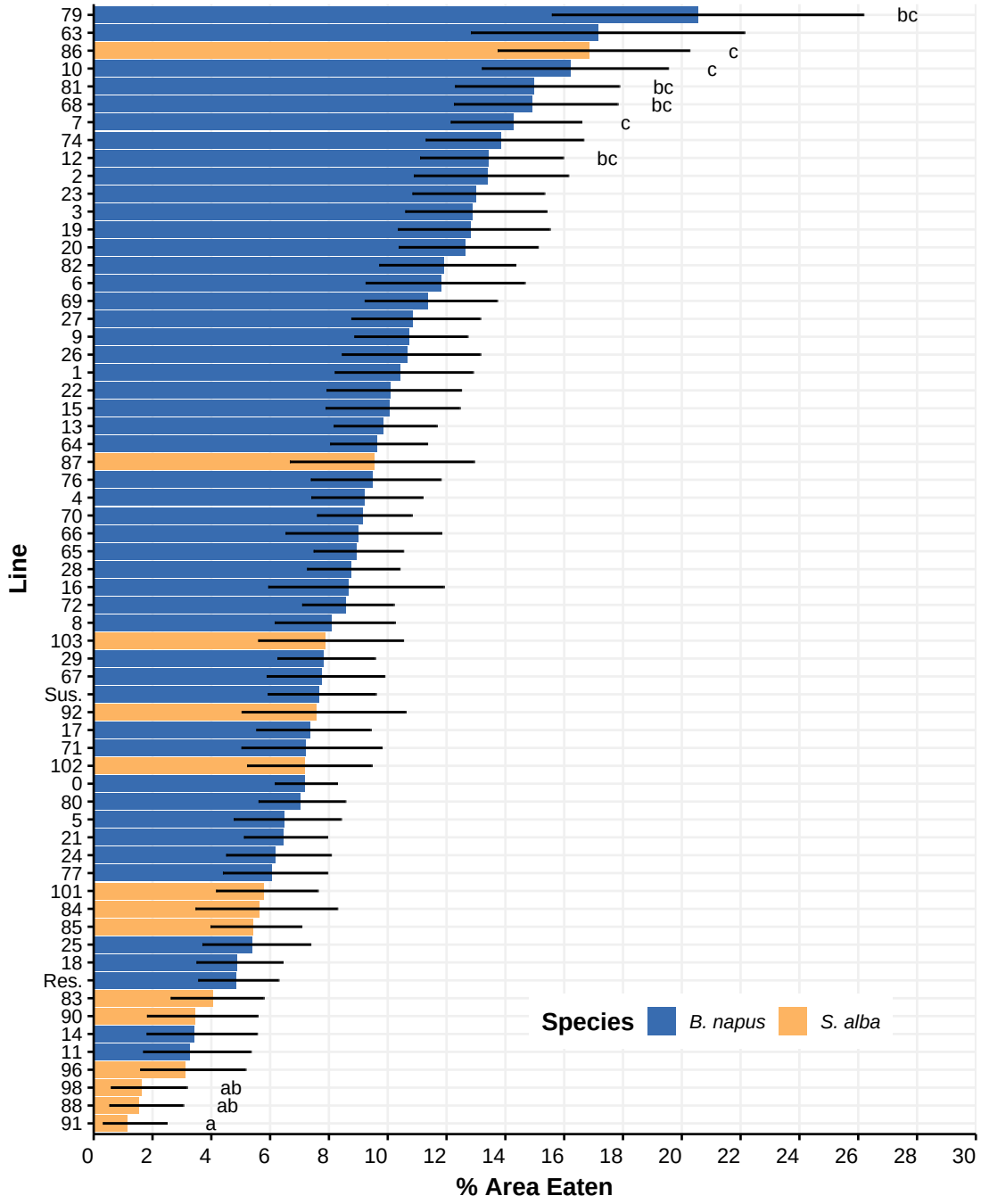

Figure 4.6: Mean area eaten for by cabbage stem flea beetles in laboratory screens for 14 accessions of *Sinapis alba*, and 49 *Brassica napus* accessions. Letters represent significant differences between lines from an unbalanced ANOVA and Bonferoni post-hoc test, where lines with the same letters are not significantly different. Only those lines that had any significant difference are displayed. Error bars represent the estimated standard error. Data is back-transformed for plotting after use of a square-root transformation. Replicate number varied due to filtering of data and was accounted for in analysis. Replicates for each line, *n=3*: 11, 14, 16, 63, 79, 84, 87, 88, 90, 91, 92, 96 98. *n=4*: 103, 6, 66, 71. *n=5*: 10, 102, 25, 83, 86. *n=6*: 1, 101, 15, 17, 19, 2, 22, 24, 26, 5, 67, 68, 74, 76, 77, 8, 81. *n=7*: 12, 18, 20, 27, 3, 69, 82, 85, Susceptible. *n=8*: 23, 4, Resistant. *n=9*: 29, 7, 9. *n=10*: 13, 21, 80. *n=11*: 28, 64, 70, 72. *n=12*: 65. *n=20*: 0,

4.5.2 Repeating resistance screens supports contrast in resistance

Non-choice experiment

The two significantly different *S. alba* lines 86 and 91, and two *B. napus* lines that were contrasting but not significant, lines 10 and 11, were screened again with more replication and in a more controlled design. There was a significant difference between the lines ($F = 9.61$, $P = 0.002$). The Bonferoni post-hoc showed that this difference was due to line 91 being eaten significantly less than the other three genotypes; both of the *B. napus* lines 10 and 11, and the other *S. alba* genotype 86 [Fig. 4.7]. Line 91 was eaten 1.2 % (± 0.4 %), and line 86 was eaten 9.8x more with 11.8 % area eaten (± 2.2 %). Interestingly, *S. alba* line 86 was equally as palatable as the two *B. napus* lines. The *B. napus* lines 10 and 11 were not significantly different from each other, which is what was observed in the initial screen.

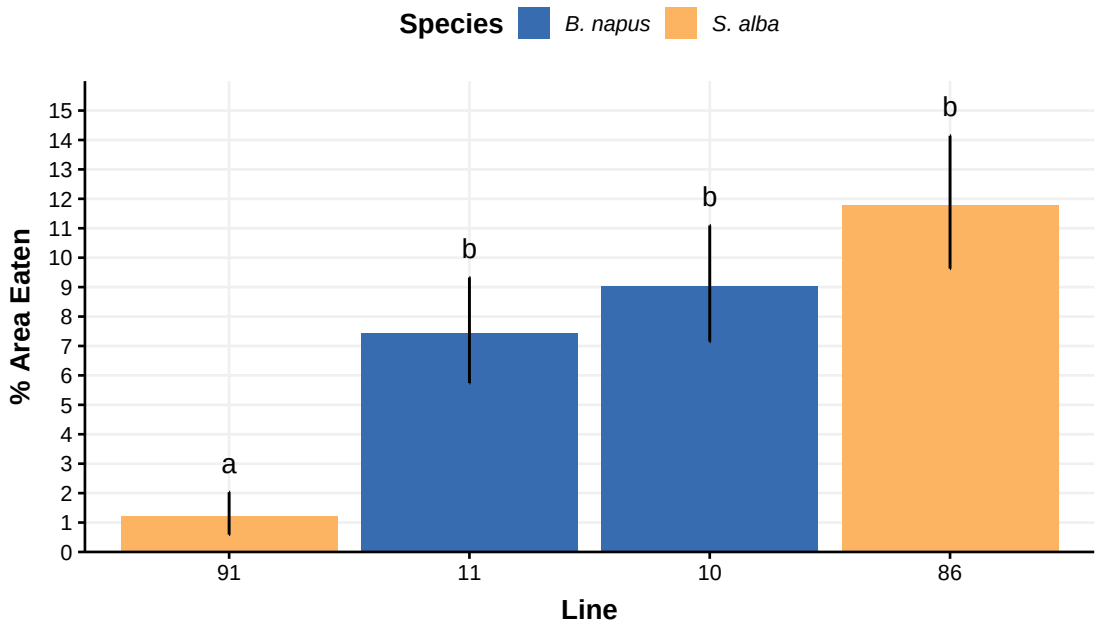

Figure 4.7: Predicted mean percent area eaten per seedling from a one-way ANOVA comparing 4 genotypes of two species in a no-choice environment. ($n = 4$) replicates per line. Significant differences between lines were calculated with a Bonferroni post-hoc, and are shown with letters, where different letters between lines represent significant differences.

Choice Experiments

Pair 86 and 91: *S. alba* line 91 was eaten significantly less than *S. alba* line 86 when scoring as both percent area eaten ($F = 7.07$, $P = 0.011$), or area eaten in mm^2 ($F =$

5.59, $P = 0.023$) [Fig. 4.8]. 2.7 % of 91 was eaten (± 1.8 %) compared to 8.5 % (± 3.0 %). 15.6 mm^2 (± 5.3 mm^2) of line 86 was eaten, compared to 6.1 mm^2 (± 3.5 mm^2) of line 91. The magnitude of the difference was slightly smaller when scoring area eaten in mm^2, with a a difference of 2.6x more eaten of line 86 than 91, compared to 3.1x more eaten of line 86 when measuring percent area eaten.

Between replicate chambers of lines 86 and 91, the area eaten varied quite considerably. This is reflected in the large impact of the blocking factor ($F = 8.23$ for mm^2 and $F = 21.48$ for % area). The magnitude in difference for 86 and 91 within the three chambers ranged from 0.5x to 27.5x % area eaten of line 86 compared to line 91, with a mean of 12.2x. There was one chamber where feeding was lower than average, and so despite the actual difference in predicted % area eaten being fairly low (just 1.11 % more of line 86 eaten than 91, where a mean of 1.15 % of line 86 was eaten compared to 0.04 % for line 91), there was a large magnitude difference between the lines (27.5x). This demonstrates the challenge of identifying contrasts when using CSFB that are not feeding heavily. There was also one chamber where the feeding quantity was high (the mean was 15.0 % for the chamber, compared to a mean of 9.4 % across all chambers of this pair of *S. alba* lines) but the contrast was reversed; line 86 was eaten just 10.0 %, whereas line 91 was eaten 19.9 %. It is not know why for this one chamber of 86 and 91 out of the four tested the contrast was flipped. However, this chamber was the final replicate for lines 86 and 91, and used beetles that had been maintained in the laboratory for 4 weeks after collection from the field. Potentially, their behaviour started to diverge from when they were first collected due a change in environmental conditions and a change in age.

Pair 10 and 11: For the *B. napus* lines 10 and 11 there were no significant differences in area eaten for either percent area eaten ($F = 0.74$, $P = 0.397$), or area eaten in mm^2 ($F = 0.18$, $P = 0.675$). The magnitude difference in % area eaten between lines 10 and 11 within the three chambers ranged from 0.9x to 1.8x more of line 10 being eaten than line 11, and for mm^2 eaten the magnitude difference ranged from 0.7x to 1.3x more of line 10 being eaten than line 11. The lines showed the same level of antixenosis across replicates. The exact area eaten varied between blocks, shown by the considerable impact of the blocking factor for both % area eaten ($F = 58.89$) and mm^2 eaten ($F = 57.33$). For example, line 10 was eaten from 3.4 % to 20.3 % across replicates. Again, this is likely due to difference between individual insects collected from the wild.

Pair 7 and 18: There was also no significant difference between *B. napus* lines 7 and 18 for either percent area eaten ($F = 1.02$, $P = 0.32$), or area eaten in mm^2 ($F = 0.2$, $P = 0.655$). As with lines 86 and 91, there was a large variance in the magnitude of the area eaten between the chambers for line 7 and 18. The magnitude in difference for 7 and 18 within the three chambers ranged from 0.006x to 1.9x of mm^2 eaten of line 7 compared to line 18, or 0.01x to 2.7x more of line 7 eaten than 18 as % area eaten. In one chamber the feeding was low for both lines (a mean area eaten of 0.7 % for the chamber, compared to 11.0 % eaten for each chamber of lines 7 and 18).

The results here support the findings of the initial screen. *S. alba* line 86 is eaten significantly more by CSFB than *S. alba* line 91, whereas the *B. napus* lines were not significantly different. As this experiment used wild-caught adults, the behaviour of the insects should be more reflective of the behaviour in the field. Therefore these results suggest that lines 86 and 91 may be contrasting for antixenosis to CSFB in a field environment. However, the lines should still be tested in field conditions to test resistance *in situ*; the need for this is also reflected in the results from the one chamber of line 86 versus line 91 where the contrast was reversed, indicating that CSFB may not always find line 86 more palatable.

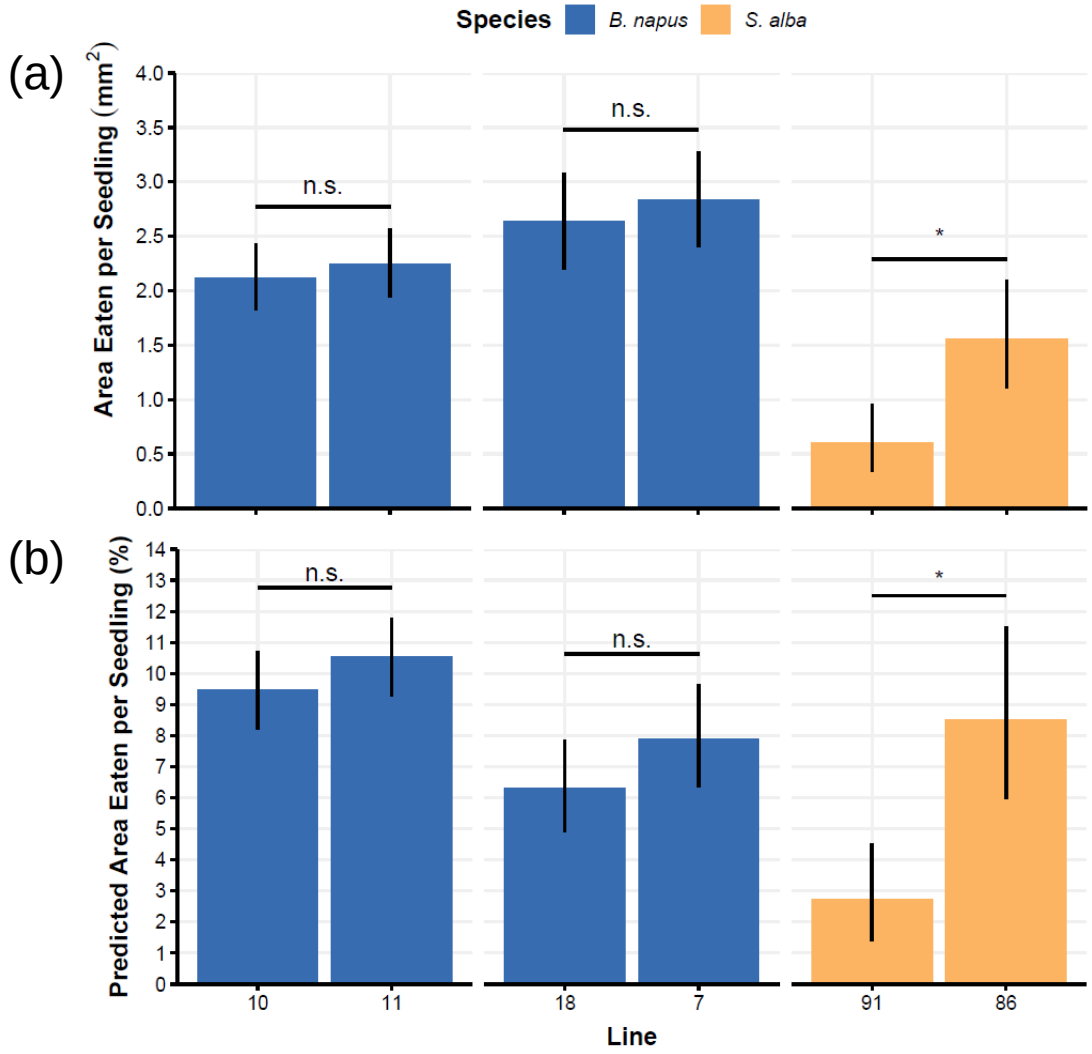

Figure 4.8: Area of leaf tissue eaten per seedling from three experiments using paired lines and analysed using t-tests. (a) scored as area eaten (mm^2). (b) scored as percent leaf area eaten (%). Three chambers of two paired lines were screened side-by-side. Pairs were 10 and 11, 7 and 18, and 86 and 91 ($n = 18$ seedlings for 7, 10, 11, 18 and $n = 24$ for 86 and 91). n.s. = not significantly different. Significant differences between lines are shown with asterisks, (*) $P < 0.05$. Note that lines outside of the pairs should not be directly compared, as they were not screened together.

4.5.3 Screening the contrasting candidates at biologically relevant age

Although in general there is a significant difference in the area eaten between the two lines ($F = 70.56$, $P < 0.001$), the age of the adult has an impact on the insects preference ($F = 6.93$, $P = 0.022$). The area eaten of 91 does not significantly change with age, but the area of 86 eaten significantly reduces for the older adults [Fig. 4.9]. 11.9 % (± 0.6 %) of 86 is eaten by the recently eclosed adults that were 11 days old, whereas just 6.3 % (± 0.6 %) was eaten by the post-aestivated adults that were 76 days old (t = -4.711). This is a 1.9x reduction in leaf area eaten by older adults on line 86. For line 91, 1.6 % was eaten at 11 days, whereas 2.7 % was eaten at 76 days in age (t = -0.992). There is no significant difference in the area eaten between lines 86 and 91 for adults ages 76 days old. This is reflected in a significant interaction of line with the age of the adult ($F = 16.26$, $P = 0.002$). However, this may be due to use of a Bonferroni post-hoc test which can be overly conservative, as the raw data indicates line 86 is eaten 2.3x more than line 91.

As the lines are not different at the CSFB age most relevant to field conditions, this results brings into question the relevance of the contrasts in antixenosis observed so far. However, the results should not be completely discounted from this one experiment. Screening these genotypes in the field against wild populations of CSFB would be the optimal way to test for contrasts in these two lines at a biologically relevant CSFB age. This screen does highlight that interesting contrasts are much more obvious when using freshly eclosed CSFB, and highlights how controlling CSFB age in this way can help to identify plant varieties with nuanced levels of CSFB resistance in the future.

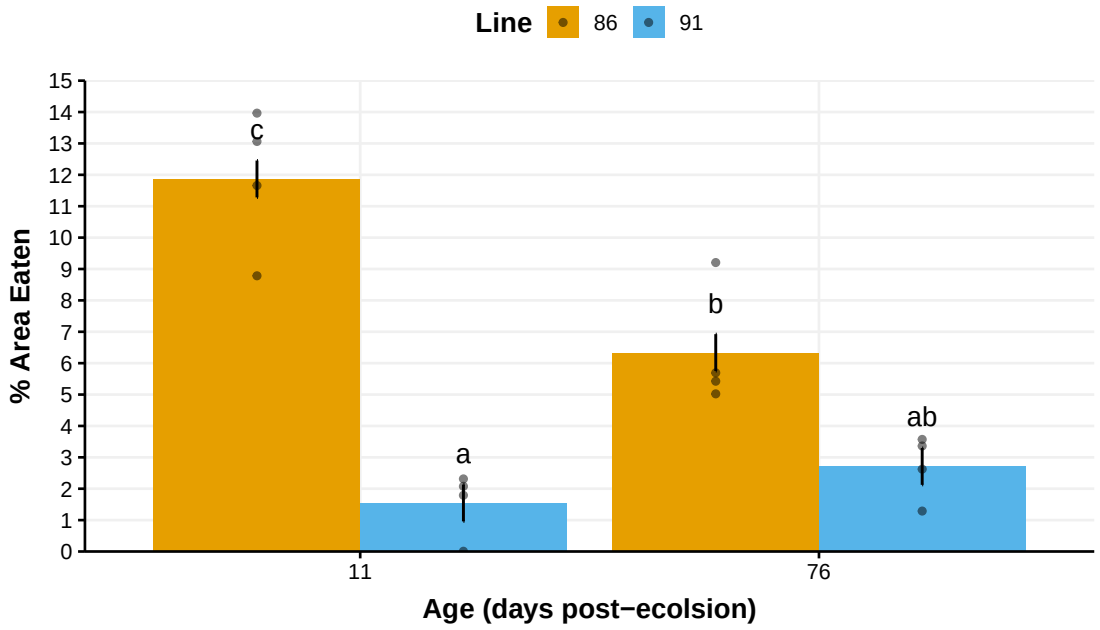

Figure 4.9: Mean percent area eaten per seedling from a two-way ANOVA comparing 2 genotypes of *S. alba* at 2 ages in a non-choice environment. ($n = 4$) replicates per line. Error bars are the standard error. Raw data is also plotted as individual points on each bar to better demonstrate the distribution of data. Significant differences between lines were calculated with a Bonferroni post-hoc, and are shown with letters, where different letters between lines represent significant differences.

4.5.4 Assessing resistance to cabbage stem flea beetles in a field trial

Adult resistance in the field

For the two scoring dates of the *S. alba* field trial utilising the ImageJ pipeline, only a small amount of damage occurred to both lines of *S. alba* [Fig. 4.10]. At 5 days after the first CSFB was noted on the trial, 0.32 % (± 0.12 %) of 86 and 0.14 % (± 0.05 %) of 91 were eaten. However, CSFB pressure was relatively high, as 7.3 % (± 0.6 %) area was eaten of the *B. napus* var. "Barbados" border material. High CSFB damage was also observed in the *B. napus* trial situated next to this one. Clearly both lines of *S. alba* are highly resistant compared to *B. napus* in the field; detection of difference between the two *S. alba* in this choice-style environment is therefore exceptionally difficult. Although the absolute difference between the lines is very small because of this low level of feeding, line 86 was eaten significantly more than 91 in at both dates ($F = 6.25$, $P = 0.013$). The area eaten of both lines at the second scoring increases significantly, which is expected as the beetles had more time to feed ($F = 50.1$, $P < 0.001$). No location effects were implicated in the analysis, as the effect of plot and it's interactions with the line was not significant. Although these analyses do demonstrate a significant difference, it is not wise to rely on

these exclusively as the feeding quantity is very low. These results do support the previous findings that line 86 is significantly more susceptible than 91 however, and together these results strengthen the evidence that these two lines are contrasting in CSFB antixenosis. These results also highlight one of the challenges in screening *S. alba* accessions against CSFB antixenosis in the field. Wild populations of CSFB are unlikely to cause a high magnitude of damage to *S. alba* when they have the choice to infest other *Brassicaceae*.

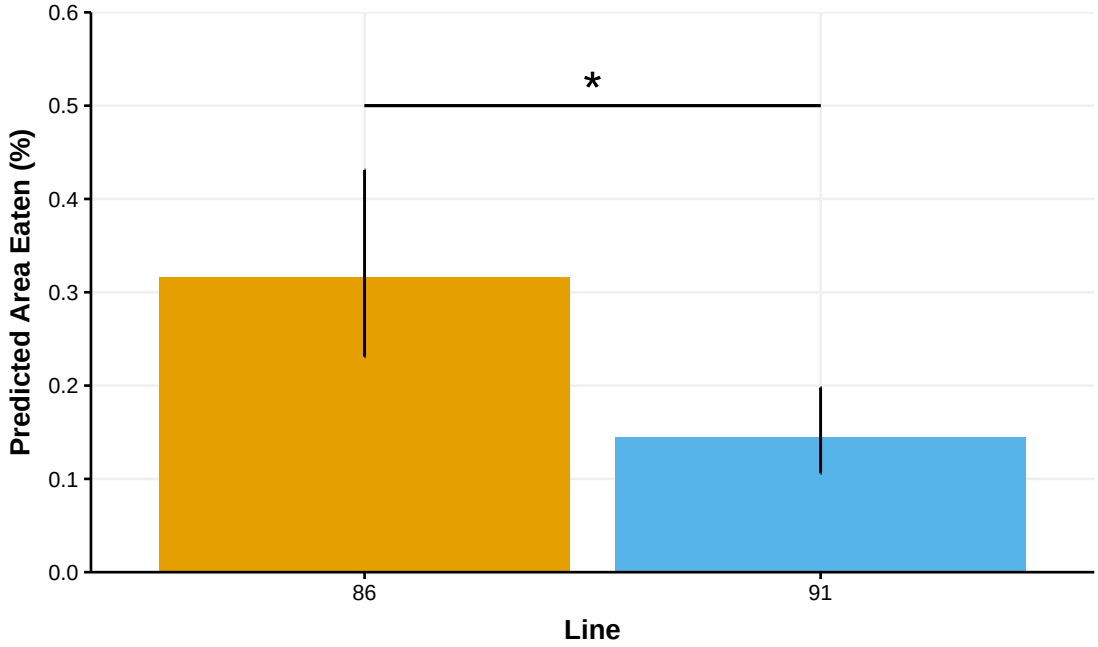

Figure 4.10: Percent area of leaf tissue eaten per seedling from two scores of a field trial. Damage was scored quantitatively using an ImageJ pipeline. (n=98) replicate seedlings for each line of *S. alba* were taken across two scoring dates. The model accounts for the effect of the sampling date using a blocking factor. A significance value of $P = 0.013$ is represented by a single asterisk (*).

The number of true leaves was the same for both lines ($F = 1.86$, $P = 0.559$), and by eye both lines appeared to be of the same size. The number of plants per plot was also not significantly different ($F = 3.84$, $P = 0.214$). Therefore phenology of these plants appeared to be equal, which aids accurate scoring of CSFB area eaten between lines. The estimated percent area eaten for the two lines was significantly different, and explains most of the variance ($F = 65.99$, $P < 0.001$). 7.4 % ($\pm$ 0.8 %) of 86 was eaten, compared to just 2.5 ($\pm$ 0.5 %) of line 91 [Fig. 4.11]. There was also a significant interaction between the plot and line, which suggests some spatial effects across the field ($F = 4.25$, $P < 0.001$). Hence the plot was included as a blocking factor in the analysis to account for this.

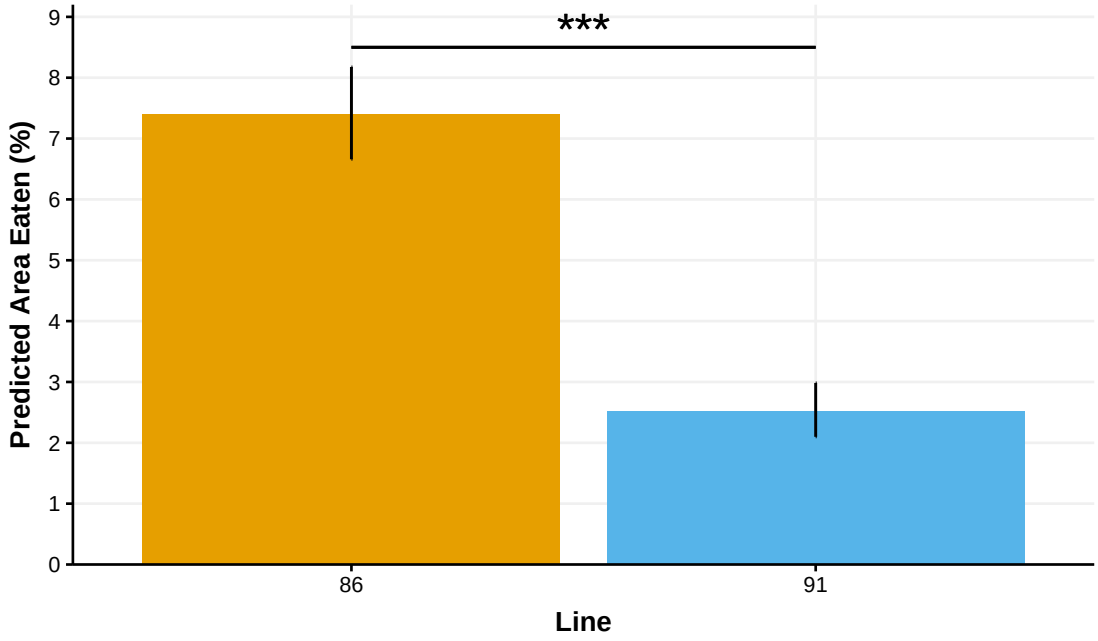

Figure 4.11: Percent area of leaf tissue eaten per seedling from a field trial, using by-eye scoring of (*n=49*) replicate seedlings for two lines of *S. alba*. The model accounts for the plots which were sampled (spatial effects). A significance value of ($P < 0.001$) is represented by asterisks (***).

In the semi-field trial controlling for plant phenology, fewer plants of 91 were damaged, with 15 plants showing CSFB damage for line 91 and 20 plants for line 86. This suggests that in this choice environment, wild CSFB preferentially feed on 86. When scoring the damaged seedlings, line 86 was eaten significantly more than line 91 ($F = 4.31$, $P = 0.047$), with 1.1 % ($\pm$ 0.4) of line 91 eaten compared to 2.1 % ($\pm$ 0.7) of line 86 [Fig. 4.12]. The absolute difference between the two lines is very small, with line 86 eaten just 1 % more than line 91. However, this small difference is expected in the field, where the level of damage to *S. alba* is typically very low. This indicates that although these two lines are contrasting for antixenosis, they are generally both very resistant against CSFB. The difference between these two lines equates to line 86 being eaten 1.8x more than line 91, which is comparable to the magnitude difference observed in controlled laboratory screens. There was also a significant difference between the four trays, though it explained less of the variance than the line ($F = 3.3$, $P = 0.034$). This may have been due to the orientation of the trays relative to the *B. napus* field trial located adjacent, which was the likely origin of the CSFB. The trays were placed together in a square, therefore two of the trays were closer to the *B. napus* trial.

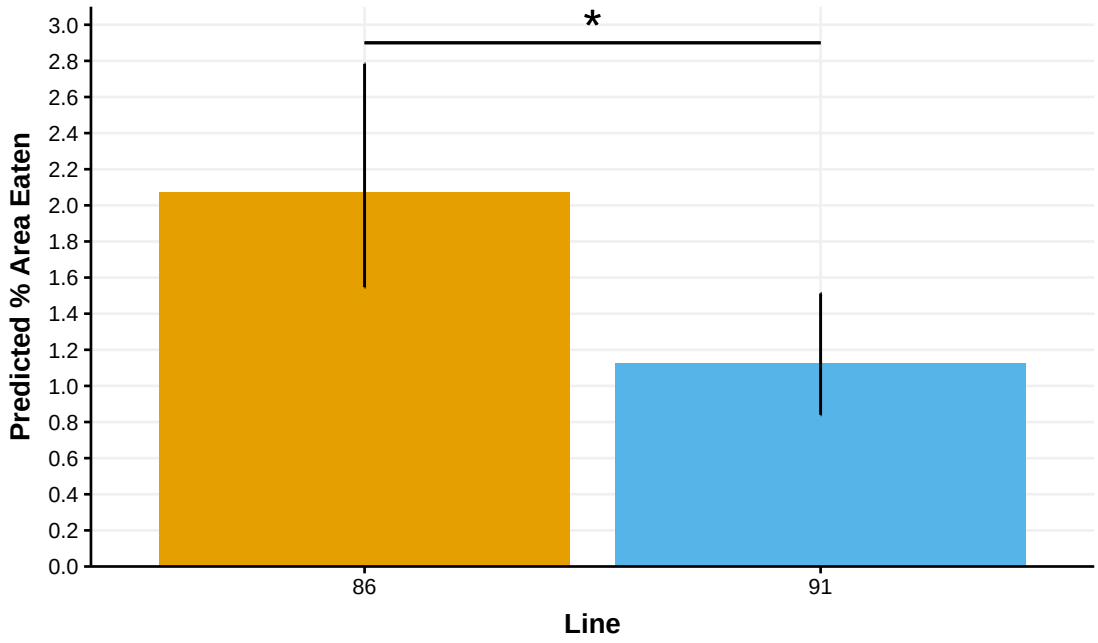

Figure 4.12: Percent area of leaf tissue eaten per seedling from a semi-field trial of *S. alba* line 86 (n=20) and *S. alba* line 91 (n=15), after 7 days at a field site, where plant number and phenology were controlled. Means are predicted from an unbalanced ANOVA, and error bars are estimated standard error. Significant differences are shown using an asterisk (*), where $P = 0.047$.

Larvae resistance in the field

Both lines of *S. alba* were much larger than the *B. napus* border, and the architecture showed much more branching and many more true leaves. The *Brassica napus* var. "Barbados" border was not included in the statistical analysis as it did not form part of the trial, but it demonstrated that CSFB larvae infestations were high. In all recording methods, the *B. napus* material had a greater number of each instar, and total larvae [Table 4.1]. When normalising the number of larvae for plant mass, *B. napus* had 170.4 (± 25.7) larvae per 100 g dry-weight, which was 5 to 6 times more larvae per dry weight than the two *S. alba* lines in the trial. There were also 2 to 3 times more larvae per plant in *B. napus* than the two *S. alba* lines despite the border having a lower mass, at just 11.4 g (± 1.4 g) per plant (dry-weight), compared to 30.9 g (± 21.0 g) for line 86, and 19.4 g (± 10.3 g) for line 91. The plant architecture of *B. napus* was quite different to *S. alba*. *B. napus* had a mean of just 7 (± 0.6) leaves, whereas line 86 had 41 (± 6.4) and line 91 had 30 (± 7.0); 4x to 6x times more leaves. Although the two *S. alba* lines had a greater number of leaves, *B. napus* leaves were larger, with larger petioles. In addition, *S. alba* had bolted, so a greater proportion of mass consisted of stem tissue, where-as the vast majority of larvae prefer petiole tissue (White and Cowlrick, 2016) (and personal

observations). This difference in architecture may have contributed to the greater number of larvae in *B. napus* seen here.

For both lines, there were significantly more first instar larvae per plant than third instars. This was the case for larvae per 100g dry weight ($F = 21.56$, $P = < 0.001$), larvae per plant ($F = 14.66$, $P < 0.001$), and larvae per leaf ($F = 25.1$, $P < 0.001$). This is likely due to the time of year, as it was relatively early in the year and so most larvae had not yet had time to develop further.

Here, CSFB per plant was considered to be the most important scoring method. The architecture between the two *S. alba* lines was quite different; *S. alba* 86 had much more stem tissue than line 91. As stem tissue is not heavily infested by larvae, the greater stem mass observed in line 86 will confound larvae counts for measurements per unit of plant mass. In addition, plant mass may be reduced where flea beetle feeding is heaviest as the plants will have fewer resources to put towards growth, and so plant mass will be correlated to the number of larvae. Flea beetles are observed to lay more eggs at the base of plants that the adult females consider to be more suitable hosts for their offspring (Maddox and Rhyne, 1975). Therefore, if each plant is infested with more larvae, it suggests that line was a more considered a more preferable host by the female CSFB. The number of larvae per plant should give a good understanding of susceptibility regardless of plant size or architecture.

There was no significant difference in the total larvae per plant between the lines ($F = 3.6$, $P = 0.094$). The current recommended treatment threshold for CSFB larvae is five larvae per plant, and yield tends to decreases in plants with more than 10 larvae (Nicholls and Ellis, 2016). The number of larvae per plant recorded in the *B. napus* here far exceeds this at 19.2 ($\pm$ 2.8) larvae per plant, and confirms observations that infestation of CSFB was high in this trial. Although both *Sinapis alba* genotypes were infested much less by CSFB than *B. napus* var. "Barbados", they both exceeded the treatment threshold. This result supports previous findings that *S. alba* is a less preferable host for CSFB larvae than *B. napus* (Döring and Ulber, 2020), though as the *B. napus* surrounded the *S. alba* it may have acted as a trap crop and exacerbated the contrast further.

There was no significant difference in the total larvae per 100 g dry weight between the lines ($F = 1.39$, $P = 0.273$). The number of larvae of each instar was also the same for the two lines ($F = 1.97$, $P = 0.173$). There was also no significant difference in the total number of larvae per plant for the two lines ($F = 3.6$, $P = 0.094$). However, there was a significant difference between line when analysing the numbers of each instar.

There was a large significant difference in the total number of larvae per leaf, with 1.4x more larvae per leaf in line 86 than 91 ($F = 72.72$, $P < 0.001$). There was also a significant difference in the number of third instar larvae between the two lines, with 2.2x more larvae in line 86 than 91 ($F = 5.4$, $P = 0.029$). As the number of leaves between the two lines was not significantly different ($F = 1.39$, $P = 0.272$), we may expect the

results for larvae per plant and larvae per leaf to be consistent, but this is not the case. Instead, the difference between lines is more pronounced when measuring larvae per leaf than larvae per plant. Scoring larvae per leaf may control for the differences in plant size within a line (i.e. maximum larvae load), without introducing confounding effects due to the difference in stem mass when measuring larvae per unit plant mass.

	Line			Statistics of 86 vs 91	
Larvae instar	*B. napus* Barb.	*S. alba* 86	*S. alba* 91	F	P
		Number of larvae per 100g dry weight			
1	104.0 ± 27.9	19.7 ± 4.0	13.9 ± 4.1		
2	50.6 ± 11.2	13.9 ± 3.2	12.5 ± 2.4	1.97	0.173
3	15.8 ± 3.3	3.2 ± 1.5	1.4 ± 0.64		
Total	170.4 ± 25.7	36.8 ± 5.8	27.8 ± 5.3	1.39	0.273
		Number of larvae per plant			
1	11.3 ± 2.6	5.8 ± 1.4	2.5 ± 0.74		
2	6.0 ± 1.7	4.1 ± 1.2	2.7 ± 0.95	6.2	0.02*
3	1.9 ± 0.59	0.87 ± 0.29	0.33 ± 0.15		
Total	19.2 ± 2.8	10.8 ± 2.6	5.5 ± 1.4	3.6	0.094
		Number of larvae per leaf			
1	1.8 ± 0.45	0.14 ± 0.018	0.091 ± 0.026		
2	0.87 ± 0.20	0.11 ± 0.033	0.084 ± 0.013	5.4	0.029*
3	0.27 ± 0.063	0.019 ± 0.0067	0.0086 ± 0.0041		
Total	2.9 ± 0.41	0.26 ± 0.040	0.18 ± 0.018	72.73	< 0.001*

Table 4.1: Mean number of CSFB larvae (± standard error) within plants of a field trial, presented as larvae per 100g dry-weight, larvae per leaf, or larvae per plant. For statistical analysis only the two *S. alba* lines 86 and 91 were compared. *B. napus* var. Barbados is included as a reference for CSFB abundance in the trial only. A one-way ANOVA was used to compare the total number of larvae for the two lines, for each type of measurement. A two-way ANOVA was used to compare both line and instar number for the two lines for each type of measurement, with the statistics presented being for the interaction of line and instar. Significant differences between lines 86 and 91 are shown with an asterisk (*) where $P < 0.05$.

4.6 Discussion

4.6.1 Development of a semi-automated pipeline to quantify CSFB resistance

Here, a semi-automated ImageJ pipeline was developed to precisely quantify the area of leaf tissue eaten by CSFB [see the Appendix for the scripts]. The challenge in full automation of this pipeline is due to herbivory present at the leaf edges. The protocol

used here required manual interpolation of leaf boundaries. While this is intuitive due to the consistent shape and smooth leaf boundaries of cotyledon, it is time consuming and introduces the potential for subjectivity from the experimenter. The need for manual interpolation could be negated by scoring of control plants without CSFB, and comparing leaf area remaining from test plants to these. However, this significantly increases the labour and space requirements for performing experiments. In addition, the *Sinapis alba* lines tested here were not fixed, as white mustard is an obligate out-crossing species. Cotyledon size could therefore be variable within a genotype, and so comparison to other individuals would reduce the precision of scoring. Leaves were also observed to shrink considerably after experiments, potentially due to water loss from heavy feeding [Fig. 4.13]. As the leaf area of these shrunken leaves was reduced quite considerably from the start of the experiment, comparison to undamaged leaves would misrepresent the level of damage.

Figure 4.13: Demonstration of how some leaves appeared to shrink from heavy flea beetle feeding. Two sets of cotyledons from two separate seedlings of *Sinapis alba* genotype 91 are shown. Seedling size was uniform at the start of the experiment. These seedlings were exposed to CSFB in a laboratory screen in the same chamber. The left most cotyledon (*) is smaller than the other three cotyledons and has experienced a high level of damage, compared to the other cotyledons with no/low levels of damage. The scale bar is 1 cm for all leaves. Seedlings are overlaid onto the processed image (red), showing the interpolated boundary.

Automated interpolation of the leaf boundary could instead be performed with techniques such as active contour models (De Vylder et al., 2011), or machine learning algorithms once a large enough dataset of images labelled for damage was collected (F. Danilevicz and Bayer, 2022). Several thousand labelled cotyledon images were produced from this project, which may be a useful resource in the development of such a machine learning algorithm. There is also scope to automatically crop images to the seedling-level, for example by using connected component analysis to identify the cotyledons in an image. Packages such as OpenCV in Python are able to perform such techniques (Bradski, 2000). Integrating these tools together would allow scanned images to be fed through a single

pipeline, and output quantified damage scores. However, these automation techniques require an advanced level of programming to develop, which was beyond the scope of this project. The ImageJ pipeline developed here was sufficient to accurately quantify CSFB feeding for a large panel in a reproducible manner to a much higher level of accuracy than has previously been reported. Though rapidity could be improved, there was not a sufficient need to further optimise the pipeline.

4.6.2 Screening a panel of *Brassicaceae* for cabbage stem flea beetle resistance

Across all lines, the mean area eaten for *B. napus* was 1.7x more than *S. alba*. The magnitude of this difference is comparable to a previous study quantifying CSFB antixenosis in non-choice assays for *B. napus* and *S. alba*. Palaniswamy and Lamb (1992a) also performed a non-choice assay using the cotyledon stage of *B. napus* seedlings, though in this case they were exposed to three to four CSFB per plant for 24 hours using just six replicate plants (compared to the experiments performed here where at least three replicates of six plants were used). In these experiments, 1.7 mm^2 of *S. alba* was eaten, and 3.0 mm^2 of *B. napus*, a difference of 1.8x more *B. napus* consumed. However, in the previous study by Palaniswamy and Lamb (1992a) this was not a significant difference. Potentially, this may be because CSFB were field-collected and perhaps more variable in their age and behaviour, or due to the limited number of replicate plants studied. As the magnitude difference between the two species is consistent for the experiment here and this previous study using field-collected adults, it suggests that CSFB behaviour for this laboratory colony and field-collected adults is consistent.

The experimental block explained the majority of the difference between lines. The factors that changed between blocks were the batch of plants and the batch of CSFB used. As the plants were grown in a consistent way (for the same amount of time, in the same conditions, and in the same location), it is much more likely that the large amount of variance between blocks is due to differences in behaviour between individual beetles. Across all blocks, insect age could vary by up to 20 days, and Chapter 1 demonstrated how CSFB feeding can change considerably with age. However, insect age did not have a significant impact on the percent area eaten here, perhaps because age was an estimate $\pm$ 7 days, or because there were other difference between beetle batches that were not controlled. The results demonstrate that beetle feeding behaviour can be highly variable even on the same line. Better controlling for insects (e.g. screening all replicates using insects from the same "batch") could significantly reduce this variance, and greater differences between the species may be observed. This was controlled as much as was possible given the constraints of screening such as large number of replicates. Future experiments would therefore focus on just the most highly contrasting lines for further replication using more controlled insects.

No lines of either *B. napus* or *S. alba* were completely resistant. These results support prior findings that resistance to flea beetles in *Brassicaceae* is not dichotemous as only quantitiative differences in flea beetle resistance have previous been identified (Heath, 2017; Kuzina et al., 2011).

Re-screening of the most contrasting lines

When re-screening the lines of interest in a non-choice assay, line 91 was eaten significantly less than the other lines 10, 11, and 86. These results confirm the results seen in the initial screen, and indicate that both the experimental design and analysis using an incomplete block design were able to analyse contrasts in antixenosis. These results also suggest that the sex of the adults did not significantly influence the results of the initial screen. This may be because the sex ratio for CSFB is approximately 1:1 (personal observations), so therefore there is a low probability of a significant skew towards either males or females for a particular line in the initial screen, and so the sex ratio in the initial screen and this repeat should be approximately the same. These results therefore do not disclose if there are differences in preference or behaviour between male and female CSFB.

The magnitude difference between lines 86 and 91 was greater when screened in a choice assay; 3.1x more leaf area (%) was eaten of line 86 in a choice environment. This supports prior findings that differences in CSFB feeding between species can be more easily differentiated in choice experiments. Palaniswamy (1992) found that 1.9 mm^2 of *S. alba* and 4.2 mm^2 of *B. napus*, magnitude difference of 2.2x, compared to 1.8x difference between species in a non-choice assay.

Differences between the lines tested here were consistent when recording damage as unit area (mm^2) or as % of leaf area. As % is a more intuitive measurement to understand the level of damage, percent leaf area eaten is used for reporting and analysis of subsequent experiments. In addition, significant shrinking of the leaf tissue sometimes occurred to seedlings, potentially due to water loss [Fig. 4.13]. Recording area (mm^2) would therefore underestimate damage. This results supports using percent area damage to score antixenosis for these lines, as leaf sizes were not different enough to influence the conclusion.

Although no differences within *B. napus* were detected, this is not surprising as many prior screens have also failed to identify flea beetle resistance within *B. napus*. Most of the available oilseed rape material is derived from only a few interspecific hybrid plants, and so genetic diversity is limited (Mason and Snowdon, 2016).

Further testing of *Sinapis alba* lines against biologically relevant insects

In Chapter 1, insect feeding reduced in adults older than 21 days, and this was also seen here for the two *S. alba* lines 86 and 91. It was not expected that the feeding preference

would vary between young and older adults, as feeding patterns of *Phyllotreta* flea beetles on oilseed rape did not change throughout their life cycle (Heath, 2017). However, the two *S. alba* lines were no longer significantly different in their level of CSFB resistance when fed upon by older adults. This experimental design may not be able to differentiate between lines when the feeding is low. During validation of this protocol, the mean area eaten for one *B. napus* line varied by ± 3.5 % (SD), which indicates that this is the level of precision of the assay. At 76 days of age there was just a 3.6 % difference between lines 86 and 91, so potentially this experimental design is unable to differentiate feeding at this level, especially in conjunction with use of a Bonferroni post-hoc test which can be overly conservative. Further replication could reduce the error and validate the result. Alternatively, the lines could be screened in the field which would give the best indication of whether the screening method used here is a useful method for identifying resistance which could hold up to wild CSFB preference. Therefore, a field trial was used to validate the results of the laboratory experiments *in situ*.

4.6.3 Field trials confirm contrasts in adult resistance for two *Sinapis alba* lines

The level of resistance in the two *S. alba* lines 86 and 91 remained consistent from laboratory screens to the field. These results indicate that lines 86 and 91 are interesting candidates for further study, and looking for contrasts between them could help determine a resistance mechanism to CSFB. The major difference between the laboratory assays and field trials was that the magnitude of feeding was substantially lower in the field for both lines. Potentially, the presence of *B. napus* in the trials acted as a trap crop as it is a much more preferable host.

The geographic origin for line 86 is Mongolia, and line 91 is from Greece. Although the prevalence of CSFB in Mongolia has not been reported, CSFB is not an important pest in China, which is the only neighbouring country for which CSFB pressure has been assessed (Zheng et al., 2020). As CSFB are prevalent across Europe (Bonnemaison, 1965), accessions from Greece may face greater CSFB pressure than those from Mongolia. Therefore, a CSFB resistance phenotype may have greater selection pressure in Greece, and help to explain why line 91 is more resistant to CSFB.

In the semi-field trial, damage was greater to both genotypes than in the field trial as only seedlings that received any damage were selected for scoring. Up until this point, it had not been assessed whether the resistance of line 91 was due to a smaller number of seedlings receiving the same level of damage as 86, or because the same number of plants were eaten to a less extent. These results demonstrate that the latter is true. This result can help to understand the resistance mechanism. As a similar number of both seedlings were eaten, either beetles feed more slowly on line 91 than 86, or beetles take longer to detect line 91 and so do not have time to feed to the same extent. It does not appear

that CSFB struggle to identify *S. alba* line 91 as a host. This could be tested further by observing how the beetles feed over time when presented with both lines in a non-choice test. If they take longer to start feeding on line 91 than line 86, it suggests that there is a feeding cue lacking from line 91 which prevents CSFB from finding the plants, such as secondary metabolites from the surface or from volatile emissions. Alternatively, there could be a repellent cue instead, such as secondary metabolites, volatiles, or mechanical defences. If they do not take longer to start feeding, it implies the resistance mechanism is due to a cue detected during feeding.

For the larvae experiments, the results here do not provide a concrete conclusion for whether there were differences in resistance to CSFB larvae between the two *S. alba* lines. Line 86 had more third-instar larvae than line 91, but due to differences in plant development it was difficult to establish the cause. This may suggest that line 86 is a better host as females may have laid eggs earlier, giving larvae longer to develop. Or, line 91 may have shown higher antibiosis, and so larvae may have developed faster in line 86. Scoring the number of larvae per leaf and per plant helped to reduce the variance in the data and control for plant size, while ignoring differences in stem mass that were not relevant.

The differences in larvae infestation between lines warrants further investigation, and could be tested in the laboratory using plants grown in controlled conditions to better control plant architecture. A choice experiment could be performed with ovipositing females to determine if CSFB females prefer to lay eggs at the base of line 86 compared to line 91 by filtering the soil around these plants and counting eggs. A non-choice assay could also be performed to test larvae performance for plant antibiosis as in (Döring and Ulber, 2020), to see if larvae do develop faster in line 86 as was implied here by there being significantly more third-instar larvae per plant and per leaf. If line 86 was more susceptible than line 91 in these experiments, studying these genotypes further could help understand mechanisms for resistance against larvae, which is currently unknown.

Although further larvae collections were planned for later in the winter, a hard frost caused lodging of line 86. The lodging was not caused by CSFB damage, as stems of plants from all plots were dissected to check for larvae and signs of previous larvae infestation such as scarring and exit holes, but no evidence of stem infestation was found.

4.7 Chapter summary

In this study, two candidate lines of *S. alba* were identified as significantly contrasting for adult CSFB antixenosis. The difference between lines was consistent between multiple laboratory screens (both choice and non-choice), and in field trials. There was also some evidence that the contrasting resistance applied to larvae development, but this needs further investigation. This study is in agreement with previous reports that *S. alba* is

more resistant to CSFB than *B. napus*, and that *B. napus* is lacking in resistance. This indicates that inter-specific crosses are likely required to introduce resistance genes to CSFB into *B. napus*. There was significant variability between CSFB batches despite use of a controlled colony in insects. This highlights the need for tightly controlled experiments to identify nuanced levels of resistance. The two lines of interest identified here can be compared to understand the resistance mechanism and causative genes.

5. Characterising candidate varieties for resistance mechanisms

Insect resistance in plants is mediated by deterrent metabolites, proteins, and physical structures (Rani and Jyothsna, 2010; War et al., 2011; Xuan et al., 2020). Specialised metabolites can be unpalatable or toxic to the insect, whereas physical structures such as thorns, trichomes, and high levels of lignification can act as a mechanical barrier (Gruber et al., 2018; Ogran et al., 2019; Rathinam et al., 2020; Santiago et al., 2013). Glandular trichomes can serve both functions by harbouring toxic metabolites and forming a barrier (Duke et al., 2000). The specific defence traits utilised can vary considerably between both the species of plant, and the species of attacking herbivore (Agrawal and Fishbein, 2006; Soler et al., 2012). Due to this complexity, more studies have identified the presence of cabbage stem flea beetle and crucifer flea beetle resistance than have found specific resistance mechanisms. Although only trichome-based resistance against CSFB has been well described, many more studies have identified varieties which are unpalatable to adult CSFB. Resistance mechanisms have been correlated to compounds such as glucosinolates, but their role has not been definitively demonstrated and the molecular pathways within the plants determining their expression are unknown (Bartlet and Williams, 1991; Brandt and Lamb, 1994; Döring and Ulber, 2020; Henderson et al., 2004). However, there is a need to understand what the mechanisms of defence are to fully utilise them (Leybourne and Aradottir, 2022).

There are many potential resistance mechanisms that may control CSFB resistance. Previously studied resistance mechanisms have been discussed in the introduction, including glucosinolates, waxes, and trichomes. However none of these mechanisms have been definitively proven to cause resistance to CSFB, and little work has focused on defences of *S. alba* against CSFB specifically. However, identification of specific resistance mechanisms would be tremendously useful for breeding resistance into CSFB. Firstly, phenotyping plant varieties for the specific trait linked to resistance could be faster than bioassays involving the live insect. For example, extraction of metabolites or proteins and quantification with chemical analysis techniques (e.g. mass spectrometry/high-performance liquid chromatography) is relatively faster and simpler than use of live CSFB (Razzaq et al., 2019). Future breeding approaches could therefore target the specific defence trait, instead of repeated testing for CSFB feeding. Therefore, the need for CSFB in the phenotyping step could be negated, and there is potential to significantly increase the throughput of CSFB resistance breeding by tracking the resistance trait in progeny instead.

Furthermore, if the traits can be identified, their presence could phenotyped within *B. napus* germplasm for the possibility to negate inter-species introgression of the resistance trait, which can be challenging (Brown et al., 1997). It may be possible to exploit native pathways for resistance using techniques such as mutagensis (Kozjak and Megli, 2012), or

potentially through genome editing techniques such as CRISPR/Cas9 which are nearing regulatory approval in the UK (Coe, 2022). For *S. alba* lines 86 and 91, characterising the traits linked to CSFB defence could direct future research in *B. napus*. To understand the potential resistance trait, it would be prudent to characterise those traits linked to cabbage stem and crucifer flea beetle resistance previously. Fortunately, due to the importance of these traits for many plant species, simple and effective protocols for their characterisation are well defined.

Trichomes are one of the best studied traits for their potential role in flea beetle resistance (Palaniswamy and Bodnaryk, 1994; Soroka et al., 2007). As a particularly hairy species, trichomes have also been implicated for flea beetle resistance in *S. alba* (Abdel Khalik, 2005; Lamb, 1980). Assessing trichome density and structure can be easily performed using microscopy, and if line 86 is significantly lacking in trichomes or has significantly altered trichome structure, this may play a role in it's susceptibility.

Similarly, waxes are also well characterised for CSFB resistance (Bodnaryk, 1992b; Bohinc et al., 2014). Epicuticular waxes form crystalline projection from the surface of the plants, and their crystal structure is determined largely by their chemical composition which directs their self-assembly (Jeffree et al., 1975; Koch and Ensikat, 2008). Therefore, visualisation of these wax crystals can be used to identify significant differences in wax profiles without requiring more arduous techniques such as mass spectrometry (MS) (Barthlott et al., 1998; Laila et al., 2017b).

Leaf thickness can also influence resistance by increasing the strength of the tissues against insect chewing (Gómez et al., 2008). In addition, the thickness is the leaf is important to consider for the method used to measure herbivory here. Quantification of damage from a two-dimension image inherently ignores the thickness of the leaf, and so the mass of tissue actually consumed may not be measured accurately using the pipeline developed here. If line 91 is thicker, it may lead to smaller shot-holes for the same level of herbivory by CSFB, and an under-estimation of damage. Therefore, measurements of leaf thickness may explain the difference in area eaten in addition to influencing resistance.

Characterising CSFB behaviour when presented with the two genotypes may also provide evidence for the resistance mechanism. For example, male and female insects can differ in their preferences due to the nutritional quality of the host, which has been found for other herbivorous leaf beetles (*Chrysomelidae*), such as *Gynandrobrotica guerreroensis*, *Cerotoma ruficornis*, and *Phratora laticollis* (Ballhorn et al., 2013; Li et al., 2020). This may be because high nutritional quality is needed for females to develop eggs, so they are deterred by nutritionally poor hosts. If similar deterrence was seen for female CSFB when presented with the resistant *S. alba* line 91, it may also indicate that nutritional quality is of importance for resistance.

5.1 Induced defences

Resistance traits can be presented to herbivores using two major strategies; they are either constitutive and present throughout the life of the plant, or they can be specifically induced by insect exposure (Kerchev et al., 2012). Inducing defences allows the plant to tailor the deployed arsenal to suit the specific herbivore, as the effectiveness of the defence is dependent on the insect species. For example, glucosinolates can stimulate specialist herbivores such as CSFB, while repelling generalists like slugs (Giamoustaris and Mithen, 1995; Pivnick et al., 1992). Induction may also reduce the energetic cost to the host in the absence of herbivores (Züst and Agrawal, 2017).

Previous studies have identified induced responses by measuring changes to defence signalling pathways after herbivory, or by quantifying the accumulation of defence traits. For example, trichome density is increased in black mustard (*Brassica nigra*) after herbivory, and the response is herbivore-specific (Traw and Dawson, 2002). However, these studies do not directly link these changes to the observed resistance. In addition, studies of induced defences can be demanding. Experimental design must be highly controlled, as both the quantity of damage and the time taken to inflict it alters induced responses (Bricchi et al., 2010). The plant developmental stage must also be controlled to reduce variance introduced from other events, such as changes to the circadian rhythm. Controlling CSFB feeding in such a manner would be demanding. Establishing if the resistance seen here is induced before undertaking these studies could reduce the time spent on characterisation.

Monitoring the rate of CSFB feeding could establish if the resistance trait in line 91 is induced. Once the defence mechanism accumulates after induction, CSFB feeding should decrease. Therefore, quantifying the rate of feeding could help to determine if resistance is induced. Videos have previously been used to record insects feeding to quantify total herbivory (Kloth et al., 2015; Thoen et al., 2016), though no previous studies are known to have have measured how the rate of feeding may be altered by induction of defences.

The time taken for defence metabolites or structures to accumulate is dependent on the specific trait. Generally, defences against insect herbivores are induced within 24 hours of feeding (Costarelli et al., 2020; De Lange et al., 2020; Saravanakumar et al., 2007). Volatiles have been seen to increase just 90 minutes after treatment with fall army worm oral secretions, (Schmelz et al., 2007), and glucosinolate profiles were significantly altered within 7 hours after flea beetle feeding (Bartlet et al., 1999a). Some metabolites are slower to accumulate, such as protease inhibitors, which take at least 24 hours to alter the feeding behaviour of thrips (Outchkourov et al., 2004). Therefore, if the cause of flea beetle resistance here is induced, it would be expected that the rate of feeding should be captured before 24 hours, and within 48 hours as within this time frame contrasts in resistance were seen in Chapter 2.

5.2 Aim and objectives

This chapter aimed to characterise traits previously implicated in insect resistance for the two *S. alba* lines, 86 and 91. This was to understand if these traits may be associated with the resistance mechanism which altered CSFB feeding. The objectives were to assess differences in plant morphology between line 91 and line 86 by comparing trichome density and structure, and wax crystal structure, using cryo-scanning electron microscopy (SEM). Differences in leaf size and thickness were also measured, which may have influenced the level of herbivory measured due to the scoring technique. Chemical analysis of glucosinolates and waxes was to be compared between the two *S. alba* lines. Finally, changes to CSFB behaviour when presented with the two *S. alba* lines were observed from video recordings to implicate potential resistance traits from their influence on the insect. Induction of defence was also tested from these recordings by quantifying the area eaten oven time; if resistance was induced the rate of feeding would have been greater nearer to the start of feeding until the resistance trait was up-regulated.

5.3 Materials and Methods

5.3.1 Preference of male and female cabbage stem flea beetles for resistant and susceptible *Sinapis alba*

A difference between CSFB male and female preferences may indicate a difference in nutritional quality between *S. alba* lines 86 and 91. Post-aestivated beetles were collected from Church Farm, Norwich, on 13th September 2022 from a WOSR crop at the seedling stage. Beetles were sexed using the first tarsal segment, and maintained in separate male and female boxes for 7 days as previously described. Seedlings were grown as previously, but transplanted into individual 2 cm x 2 cm plug pots at 6 days old.

A rotary drill was used to cut a 1mm notch into the lid and base of 35x10mm Nunc™ Petri dish (Thermo Fisher Scientific, Inc.). The notch was sealed with micropore tape, and the lid was filled with 1.5 % water agar. Once set, the tape was removed and a slit cut into the agar using a razor blade. The stem of the 7-day-old whole *S. alba* seedling was gently pressed into the slit, resulting in a modified "clip cage" that allowed the beetles to feed upon the cotyledons of the intact plant. The plug pot was covered in cling film to prevent the compost from drying out during the experiment. A single flea beetle was added to each seedling, and the lid was secured by lining up the notch on the base, and inserting it into the agar until flush with the lid. ($n = 20$) females and ($n = 19$) males were screened per line, for a total of 78 beetles screened in a single block. Beetles were starved for 24 hours, then placed inside the dishes and allowed to feed for 48 hours. After 48 hours, beetles were removed and the cotyledons were destructively quantified using the ImageJ pipeline described previously. An unbalanced ANOVA was used to compare the

percent area eaten to both line and sex, with an interaction between these two factors. A square-root transformation was used to control for unequal variances.

5.3.2 Leaf size

Previous quantification of damage for *S. alba* lines 86 and 91 used percent damage measurements, so it was important to ensure that the leaf sizes between the two lines were not different. If line 86 had significantly smaller cotyledons, the same level of herbivory would be over estimated.

Leaf size was measured from seedlings that had been included in phenotyping experiments in Chapter 2, but had not received any damage. This allowed for utilisation of data that was previously collected, and also prevented bias as these seedlings were a random and representative sample of the plants that were included in previous experiments. The previous experiments that these seedlings were used in were from re-screening the lines of interest in choice petri-dishes, re-screening in non-choice phytatrays to compare feeding versus CSFB age, and screening individual seedlings to compare preference with CSFB sex. All data was filtered to include only seedlings of line 86 or 91 where 0 % leaf area was eaten. This gave 40 seedlings, with (*n=19*) for line 86, and (*n=21*) for line 91. An unbalanced one-way ANOVA was performed to compare leaf size (mm^2) to the two lines, and included the experiment as a blocking factor. Data was squared for analysis to stabilise variances.

5.3.3 Leaf thickness

Leaf thickness was measured using two methods, modified from methods previously described (Galdon-Armero et al., 2018; Pullen et al., 2019). Genotypes 86 and 91 were sown as previously described, and 7 days after sowing one cotyledon from (*n=3*) plants was cut from the seedling and immediately secured in between two polystyrene sheets each approximately 1 cm in thickness. A scalpel with a fresh blade was used to slice through the top sheet and centre of the leaf in two parallel lines, approximately 1mm apart. The top sheet was removed and the bottom sheet, with attached sample, was rotated 90 °. The cut section was gently removed from the polystyrene sheet using a pair of pointed forceps and transfered to a microscope slide. The section of cotyledon was imaged using a Leica S9D stereo-zoom microscope (Leica Microsystems, Germany) and leaf thickness was measured at 10 points along each cotyledon, excluding the mid-vein, using GXCapture-T software (GT Vision version x64), calibrated with a stage micrometer. For each cotyledon, the mean of the 10 measurements was calculated.

A single cotyledon per plant was also measured using a digital IP67 coolant proof ABS calliper (Mitutoyo, UK) (0.01 mm step ± 0.0254 mm). For (*n=10*) whole plants, the callipers were used under the Leica S9D microscope and clipped onto the middle of one

lobe of a cotyledon, then slowly tightened until there was no visible gap between the leaf surface and calliper. The measurement of thickness was taken blind (i.e. viewed only after the callipers were tightened) to prevent bias. The calliper was known to be overtightened if there was an impression on the leaf surface; if this happened the sample was discarded.

Thickness was compared between the two lines using a one-way ANOVA for each measurement method, where line was considered as a factor and thickness (mm) as a variable. Data was squared for calliper measurements, and log-transformed for microscopy measurements to stabilise variances.

5.3.4 Trichome density and structure, and epicuticular wax structure

Cryo-Scanning electron microscopy (Cryo-SEM) was performed at the John Innes Centre by Jake Richardson of the Bioimaging platform to observe trichome morphology and density, and epicuticular wax structure, as previously described (Barthlott et al., 1998; Zheng et al., 2022). 7 day old cotyledon tissue was cut and mounted on an aluminium stub with Tissue Tek OCT (Agar Scientific Ltd, Essex, UK) and plunge frozen in slushed liquid nitrogen to cryo-preserve the material before transfer to the cryo-stage of a PP3010 cryo-SEM preparation system (Quorum Technologies, Laughton, UK) attached to a Zeiss Gemini 300 field emission gun scanning electron microscope (Zeiss UK Ltd, Cambridge, UK). Surface frost was sublimated by warming the sample to -90 °C for 3 minutes, before the sample was cooled to -140 °C and sputter coated with platinum at 5 mA for 50 seconds. The sample was loaded onto the cryo-stage of the main SEM chamber and held at -140 °C during imaging at 3 kV using an Everhart-Thornley detector and digital TIFF files were stored for analysis. Multiple high magnification images were also obtained for each sample to observe trichome structure and vestiture was categorised according to (Simpson, 2019), and assess epicuticular wax structure.

For line 86, images were analysed from (n=5) seedlings for the abaxial surface, (n=6) for adaxial surface, and (n=7) for stem. For line 91, (n=6) for the abaxial surface, (n=5) for the adaxial surface, and (n=7) for the stem.

Once images were received, trichomes were counted for the images which were taken between 85 to 150x magnification, with the majority taken at 100x, using ImageJ. The grid function was used to overlay a grid across the image. The number of trichomes within each grid was counted, with 3 to 12 grids per image. The mean number of trichomes per grid square was calculated for each image. Two images were analysed per seedling and per surface, and the mean was taken for each seedling. Trichome density per mm^2 was compared between the two lines statistically using a one-way ANOVA for each of the three surface types, with line as a factor. A square-root transformation was used to stabilise variances for the abaxial and adaxial data only.

5.3.5 Chemical analysis of cotyledon glucosinolates

To note, the preparation of materials for analysis was undertaken but the results were not returned from the collaborators; it is not known if the chemical analyses were performed. For reference, the methods are included but no results section is presented.

As discussed in the introduction to this thesis, flea beetles are attracted to isothiocyanates and may be deterred by other glucosinolates (Bartlet et al., 1996; Pivnick et al., 1992; Soroka and Grenkow, 2013; Tóth et al., 2007). Therefore, an experiment was planned to analyse the specific glucosinolate profiles of the *S. alba* lines screened for CSFB feeding in this project, and to correlate glucosinolates to the quantity of feeding. The total glucosinolates, the glucosinolate type (aliphatic, aromatic and indole), and specific glucosinolates (mainly sinalbin, but potentially others depending on the chemical profile observed) were to be correlated to CSFB resistance. It was hypothesised that either the total, type, or specific glucosinolates may mediate CSFB resistance.

For analysis of glucosinolates, plants were to be subject to high performance liquid chromatography (HP-LC) using a method previously developed by the collaborator for Brassicaceae, including *S. alba* (Doheny-Adams et al., 2017). The specific glucosinolates would have been identified and quantified by the collaborator using a reference database they had previously developed using standards and their analysis method. Plants of *S. alba* lines 84, 86, 87, 88, 91, 92, 98, 101 and 103 were selected, as they had previously been screened for CSFB resistance here. In addition, *B. napus* var. "Barbados" was also included. The glucosinolate composition of *S. alba* cotyledons has not been published to date, so it was also of interest to see if there were glucosinolates present in all lines of *S. alba* that were absent or minor in *B. napus*, which may influence the increased resistance to CSFB generally observed for *S. alba*.

Seedlings were grown to the cotyledon stage (1 week old) as previously described in the general materials and methods. Five replicates per line, consisting of approximately 20 cotyledons each, were sampled. For each replicate, cotyledons were removed at the petiole with a cut along the lamina base and added to a tinfoil packet, which was folded closed then immediately flash-frozen in liquid nitrogen. Cotyledons were then transferred to a pre-labelled and pre-weighed 15 mL centrifuge tube (Merck, CLS430055) and freeze dried for 48 hours. Samples were removed from the freeze drier and sealed with the lids and Parafilm, and shipped to the collaborator the same day for extraction and HP-LC analysis. The collaborator would have returned a digital file containing the identity of specific glucosinolates and their quantity (per unit dry weight) for each replicate.

If the results were received, a principal component analysis would have been performed on the data from chemical analysis to assess if lines with similar levels of feeding resistance to CSFB had similar glucosinolate profiles. Principal component analysis is a clustering method which reduces the dimensionality of multivariate data, and the output can be viewed graphically with a "scores" plot (Goodacre et al., 2000). This method is fairly

well established and has previously been performed for correlating glucosinolates to resistance against other insects, such as for *Spodoptera exigua*, *Plutella xylostella*, and *Mamestra brassicae* in *Arabidopsis thaliana*, in addition to pathogens such as *Sclerotinia sclerotiorum* in cabbage (Abuyusuf et al., 2018; Arany et al., 2007; Beekwilder et al., 2008). The quantity of specific glucosinolates of interest (e.g. sinalbin) could also have been plotted directly against the area eaten by CSFB to assess for correlations, and significant differences in their quantities could have been assessed statistically using an ANOVA. If correlations were observed, these glucosinolates could have been assessed further for palatability in feeding experiments, or the differences in genetic control of their synthesis could have been investigated.

5.3.6 Chemical analysis of cotyledon surface waxes

Similarly, the chemical composition of the wax profiles of the cotyledons were to be correlated to *S. alba* resistance. The quantity of cuticular waxes has previously been correlated with increased resistance to Brassicaceae-specialist flea beetles, and particular waxes are deterrent to other insects (Bodnaryk, 1992b; Bohinc et al., 2014; Jones et al., 2002; Munaiz et al., 2020; Negin et al., 2021). Therefore, it was of interest to assess if differences in waxes between *S. alba* lines 86 and 91 could explain the contrasting levels of CSFB feeding observed. This work was performed on only lines 86 and 91 rather than all of the available *S. alba* lines, as the wax analysis protocol was not optimised and so data analysis would have been quite complex for a greater number of samples. The experimental work here was undertaken but the results were not returned from the collaborators site. For reference, the methods are included but no results section is presented.

The lines tested were *S. alba* 86 and *S. alba* 91, and *B. napus* var. "Barbados" was also included as an additional reference. The wax composition of *S. alba* cotyledons has not been published to date, so it was also of interest to see if there were waxes present in both lines of *S. alba* that were absent or minor in *B. napus* which may influence the increased resistance to CSFB generally observed for *S. alba*. The intention was to look for differences in the total quantity of wax, and for significant differences in individual wax types, between the two *S. alba* lines. It was hypothesised that wax quantity may be greatest in the CSFB resistant *S. alba* line 91, or that this line may have a significantly different quantity of a particular wax which may be repellent or attractive to CSFB. Data would have been analysed statistically using an ANOVA to assess significant differences in the total quantity of wax, and of individual waxes between the two *S. alba* lines. Wax total quantity and composition have previously been assessed in Brassicaceae using gas chromatography–mass spectrometry (GC–MS) analysis, as was to be performed here (Cao et al., 2021; Laila et al., 2017a; Zhu et al., 2019). This technique has also been used to correlate specific waxes to insect resistance, such as several wax components in cabbage to *P. xylostella* resistance (Eigenbrode et al., 1991).

For analysis of waxes, fresh cotyledons from 1-week-old plants were subject to GC-MS analysis using a method previously developed for use with *Brassica* (modified from Liu et al. (2021)). Plants of *S. alba* line 86 and 91, and *B. napus* var. "Barbados" were grown to the cotyledon stage as previously described in the general materials and methods. Six replicates were produced for each line, and each replicate consisted of 1 g of cotyledons (approximately 22 cotyledons/11 seedlings). Cotyledons were cut at the base of the petiole and placed into a glass beaker. Polar compounds were extracted by adding 10 mL method containing 10 μM cholestanol (Supelco, 47129), and samples were swirled gently for 30 seconds. The methanol was moved to a clean, 15 mL amber glass vial (Merck, 27003) using a glass Pasteur pipette. To extract less polar compounds, 10 mL of chloroform containing 10 mM of each docosane (Merck, 43942-1G) and docosanol (Merck, L3507-25MG) was added to the same cotyledons and swirled gently for 30 seconds. The chloroform was moved to a clean, 15 mL amber glass vial (Merck, 27003) using a glass Pasteur pipette. The methanol and chloroform was evaporated from each tube using a stream of nitrogen gas until completely dry (overnight). Tubes were closed with a lid that contained a PTFE insert, sealed with Parafilm, and kept in a -20 °C freezer. For both methanol and chloroform extracts, free hydroxyl groups were derivatized by adding 200 ml N,O-bis (trimethylsilyl)trifluoroacetamide):trimethylchlorosilane (99:1; BSTFA/TMS) and heating at 85 °C for 1.5 hour in an oven. BSTFA-TMCS was evaporated using a stream of nitrogen gas, and the samples were dissolved in 75 μL hexane for analysis.

At this point, samples were provided to the collaborator for chemical analysis. The silylated samples were analysed by GC-MS to identify specific waxes. Samples were run for 49.13 minutes on an Agilent GC-MS using a 30-m, 0.25-mm HP-1 capillary column with helium as the carrier gas. Columns were washed with ethyl acetate and hexane at the start of each run. The total waxes were also quantified using a flame-ionization detector (FID), connected to a GC with the same column. The quantification of each compound was based on peak areas relative to the internal molecular standards, expressed per unit of tissue (fresh weight). Although this experimental work was completed, the produced data was not returned for analysis.

5.3.7 Cabbage stem flea beetle feeding dynamics

To record the rate of feeding, a 35x10mm Nunc™ Petri dish clip cage was used as above, though only a single cotyledon from the intact seedling was presented to each cabbage stem flea beetle using a through a gap in the petri dish [Fig. 5.1a].

Four CSFB age 14 days old or less were recorded simultaneously (two per line) for 48 hours, and each batch of four CSFB was treated as a separate block. Overhead panel lighting illuminated the experiment for 12 hours, and to be able to continue recording through the night, two 5-LED strip lights were also used which provided sufficient illumination to observe the damage. Four Celestron 44308 5 MP Handheld Digital

Microscope Pro microscope cameras were used in tandem, and one was placed above each dish using a separate clamp and stand at equal heights. The cameras and were connected to a desktop computer to record video footage at 60 fps at a resolution of 1552x1166 (width and height). Videos were captured simultaneously in a single frame using OBS Studio v. 28 software (Bailey, 2017). The recording was started and insects were added into chambers and placed under the cameras within 60 seconds of each other. For analysis, *t=0* was considered to be when all chambers were placed under the microscopes.

In total, 551 GB of video footage was collected, and 32 insects across 8 blocks were recorded. One insect escaped and the replicate was excluded, leaving (*n=16*) replicates for line 86 and (*n=15*) for line 91.

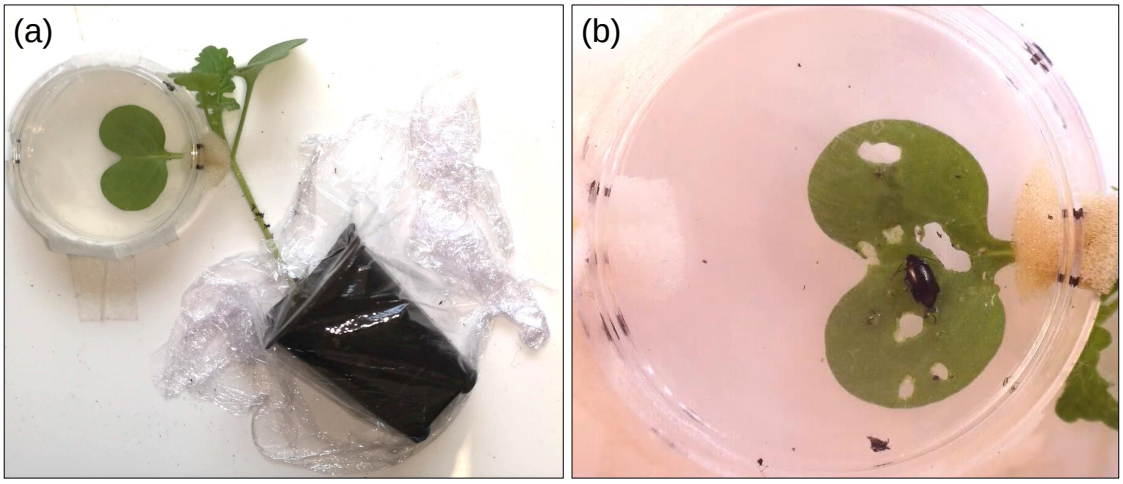

Figure 5.1: Images showing the experimental set-up used for observing cabbage stem flea beetle behaviour on 7-day-old seedlings of *S.alba* line 91 (resistant) and line 86 (susceptible). (a) The "clip-cage" developed using a petri-dish with a hole drilled on either side to allow a single cotyledon to be placed inside the chamber, and for a single a cabbage stem flea beetle to be introduced. (b) A still frame from the video captured using a USB-microscope camera recording the clip cage. A cabbage stem flea beetle can be seen feeding on the cotyledon, with a shot-hole developing on the leaf. Videos were recorded for 48 hours and damage was quantified computationally from still frames.

Videos were imported into Shotcut v. 21.03.21 video editing software (Meltytech, 2011). Videos were paused at *t=48* hours, and still frames were exported for quantification of damage. This was to check the mean level of feeding for each block. The ImageJ pipeline was used to quantify damage computationally, but as the leaf at *t=0* was present, images were not interpolated but instead the total leaf area remaining at each time point was subtracted from the starting leaf area at *t=0*. This was not exactly precise due to differences in lighting conditions, and potentially from slight growth of leaves during the experiment. For seedlings with no damage the greatest variance from 100 % leaf area remaining was 3 %, and so quantification was estimated to be approximate to ± 3 %. There were some instances where the CSFB was sat on the leaf for the still frame that

was captured, such as in Fig. 5.1b. In these instances, the area covered by the beetle was treated as uneaten.

Replicates were filtered to remove blocks where mean feeding was below 2 % of total leaf area. This removed two blocks (8 replicates), where the mean feeding was 1.1 % and 0.3 %. This left (*n=11*) for line 91, (*n=12*) for line 86.

Mean area eaten

The percent area leaf eaten at 48 hours was used to compare the total leaf area eaten for each line. An unbalanced ANOVA was used to compare % cotyledon area eaten between the two lines, with the week as a blocking factor. A square-root transformation was used to stabilise residuals.

Time until first bite

To record the time taken for the first bite, just the individuals that fed were included for analysis which was (*n=11*) for 86, (*n=9*) for 91. Video footage was played back a 10x speed until feeding was noted. The initial bite was often obscured by the beetles body, but the back and forth motion of the head was an indicator that feeding was taken place. Once this was observed, videos were played back at normal speed to note the precise time taken in hours for the beetle to take it's first bite.

An unbalanced ANOVA was used to compare the time taken for the first bite between the two lines of *S. alba*, with the beetle batch considered as a blocking factor. A log transformation was used to stabilise variances.

Feeding dynamics over time

To compare the rate of feeding, additional still frames were exported for quantification as above. The time points selected were t = 0, 1, 4, 8, 12, 18, 24, 30, 36, 42 and 48 hours. Damage earlier in the experiment was measured more frequently to try and capture any early changes in feeding dynamics that may be caused by induced resistance.

A repeated measures ANOVA was used to compare the rate of feeding over time by assessing the difference in the slopes and intercepts between the two lines. The percent area of leaf tissue remaining was compared, where the subjects were the unique beetles for each chamber, the time points were the time point of each still frame in hours, and the fixed model was the interaction between the line and the time point. The same time point was used for each subject, and a power model (city-block) was used to account for heterogeneity across time, and additional uniform correlation between subjects. For model converge a maximum of 10,000 iterations was specified. A significant interaction between line and time indicates that the slope of the lines differ.

Fixed model:

$$Y = \beta + L + L \times T$$

Random model:

$$Y = B + B \times T + \epsilon$$

Where β is the random intercept L is the plant line, T is the timepoint, S is the individual seedling, B is the individual beetle, and ϵ is the random error term.

5.4 Results

5.4.1 Preference of male and female cabbage stem flea beetles for resistant and susceptible *Sinapis alba*

In alignment with previous results, *S. alba* line 86 was eaten significantly more than line 91 ($F = 21.74$, $P < 0.001$). Each beetle ate a mean of 6.8 % ($\pm$ 0.06) for each seedling of line 86, and 2.1 % ($\pm$ 0.06) for line 91. There were no significant differences in the area eaten for male and female CSFB ($F = 0.17$, $P = 0.68$) [Fig. 5.2]. Females ate a mean of 4.3 % ($\pm$ 0.06 %) across the two lines, and males ate 3.9 % ($\pm$ 0.06 %) across the two lines. There was also no significant interaction of sex with line, demonstrating that both males and females preferred line 86 over line 91, and to an equal extent ($F = 1.25$, $P = 0.267$).

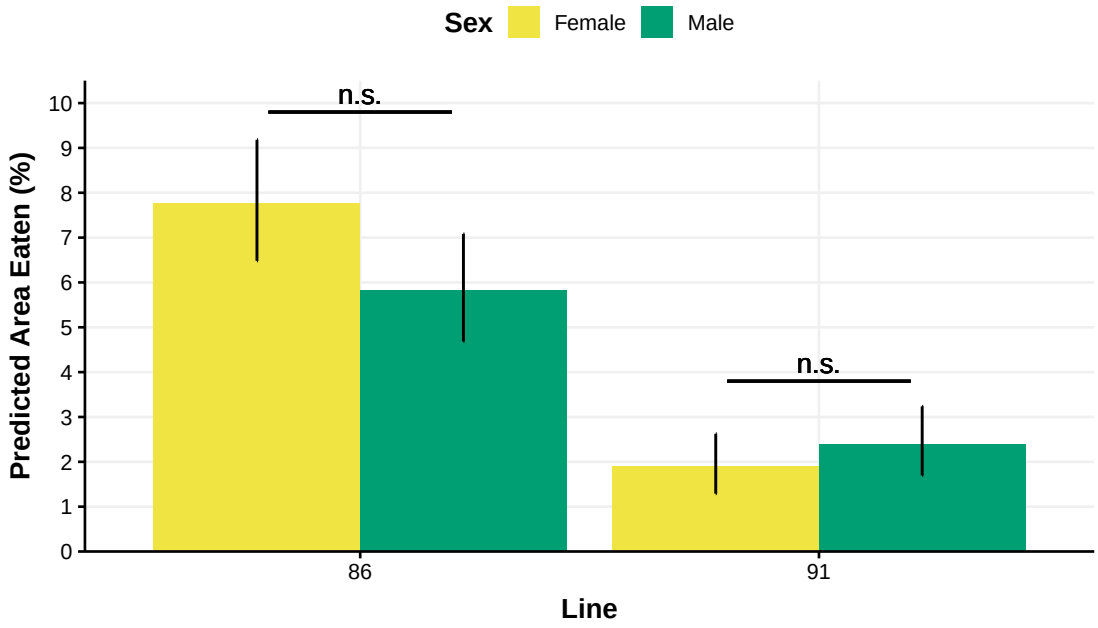

Figure 5.2: Mean percent area eaten in a laboratory experiment for (*n=19*) male and (*n=20*) female cabbage stem flea beetle feeding on two *Sinapis alba* lines, 86 (susceptible) and line 91 (resistant). An unbalanced ANOVA was used to determine if there was statistically different quantity of leaf area eaten between the sexes, and between the lines. n.s. = not significantly different. Error bars represent estimated standard error.

5.4.2 Leaf size

There was no significant difference in leaf size between the different experimental designs for which seedlings were obtained ($F = 0.61$, $P = 0.55$). This is as expected, as these plants were grown in the same conditions for each of the experiments despite the experimental designs used to present them to flea beetles being different. This result shows that between experiments, the method of scoring percent area eaten should not be significantly altered due to the experimental design.

There was also no significant difference in leaf size between *S. alba* lines 86 and 91 ($F = 1.28$, $P = 0.266$). The two 7 day old cotyledons of line 86 were 13.9 mm^2 ($\pm$ 0.92 mm^2) in surface area, and line 91 was 12.8 mm^2 ($\pm$ 1.0 mm^2). This suggests that scoring the area eaten as percent will not significantly misrepresent herbivory. Although percent area eaten is relative to leaf size, because there is no difference between leaf size between the two lines it is fair to compare percent area between the two lines.

Interestingly, there were two instances where cotyledons of line 91 were much smaller than the mean, measuring at 2.3 mm^2 and 4.7 mm^2. These were just 18 % and 37 % the size of the mean cotyledons of line 91 respectively. In both cases, the main stem of the seedlings had been eaten through despite the cotyledons remaining untouched. These

cotyledons were wilted and delicate, possibly due to water loss from the severing of the main stem. Water loss caused the cotyledons to become flaccid and significantly shrunken. It was observed that the stems and/or petioles of at least one plant were damaged by CSFB feeding in the majority of replicates, and so these were not discarded. Measuring damage relative to the leaf size by scoring percent eaten negates this shrinkage. Scoring absolute area eaten would underestimate damage in the case of seedlings that shrink during the experiment, such as these.

It is important to note that the conclusions drawn from this experiment are limited, as the specific seedlings selected were determined by the non-preference of CSFB in other laboratory experiments. The reason these seedlings were not selected by CSFB is unknown; potentially, it is due to the availability of plants being greater than the hunger of the insects, or that these specific seedlings were different in some way and avoided. Therefore, they may not be representative of all seedlings across experiments. However, as the increased resistance of *S. alba* line 91 was also observed in repeated experimental designs including at different life stages a field environment, it is unlikely that differences in cotyledon size between the two lines are the reason for quantifying differences in CSFB resistance.

In addition, it would be preferable to compare the results of feeding experiments quantified as both percentage area and absolute area. However, the scoring method using ImageJ developed here was unable to record absolute area. Therefore, the best method cannot be determined from the results collected here.

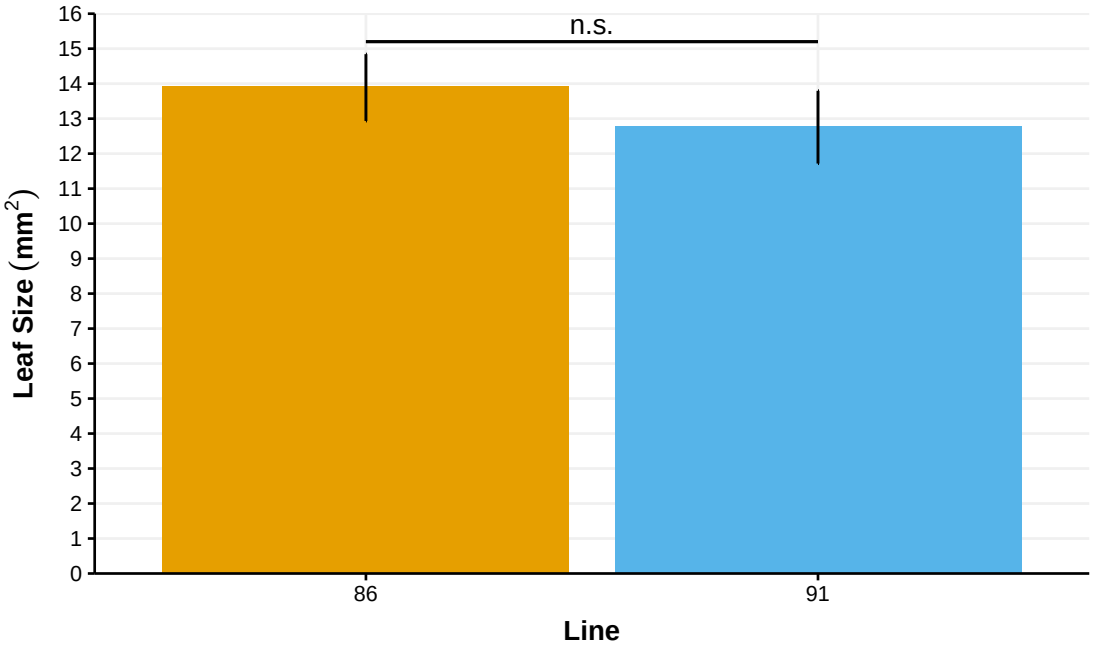

Figure 5.3: Comparison of leaf size of 7 day old undamaged seedlings of *S. alba*, for (*n=19*) seedlings of line 86 (susceptible), and (*n=21*) seedlings of line 91 (resistant). A one-way ANOVA was used to compare leaf size to the line, with blocking factor accounting for the experimental origin of the particular seedlings. n.s. = not significantly different. Error bars represent estimated standard error.

5.4.3 Leaf thickness

Two scoring methods were used to measure cotyledon leaf thickness for both lines of *S. alba*. Variance was higher when scoring using sectioning, likely because there were fewer replicates, and so the natural variation in thickness between individuals of the same line was more prominent. There was no significant difference in leaf thickness between the two lines when measured using sectioning ($F = 0.29$, $P = 0.616$) [Figs. 5.4, 5.5].

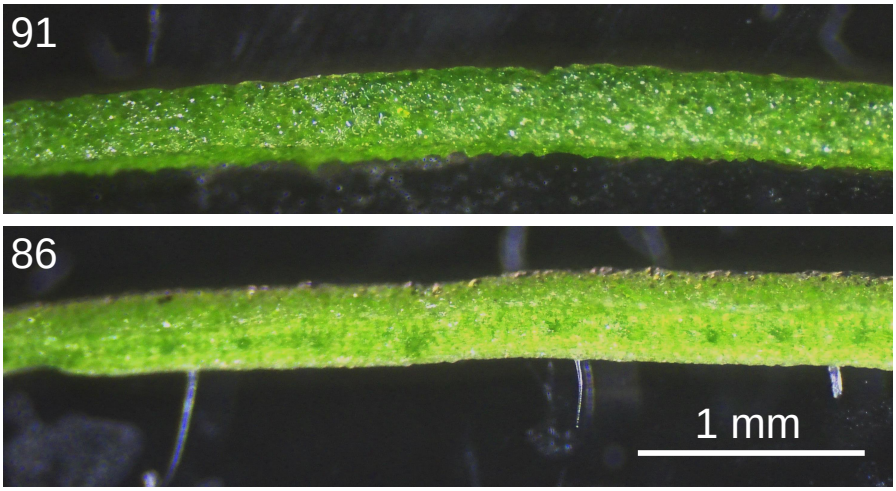

Figure 5.4: Representative cotyledon cross sections demonstrating thickness of two *Sinapis alba* genotypes, line 91 (resistant), and line 86 (susceptible), as viewed under a stereo-zoom microscope. Trichomes are visible on the abaxial (lower) cotyledon surface of line 86. Scale bar is 1 mm for both images. Leaf thickness was measured from images such as these, in addition to digital callipers.

A significant difference was seen between the thickness of the two lines when measured using the callipers ($F = 4.56$, $P = 0.047$) [Fig. 5.5]. Line 86 was significantly thicker, and had a cotyledon thickness of 0.27 mm ($\pm$ 0.011), whereas line 91 had a thickness of 0.24 mm ($\pm$ 0.013). Line 86 was 0.026 mm (10 %) thicker than line 91.

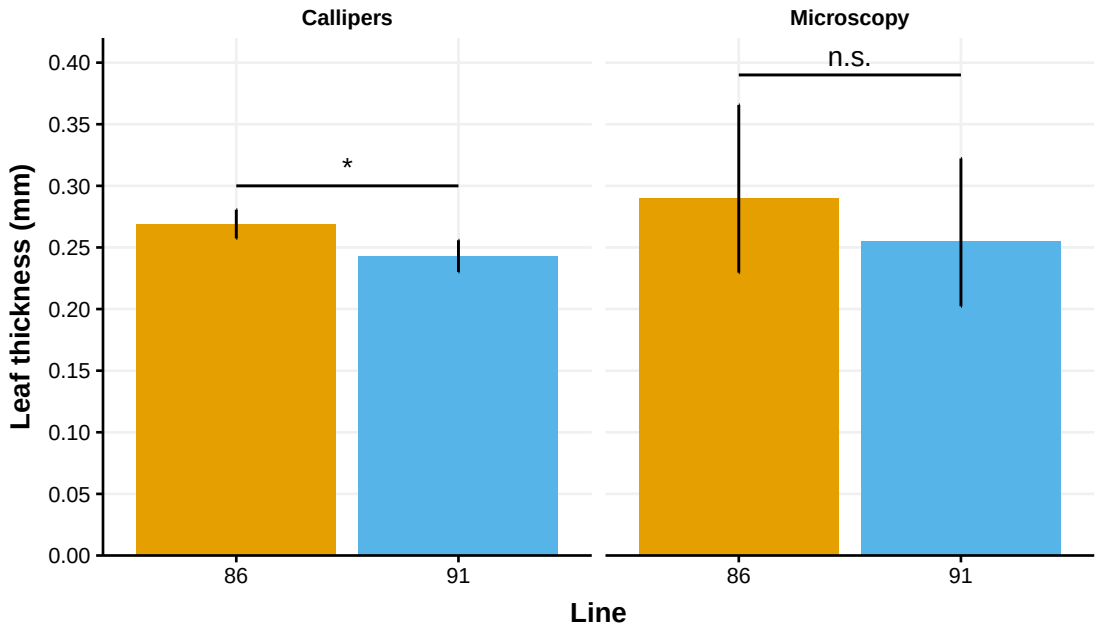

Figure 5.5: Mean leaf thickness of two *Sinapis alba* lines, 86 (susceptible) and 91 (resistant) using two measurement techniques. Callipers: The results of scoring leaf thickness using a digital calliper. Micrscopy: The results of scoring leaf thickness using sectioning and a stereo-microscope camera. Two one-way ANOVAs were used to determine statistically significant differences in the mean thickness for the two lines. A significance of $P = 0.047$ is shown using one asterisk (*). n.s. = not significantly different. Error bars represent estimated standard error.

5.4.4 Trichome density and structure, and epicuticular wax structure

Trichomes were characterised by their structure. All trichomes observed across the three observed surfaces of both *S. alba* lines were simple trichomes. Specifically, they were smooth, uniseriate, subulate and non-glandular trichomes with pulvinate bases [Fig. 5.6]. This simple trichome structure has previously been recorded for different tissues of *S. alba* (Abdel Khalik, 2005).

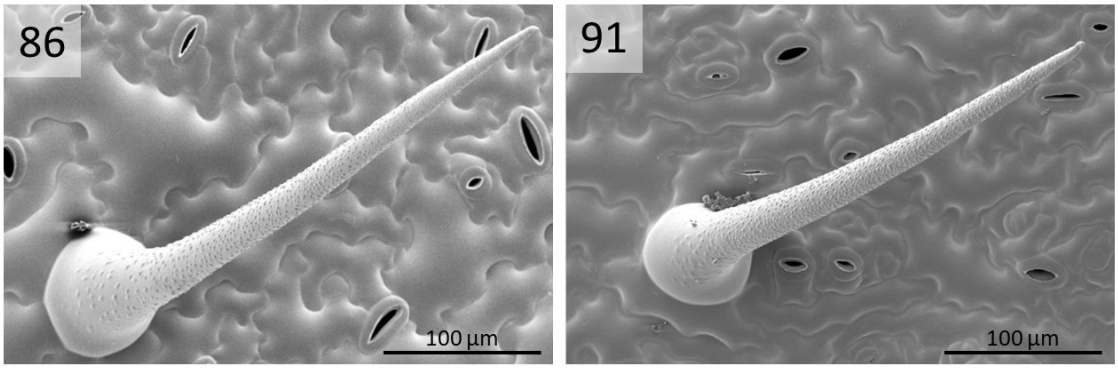

Figure 5.6: Representative cryo-SEM images comparing trichome structure between the two *S. alba* lines, showing two representative trichomes from the abaxial (lower) surface. The line is shown in the top left of each image. This structure was conserved across the observed tissues (stem, abaxial and adaxial (upper) cotyledon surfaces). The scale bar is 100 μm for both images, and both images are at the same scale.

The abaxial and adaxial cotyledon surfaces were both sub-glabrous, with much sparser trichome density than the stems which had hirsute vestiture [Fig. 5.7]. The length and angle of the trichome were not measured here, though it appeared consistent between the two lines. Generally, the stems had longer trichomes than the cotyledon surfaces.

There were no significant differences in the trichome density between the lines for the three surfaces [Fig. 5.8]. Abaxial (lower) cotyledon surface for line 86 had 2.6 ($\pm$ 0.54) trichomes per mm^2, and line 91 had 2.6 ($\pm$ 0.53) trichomes per mm^2 ($F = 0.01$, $P = 0.921$). Line 86 had 0.29 ($\pm$ 0.25) trichomes per mm^2, and line 91 had 0.013 ($\pm$ 0.086) trichomes per mm^2. However, this was not a significant difference ($F = 4.4$, $P = 0.65$). Stems were much hairier than the cotyledons, but again there were no significant differences between the two lines ($F = 0.85$, $P = 0.374$). Line 86 had 9.0 ($\pm$ 0.65) trichomes per mm^2, and line 91 had 9.6 ($\pm$ 0.65) trichomes per mm^2.

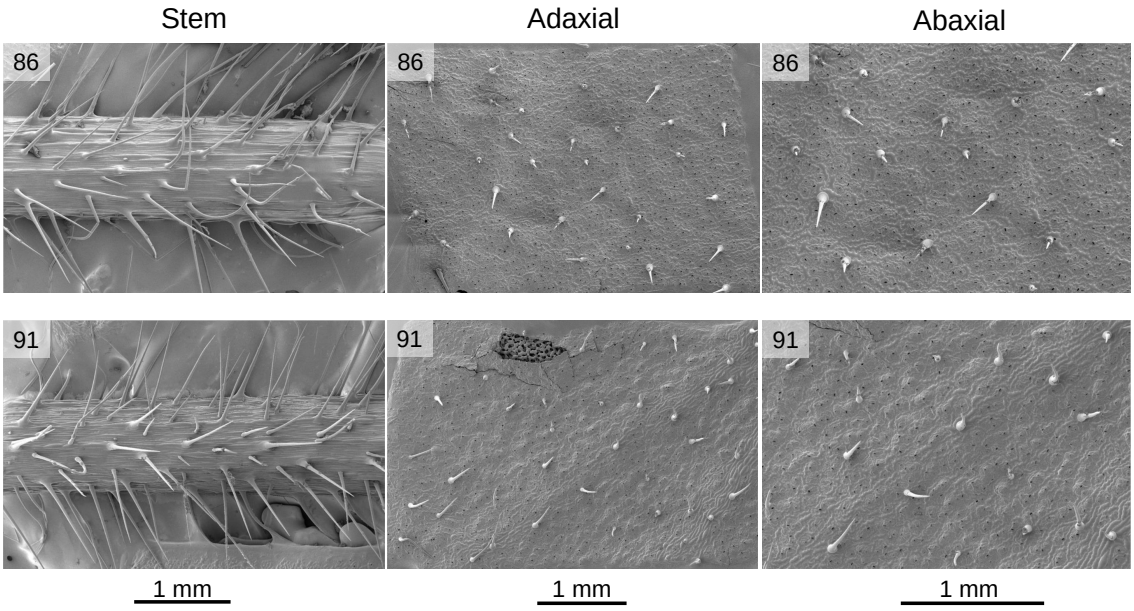

Figure 5.7: Representative cryo-SEM images comparing trichome density across three surfaces (stem, adaxial (upper) cotyledon, and abaxial (lower) cotyledon) of 7-day old seedlings of the two *S. alba* lines. The line is shown in the top left of each image, with line 86 as the top three images and line 91 the bottom three. The scale bar is the shown under the images, and is the same for the same surface.

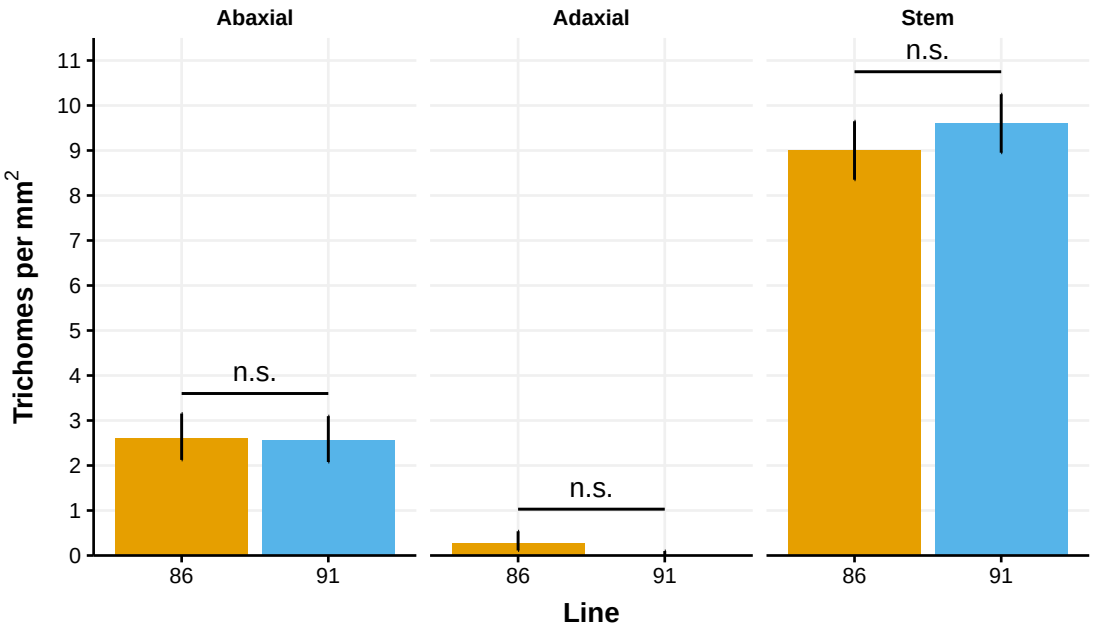

Figure 5.8: Mean trichome density across different surfaces for the two *Sinapis alba* lines contrasting for CSFB antixenosis, line 86 (susceptible) and line 91 (resistant). Trichome density was counted from cryo-SEM images using ImageJ software with an overlaid 1 mm^2 grid. Stem, and abaxial (lower) and adaxial (upper) surfaces of 7-day old cotyledons were measured. A one-way ANOVA was used for each surface to compare density to the *S. alba* line. n.s. = not significantly different. Error bars represent estimated standard error.

Higher magnification images were also taken to observe the structure of the epicuticular waxes on the two cotyledon surfaces and the stem. The epicuticular waxes present on all surfaces for both *S. alba* lines were amorphous; they lay flat on the cuticle surface in sheets [Fig. 5.9]. The presence of these waxes was verified by observing the stomata at higher magnification, where the sheets were found to over hang at the edges of the stomatal pore [Fig. 5.10].

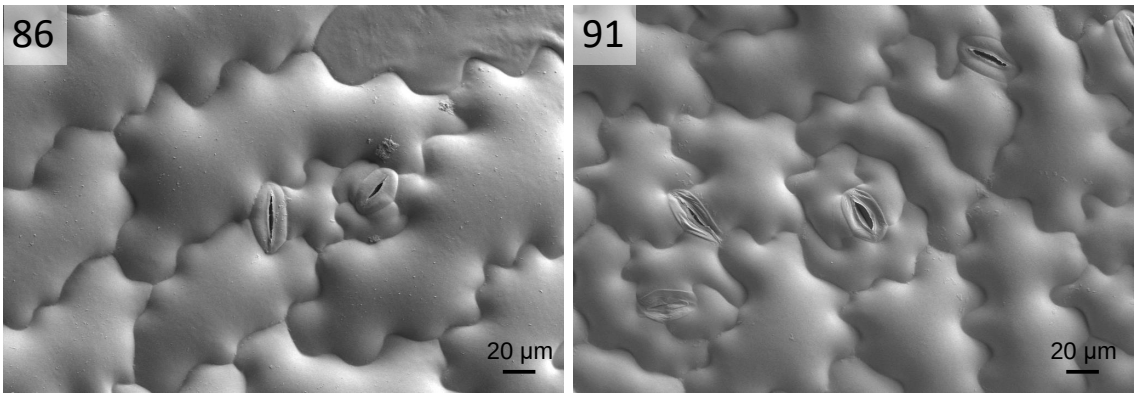

Figure 5.9: Two representative images obtained using cryo-SEM of the abaxial cotyledon surface of 7 day old seedlings of *S. alba* lines 86 and 91 showing amorphous wax films. No differences in the epicuticular wax crystal structure were observed between the two lines, or for the adaxial cotyledon surface and the stem.

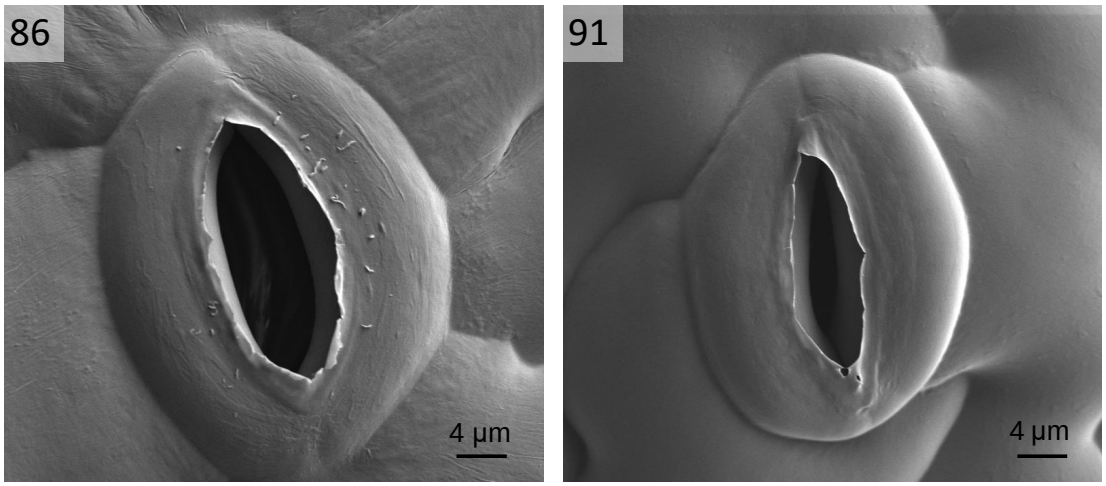

Figure 5.10: Two representative cryo-SEM images of the stomata of *S. alba* lines 86 and 91 from the abaxial surface of 7 day old cotyledons. The lamina epicuticular wax can be seen as a rugged sheet overhanging the stomatal pore in both images. This wax structure was also seen for the adaxial cotyledon surface, and the stem.

5.4.5 Cabbage stem flea beetle feeding dynamics

Mean area eaten

Significantly more leaf area of line 86 was eaten than line 91 ($F = 4.67$, $P = 0.046$). However there was a large amount of variance between blocks, i.e. between beetle batches, which explained most of the variance in the experiment ($F = 7.44$, $P < 0.001$). Because the contrasts between lines weren't always obvious, it may be difficult to pick apart differences in behaviour. Therefore, specific behaviours (e.g. time spent grooming or in time spent in contact with the leaf) were not assessed here, as it was unlikely to be a time-efficient way to reveal information about the resistance mechanism.

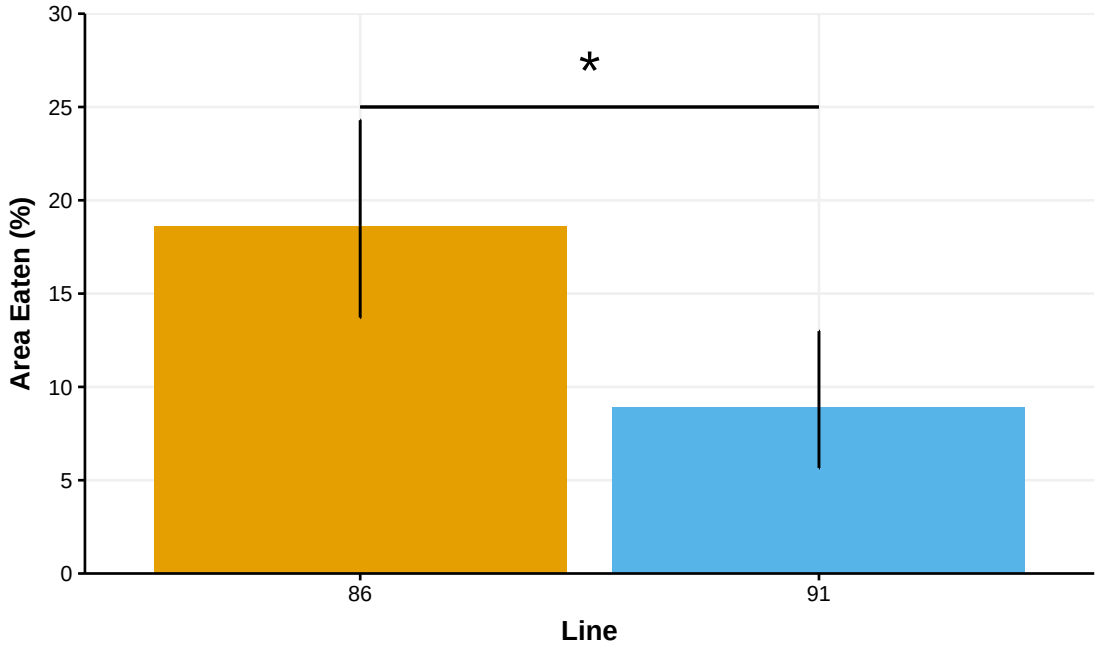

Figure 5.11: Mean percent area of leaf tissue eaten per cotyledon for two lines of *S. alba* in the experiment monitoring cabbage stem flea beetle behaviour using video footage. Each cotyledon was presented to a single flea beetle for 48 hours, for *n=12* seedlings of line 86 (susceptible), and *n=11* seedlings for line 91 (resistant). An unbalanced ANOVA was used to compare % area eaten for the two lines, with the week the experiment was performed as a blocking factor (with 2 replicates per line per block). Error bars represent standard error. A significant difference between the lines of $P = 0.046$ is shown with an asterisk (*).

Time until the first bite

There was no significant difference in the time taken for the first bite ($F = 0.19$, $P = 0.668$). It took 54 minutes for beetles to begin feeding on line 86, and 68 minutes on line 91 (approximately 20 % more time). Generally beetles spent much of the initial hour exploring the petri dish, and showed behaviour aligned with escape attempts such as attempting to squeeze between the agar and petri dish. As when measuring area eaten, there was a significant impact of block, demonstrating there were differences between beetle batches which contribute to the large amount of variance observed ($F = 3.1$, $P = 0.046$). This could be minimised by increased replication.

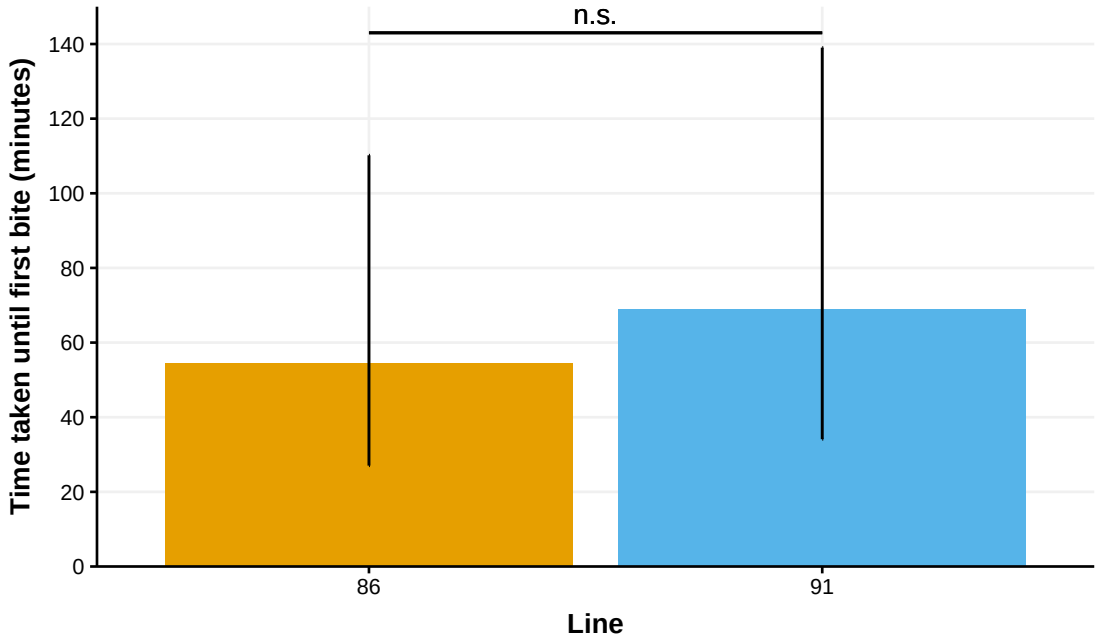

Figure 5.12: Mean time taken for a cabbage stem flea beetle to take the first bite of 7 day old seedlings of either *S. alba* line 86 (*n=11*) or line 91 (*n=9*), measured from video recordings in a non-choice environment. An unbalanced ANOVA was used to compare the time taken until the first bite to the *S. alba* line, with the week the experiment was performed as a blocking factor (6 blocks). n.s. = not significantly different. Error bars represent estimated standard error.

Feeding dynamics over time

Changes to the rate of feeding were assessed by measuring the rate of feeding on each genotype. It was expected that if the resistance response was induced, the rate of feeding would be significantly altered for line 91 only.

The repeated measures ANOVA demonstrated that there was a strong and significant interaction between line and time, i.e. the slope of the line ($F = 12.33$, $P < 0.001$). This demonstrates that the slopes for the two lines were significantly different, and therefore the rate of feeding for the two lines was different for the 48 hour experiment [Fig. 5.13]. As the area eaten over time was quite variable between blocks there was a large amount of variance towards the end of the experiment as some beetles were much more hungry than others in a particular week.

As expected, there was no significant impact of line ($F = 2.13$, $P = 0.162$), which indicates the intercepts of the two lines were the same. This was not expected to be different as the time taken until the first bite was not significantly different. Therefore the intercept was at approximately $t = 0$ for both lines, demonstrating that there was no difference in when beetles started feeding. Also as expected, there was a significant

impact of time, indicating that the leaf area remaining significantly decreased over time ($F = 108.14$, $P < 0.001$).

For both *S. alba* lines 86 and 91 the rate of feeding was very close to a linear correlation for the entire 48 hour duration of the experiment [Fig. 5.13]. It is clear that beetles are feeding at a consistent rate on both lines, however, the gradient of the slope is greater for line 86, and therefore the rate of feeding is higher. This suggests that the resistance mechanism is not induced.

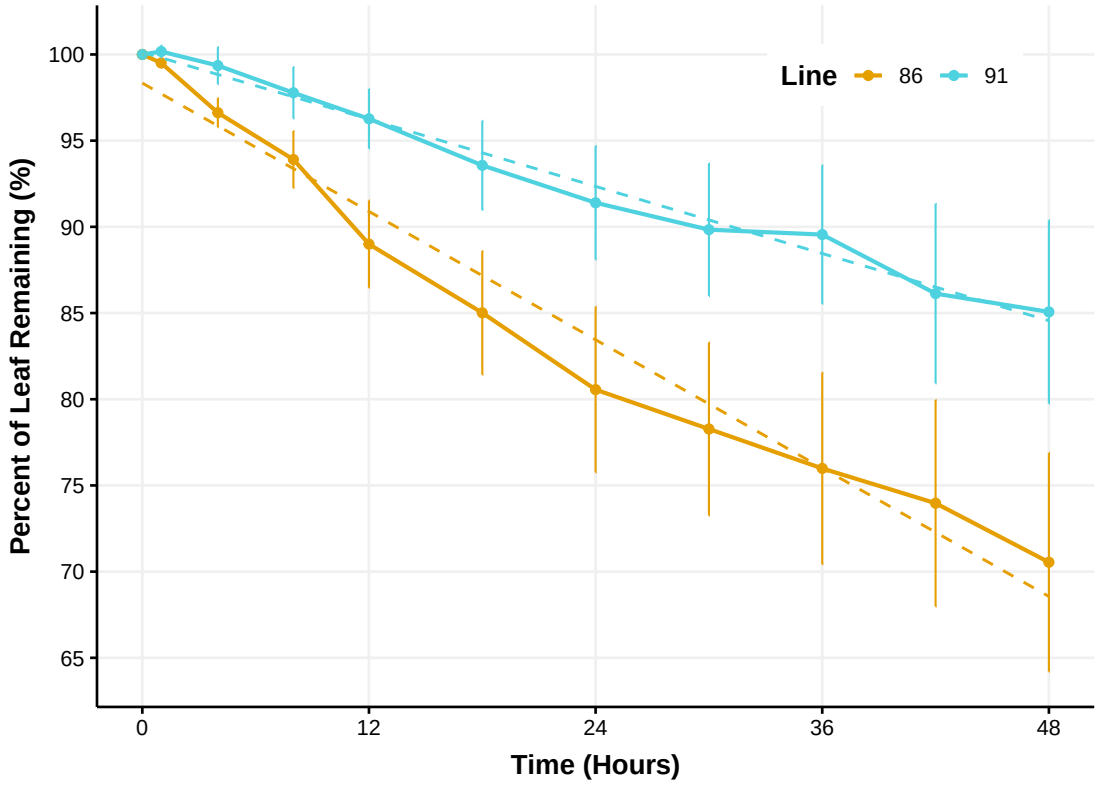

Figure 5.13: Mean leaf area remaining as a percent of the original leaf area over time, for cotyledons of two lines of *S. alba* when fed on by a cabbage stem flea beetle. (*n=12*) seedlings for line 86, and (*n=11*) seedlings for line 91. The solid line represents the mean leaf area remaining at each time point for each genotype. The dashed line is a linear correlation fitted to the data for each genotype. Error bars represent estimated standard error.

5.5 Discussion

5.5.1 Preference of male and female cabbage stem flea beetles for resistant and susceptible *Sinapis alba*

It was not previously known if there is a difference in magnitude of feeding between male and female CSFB, or if there is a preference between the sexes for a particular host.

Although male and female cabbage stem flea beetles are no different in size (Bartlet et al., 1999b), females may have additional energy requirements for egg laying which could have led to greater herbivory. However, no significant differences were observed between the sexes, and this was not altered by the two *S. alba* lines tested here; both males and females preferred *S. alba* line 86 more than line 91. It is not known if these results extrapolate to other species such as *B. napus*, where male and female CSFB may eat different amounts or have specific preferences for certain accessions.

Testing preference for each sex could have provided evidence for the resistance mechanism being linked to host nutritional quality. The quality of the host has previously been found to impact female reproduction of the mustard leaf beetle; females had a stronger preference for the higher quality host (cabbage) than males, and they laid more eggs after feeding on this host (Müller and Müller, 2016). It was hypothesised that if female CSFB had a significant preference for line 86, but males did not have a strong preference, it could indicate differences in nutritional quality between the two lines. As no significant differences were observed here it would suggest that lower nutritional quality does not cause the resistance observed for *S. alba* line 91. However, it does not rule it out completely, as a poor nutritional profile of *S. alba* line 91 may also deter male CSFB equally to females.

This experiment utilised adults collected from the field on a young oilseed rape crop; this is the stage in the CSFB life cycle where females start to lay eggs. An experiment in Chapter 2 found that older adults (i.e. at an age similar to the ones used here) do not have a significant preference for line 86 over line 91, but here a significant difference was clearly observed. The experiment in Chapter 2 was lower in replication than the experiment performed here, which suggests the variance was previously too great to discern differences. The result here should be given more weighting when considering how insects behave *in situ*, as the adults utilised here were only maintained in the laboratory for 7 days after capture from the field, whereas in Chapter 2 the adults were reared in the laboratory for their whole lives. Therefore, this result also serve to demonstrate that age of the insect does not appear to influence CSFB preference for these two lines, and *S. alba* line 86 is susceptible to both pre- and post-aestivated CSFB. This experiment also demonstrates that sexing CSFB is not necessary for phenotyping CSFB resistance for these two particular lines of *S. alba*, as the quantity of herbivory is the same for both males and females.

5.5.2 Leaf Thickness, trichome density and structure, and epicuticular wax structure

When using callipers to quantify leaf thickness, *S. alba* line 86 was significantly thicker than *S. alba* line 91. this may indicate that the physical structures of the cotyledons are altered. For example, the tissue may contain larger cells, additional cells, or an altered

quantity or arrangement of structural components such as lignin. Any altered structure of the cotyledon tissue may alter resistance, as this is the physical structure that herbivores must bypass to extract nutrients.

Although line 86 is significantly thicker, this does not alter the conclusion that line 86 is eaten more than line 91. Rather, the CSFB will need to eat slightly more mass of line 86 to cause the same quantity of damage when scored from a 2-dimensional image, as in Chapter 2. This suggests that the lines are slightly more contrasting than has been calculated, and establishes that line 86 is more susceptible to CSFB feeding than line 91. Therefore, these results suggest that potential differences in leaf thickness do not explain the contrasts in antixenosis quantified in Chapter 2.

Both the abaxial and adaxial cotyledon surfaces for the two *S. alba* lines possessed trichomes. This is unusual for *Brassicaceae*, where the cotyledons are usually glabrous (lacking trichomes). For example *B. rapa* (Li et al., 2022), *B. napus*, and the otherwise very hairy *B. villosa* (Nayidu et al., 2014) all lack trichomes on their cotyledons. Even transgenic *B. napus* modified to have significantly hairier tissues have glabrous cotyledons (Gruber et al., 2006). *S. alba* is therefore a potential resource for introgression of trichome-based resistance genes into the cotyledons of oilseed rape. As flea beetles are most destructive to the seedling stage, expression of trichomes at the cotyledons may have the greatest economic impact.

As there are no significant differences in trichome density or structure between the two lines, trichomes do not appear to be the cause of the increased resistance of *S. alba* line 91 compared to line 86. In addition, the trichome density of the cotyledons of these two *S. alba* lines is relatively sparse compared to *Brassica* where trichome-mediated flea beetle resistance has been demonstrated; for *B. villosa*, trichome density of the resistant true leaves was approximately x10 greater (21.7 trichomes per cm^2). Interestingly, the regulatory control of trichomes has been demonstrated to also control other pathways in *B. napus* which may also influence flea beetle resistance (Gruber et al., 2018). Therefore, the trichomes present on the cotyledons of the *S. alba* lines tested here may not be the primary resistance mechanism of this species, but instead they may indicate expression of other pathways influencing CSFB resistance.

This study did not find differences in the structure of epicuticular wax crystals between *S. alba* line 86 and 91, or between any of the tested tissues (abaxial and adaxial cotyledon surfaces, and the stem). Both lines had a "smooth film" of epicuticular wax, as defined by Barthlott et al. (1998). At low magnification fissures could be observed to the cotyledon surface. However, these fissures were much deeper than the thickness of the wax film overhanging the stomatal pore, and so were likely fractures formed after freezing during sample preparation. Therefore these waxes are not defined here as forming a "fissured layer".

The crystal morphology of epicuticular waxes is closely related to their chemical

composition (Jeffree et al., 1975). In lettuce (*Lactuca sativa*), epicuticular waxes form films due the presence of C22, C24, and C26 primary fatty alcohols (Bakker et al., 1998). Interestingly, *Brassicas* have epicuticular waxes with significantly different structures to that observed here; for example *B. oleracea* and *B. napus* both form rodlets composed mostly of ketones and alkanes (Baker, 1974; Holloway et al., 1977). Information on the composition of waxes from *S. alba* has not been published, but the presence of smooth films suggests that the composition is different from such *Brassica* species and may be due to a greater presence of primary fatty alcohols.

Previous research has found that wax composition can alter the resistance to feeding by insect pests (Bodnaryk, 1992b; Eigenbrode et al., 1991; Munaiz et al., 2020; Razeq et al., 2014). For primary alcohols specifically, a negative correlation was found for fatty alcohol content in leaves of *far* mutants of *Nicotiana glauca* (tree tobacco), and the growth and feeding of multiple caterpillar species (*Spodoptera exigua*, *Spodoptera eridania*, *Trichoplusia ni*, *Heliothis virescens*, and *Manduca sexta*) (Negin et al., 2021). In *Brassica oleracea*, adding additional C24 or C25 alcohols to wax extracts from leaves reduced the number of *Plutella xylostella* caterpillars attempting to feed (Eigenbrode et al., 1991). Therefore, the potential difference in wax composition for *S. alba* compared to *Brassicas* such as *B. napus* and *B. oleracea* may be important for the increased resistance generally shown by *S. alba* (Brandt and Lamb, 1993; Gavloski et al., 2000; Hiiesaar et al., 2006). It may be worth studying the identity of waxes from *S. alba* further for this reason. Mass spectrometry would be useful to verify if these two *S. alba* lines are indeed contain greater levels of primary fatty alcohols compared to *Brassicas*. As purified waxes, including primary fatty alcohols, are commercially available for purchase they could be subsequently be used to test CSFB preference. For example, painting different waxes onto the surface of cotyledons and measuring if herbivory is decreased could be used to indicate if specific waxes are an important mechanism for CSFB resistance in *S. alba*.

As the major wax components dictate the crystal structure of epicuticular waxes, differences in more minor waxes may have been overlooked (Ensikat et al., 2006). Therefore, although no differences in wax structure were observed between the two *S. alba* lines, wax composition may still be significantly altered between them. In addition, the quantity of smooth films of epicuticular wax cannot be easily measured using microscopy unless the difference is substantial. Furthermore, these observations do not reveal changes to the composition of intracuticular waxes; intracuticular waxes are embedded into the cuticle and so not visible in the micrographs obtained here, and their composition can be significantly different to epicuticular composition (Buschhaus and Jetter, 2012; Jenks et al., 2002; Koch and Ensikat, 2008). Therefore, although no major differences were observed using SEM, wax composition should be studied further to fully understand their potential role in the contrasting CSFB resistance between these two lines. Waxes could be identified and quantified by extraction with solvents and subsequent gas chromatography mass spectrometry (GC/MS) (Laila et al., 2017b).

5.5.3 Cabbage stem flea beetle feeding dynamics

As flea beetles did not take significantly longer to start feeding on line 91, it does not appear that the resistance mechanism is linked to identification of *S. alba* line 91 as a suitable host. Cues that can be detected without the need to feed include volatile metabolites, surface metabolites, or other surface structures. These could be detected with the antennae, tarsi, and mouthparts (Bartlet et al., 1999b; Soroka et al., 2011). This result in particular suggests that potential differences in the profile of volatiles between *S. alba* lines 86 and 91 does not cause the resistance of line 91. However, cues such as surface metabolites may play a role as they are ingested once feeding begins, which may provide more information to the insect to reduce herbivory upon line 91.

Some work was performed during this project to assess the attraction of CSFB to these two varieties using volatiles. This was attempted using a "Y-tube" assay, but no attraction could be induced by either *S. alba* lines 86 or 91, or to a *B. napus* line when both damaged or undamaged. Although particular volatiles have been shown as attractive and repellent to other flea beetle species (Gruber et al., 2009), it is not known if flea beetles use volatiles to determine host quality. To date, the only volatile directly associated with CSFB attraction to plants are isothiocyanates, which are used by CSFB to locate OSR crops from long distances (Tóth et al., 2007). However, volatiles may not be as important at short distances for finding hosts and establishing feeding.

The rate of feeding is consistently lower in line 91 at all time points past *t=0*. Potentially, a metabolite that is repellent is being ingested from internal plant tissues, such as alkaloids (Chapman and Bernays, 1989). Or, the plant lacks metabolites that make it more palatable, such as particular nutrients like sugars or protein (Raman and Annadurai, 1985). Alternatively, the physical structure of the cotyledon tissue may be repellent in some way, such as having a tougher composition that takes longer to chew through (Santiago et al., 2013).

The rate of feeding is linear for both genotypes of interest. As the rate of feeding is consistent over time, it does not appear that the resistance changes over time. If resistance was induced in response to feeding, the rate of feeding is expected to reduce once the resistance mechanism accumulates. Instead, the resistance mechanism is likely constitutive. This suggests that there are differences in gene expression earlier in development which alter the level of resistance. This difference could be captured through use of transcriptomics such as RNA-seq, and would not require CSFB to feed upon the plants to detect it (Wang et al., 2009). A reduction in the rate of feeding could be due to other parameters too, such as the beetles becoming satiated, but this would be observed for both lines.

A large quantity of video footage was captured here, and it could certainly be exploited further to understand more about the resistance mechanism. For example, excessive grooming of the antennae may indicate a greater response to volatiles (Böröczky et al.,

2013), or grooming of the feet can indicate responses to surface metabolites (Hosoda and Gorb, 2011). Or, shorter feeding sessions may indicate that something the beetles taste is repellent. These were not assessed here as there was considerable variation between individual insects, so significant differences would be challenging to identify from this number of replicates. Although additional replication could be performed, this would be very time consuming to analyse.

5.6 Chapter summary

This chapter characterised two of the major traits previously linked to flea beetle resistance; trichome density and wax composition. No difference in trichome density or structure were observed, demonstrating that trichome density is not the cause of the increased resistance of *S. alba* line 91. No difference in epicuticular wax structure was observed either, suggesting no major changes to wax composition. However, the full profile of wax composition and wax quantity were not studied here, and should be characterised further to either rule out or implicate waxes as the cause of resistance. The composition of *S. alba* surface waxes is unknown, but appear to be different to those of *Brassica* species as they have a significantly altered structure. These waxes may be important for the resistance seen in *S. alba* to CSFB herbivory compared to these *Brassicas*. As no differences were observed for the traits characterised here, the increased resistance of line 91 may be due to a novel mechanism not previously studied for CSFB resistance.

The experiment monitoring CSFB behaviour suggests that the resistance mechanism is due to something detected upon ingestion, such as secondary metabolites or the physical structure of the cotyledon tissue. The resistance response also appears to be constitutive, and so future work characterising the resistance mechanism of these two *S. alba* lines can be performed without involving CSFB to induce it.

6. Identification of gene candidates controlling cabbage stem flea beetle resistance in *Sinapis alba*

With two lines of *Sinapis alba* identified for contrasting CSFB antixenosis, studies of the genetic mechanisms influencing the phenotype were performed.

6.1 Quantitative genetics of insect-resistance

A quantitative trait has a continuous distribution in phenotypic variation, owing to interactions with environmental or genetic influences. The variation in the antixenosis to CSFB observed here is clearly quantitative, as no completely resistant varieties were identified. This is typical for chewing insects, where resistance is usually controlled by complex polygenic quantitative systems (Kliebenstein, 2014). Genes at multiple loci may influence the trait, and generally they each contribute small additive effects to the phenotype. These quantitative trait loci (QTL) can therefore be challenging to identify, and clusters of closely linked genes may also be detected as one QTL if they are not often separated by recombination events.

To date only one study has identified QTL for flea beetle resistance, where six unique QTL were identified in *B. napus* (Heath, 2017). However, this study did not investigate what the genes in this region were, or what their potential function could be. As QTL are defined only by their effect on resistance phenotype and the position on the genome, they cannot be used to discern the mechanism of resistance when considered alone. They must therefore be combined with genomic annotations and additional phenotyping to elucidate the mechanisms of resistance.

6.2 Identification of genes controlling quantitative resistance

The expansion of genomic and molecular techniques has facilitated the mapping of QTL, and there are numerous techniques to do this. For example, genome-wide association studies (GWAS) are a population approach for large populations with a range of phenotypes, as the technique can identify small genomic regions associated with the phenotype Hatzig et al. (2015). However, GWAS requires a genetically diverse population with a distribution of phenotypes, whereas here only a limited population of *S. alba* was screened. Therefore, it is necessary to choose a technique appropriate for the population available. Here, a technique suitable for comparisons between the two most contrasting *S. alba* lines would be most appropriate. QTL-mapping can be performed by cross-breeding a limited number of individuals, then phenotyping and genotyping the progeny. The simplest population to generate is an F2 population from a bi-parental cross.

The size and number of QTL identified from QTL-mapping is dependent on recombination events, phenotyping accuracy, and the impact of the QTL on the phenotype Lindhout (2002). Although mapping larger populations can give a higher resolution, it can significantly increase work load. Therefore, coarse mapping is generally utilised first to identify the most significant QTL, before fine-mapping techniques that focus on phenotyping only individuals with genetic differences at the QTL of interest to increase the resolution. QTL-mapping is generally not specific enough to identify a single gene or causative SNP, but instead identifies a general area where the resistance gene may reside. Therefore, combining QTL mapping with additional techniques could narrow down the candidate genes. For example, Broekgaarden et al. (2018) used QTL mapping with transcriptome and metabolome analyses to identify QTL and resistance mechanisms in *Brassica oleracea* against whitefly.

6.2.1 Bulked segregant analysis

Bulked segregant analysis (BSA) is a QTL-mapping technique which utilises sample bulking to significantly reduce the required quantity of sequencing Li and Xu (2022). Two bulks are created by pooling individuals from a segregating population displaying extreme opposing traits (e.g. resistant and susceptible). Individuals within each bulk will be similar at the loci controlling the phenotype, and heterozygous at all unlinked regions. In contrast to genotyping each individual plant, pooling reduces the workload and sequencing cost, in addition to increasing efficiency of genomic data analysis.

BSA as a QTL-mapping technique (also called QTL-seq) has grown in popularity since it was first used to identify markers linked to downy mildew resistance in lettuce (Michelmore et al., 1991). Since, robust statistical and computational tools have been developed, and BSA has since been utilised to identify QTL in plants (Tudor et al., 2020), microbes (Ehrenreich et al., 2010), and animals (Pascoal et al., 2014). When combined with next-generation sequencing (NGS) techniques, BSA can not only identify QTL but can pinpoint specific single nucleotide polymorphisms (SNPs), and be paired with genome annotations to infer a potential resistance mechanism.

Multiple algorithms have been developed for statistical determination of QTL from BSA data, reviewed in (Li and Xu, 2022). SNP-index is recognised as a robust algorithm to detect major QTL, and though it can be more conservative than other algorithms such as *G'*, it is less mathematically complex (Shen et al., 2019). SNP-index can be calculated using user-friendly means such as the R package QTL-seq, which pipelines together additional genomic processing tools such as the Burrows-Wheeler Aligner for mapping sequences against the reference genome (Takagi et al., 2013). Calculation of SNP-index generally requires sequencing data from the two parents, in addition to the pools at a sequencing depth of at least 20x coverage.

Currently, there are few published instances where BSA had been used to identify insect

resistance genes. BSA requires phenotyping a large population, and therefore requires a high-throughput phenotyping technique which insect studies are not readily amenable to. Some previous BSA studies for insect resistance have not used NGS for identification of specific genes and mechanisms, but instead focused on identification of markers linked to resistance for use in breeding. For example, markers linked to brown leaf hopper resistance in rice (Kim and Sohn, 2005), and cabbage seedpod weevil in *B. napus* (Lee et al., 2014). One study to date has used BSA with NGS to assess insect resistance, and successfully identified novel QTL linked to Colorado potato beetle in wild potato (*Solanum chacoense*) (Kaiser, 2021). Use of BSA and NGS presents a novel approach to identify resistance genes to CSFB.

6.2.2 RNA-seq

As with other QTL-mapping techniques, BSA can be combined with additional techniques to increase the evidence for the role of specific genes. RNA-seq is a technique which compares gene expression at the whole-genome level for assessment of changes to entire metabolic pathways, which can be used elucidate resistance mechanisms. The use of RNA-seq has accelerated gene expression profiling and identification of causative SNPs significantly (Martin et al., 2013). As CSFB resistance is a complex phenotype, changes to entire pathways may contribute to the resistance phenotype, opposed to single genes. These organism-wide changes could be identified by comparing gene expression between susceptible and resistant lines. BSA has previously been combined with RNA-seq in maize, coined BSR-seq (Liu et al., 2012). Combining BSA with RNA-seq facilitates correlation the resistance phenotype to associated SNPs and their expression patterns.

Interestingly, there have been two RNA-seq experiments identifying changes to gene expression due to flea beetle feeding. Gruber et al. (2012) identified many genes in *Brassica napus* which were up-regulated by crucifer flea beetle feeding, such as those involved in primary metabolism and energy, defence, cell rescue, and stress. However, whether these genes are linked to resistance is not clear. Gruber et al. (2012) also assessed transcriptomic changes of transgenic *Brassica napus* which were resistant to flea beetle feeding. Differentially expressed genes in the transgenic lines were involved in synthesis of flavonoids, phenolics, and indole alkaloids, in addition to structural components including cell wall carbohydrates, lignin, and wax (Gruber et al., 2018). However, no additional studies have been performed to implicate specific genes.

Although RNA-seq experiments typically utilise time-courses to monitor temporal shifts to gene expression due to a stimuli, the resistance phenotype here does not appear to be induced. In the previous chapter, the dynamics of feeding were not altered in response to CSFB feeding, and therefore the resistance trait here appears to be constitutive and already expressed before feeding. Therefore, performing transcriptomics at a single time-point would significantly reduce workload and should still identify

differential expression of defensive pathways.

6.3 Aims

The aim of this chapter was to undertake a preliminary investigation into which areas of the *S. alba* genome may be linked to CSFB resistance. The objectives were to construct molecular resources for *S. alba* to compare the genetic differences between resistant and susceptible lines. A whole genome sequence and annotation of *S. alba* was generated to facilitate identification of specific genes for subsequent analysis. To gain a preliminary understanding of which regions of the genome may be associated with CSFB resistance, bulked segregant analysis (BSA) was performed using an F2 population from a bi-parental cross between two *S. alba* lines contrasting for CSFB antixenosis. This data was combined with RNA-seq data from the two parental lines to identify differentially expressed genes in regions of interest. The genes which were implicated from these two studies were researched to understand if they could have a potential role in the resistance mechanism observed in *S. alba* line 91.

6.4 Materials and Methods

6.4.1 Sequencing and annotation of the resistant *Sinapis alba* parental line

To date, *S. alba* lacks a publicly available whole genome sequence and functional annotation. In order to compare the genetic difference between the resistant and susceptible lines, and understand what pathways these differences may influence, the genome of *Sinapis alba* line 91 was sequenced and annotated *de novo*. The work for sequencing and annotating *S. alba* line 91 were performed by collaborators at Innolea (Mondonville, France).

PacBio sequencing and optimal map generation

A whole genome sequence of *S. alba* was generated for line 91. A seed sample was provided to the INRAE-CNRGV (https://cnrgv.toulouse.inrae.fr/fr) for high molecular weight DNA extraction and sequencing. DNA was extracted from a single seedling (4 pairs of leaves) using a plug method. The genome was sequenced by PacBio HiFi sequencing with a SEQUELII with one SMRTCell, and HiFiasm software was used to assemble the PacBio reads. To obtain hybrid scaffolding, an optical map was produced using Bionano genomics Saphyr. The hybrid scaffolds were organized into chromosomes by merging and reversing some scaffolds, in addition to use of a reference genome (kindly provided by

Isobel Parkin, AAFC Saskatoon), and a genetic map generated from F2 crosses between 86x91 and the Parkin *S. alba* reference.

To aid generation of the genetic map, an F2 population was created from *S. alba* lines 86 and 91, and was genotyped with the 10k Seq-SNP array. The SNPs were filtered to keep only segregating SNPs in the population with a heterozygous rate between 35 % and 65 % (i.e. with a distribution around 50 %) and a NA rate under 5 %. The genetic map between 86x91 was obtained using ASMap R package (v.1.0-4) on 247 plants from the F2 population and 1131 segregant SNPs in the population. The genetic distance between individuals was calculated with the Kosambi method. The gene completion BUSCO score was 99.6 %, and 7 % heterozygosity was detected across the genome.

Genome annotation

Structural genome annotation for *S. alba* line 91 was performed using Augustus v. 3.3.3, with *ab-initio* gene predictors on a soft-masked genome (Hoff and Stanke, 2019). The genome was masked with RepeatMasker v. 4.1.1 to filter out repetitive regions and focus primarily on genes, with a repeats library from I. Parkin. The Augustus model was trained according to Hoff and Stanke guidelines, and the species used for protein-homology-based predictions were: *Arabidopsis thaliana* TAIR V10, *Brassica nigra* C2, *Brassica rapa* CHIIFU, *Brassica napus* DARMOR-bzh V10, *Brassica oleracea* TO1000, and the Parkin *Sinapis alba* genome.

Functional genome annotation was performed using eggNOG-mapper v. 2.1.5 (Cantalapiedra et al., 2021). All the protein-coding genes were searched against eggNOG database sequences v. 5.0.2 (Huerta-Cepas et al., 2019) using Diamond v2.0.11 (Buchfink et al., 2021). The results considered Gene Ontology (GO) terms, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (Kanehisa et al., 2014), Enzyme Classification (EC), CAZy modules (Levasseur et al., 2013), SMART/PFAM domains (Letunic and Bork, 2018) and clusters of orthologous groups (COGs) with their associated functional categories (Galperin et al., 2015).

A BLASTP v. 2.9.0 analysis was performed with the predicted proteins of line 91 against *A. thaliana* TAIR V10 proteins and *B. napus* DARMOR-bzh V10, with a threshold on the evalue at 1e-20 (Altschul et al., 1990). The genes with best similarity to *A. thaliana* and *B. napus* for each *S. alba* line 91 protein were used to annotate the function of the genes. Only for those genes either lacking a homolog or with a low-confidence homolog from *Arabidopsis* or *B. napus* were annotations from eggNOG used.

6.4.2 Bulked Segregant Analysis

To identify QTL that were associated with contrasting antixenosis to CSFB, the F2 population generated from lines 86 and 91 was used to perform bulked segergant analysis

(BSA). One-week-old plants from this population were phenotyped for antixenosis using a high-throughput design, and the most contrasting individuals were pooled and sequenced.

Insect preparation

To phenotype the required number of individual F2 plants, a large number of insects were required at the same time. Unfortunately, the laboratory culture could not be used as the time and resources taken to obtain thousands of adult insects simultaneously was unreasonable. Therefore, flea beetles used for this experiment were collected from the wild rather than the colony. Approximately 2000 adult CSFB were collected from a grain store of WOSR on 12th August 2022. Harvesting of the crop had started several days prior, and large numbers of CSFB were also collected with the crop by the combine. A broom was used to sweep insects from the walls of the store into a high-sided plastic bucket, and an insect aspirator was used to transfer the insects to 50 mL corning tubes for transport back to the laboratory. Up to 50 beetles per box were maintained in boxes as previously described (16 °C day:10 °C night 16:8 L:D). All adults were sexed using the first tarsal segment, and only females were retained for experimentation. Although no significant differences in the area eaten between the sexes were observed previously, only colony insects at a particular age had been tested. The variation in feeding between the sexes in these wild-caught insects could be significant.

Preliminary feeding experiments with these adults demonstrated little feeding (all adults eating < 5 % of leaf discs), and indicated that the insects were collected during their aestivation period, which is expected at this time of year (Såringer, 1984). Therefore, they could not be used for experimentation right away. Insects were maintained using the rearing protocol described in the general materials and methods until feeding increased. The quantity of feeding was measured every 7 days by cutting a 12 mm disc of *Brassica rapa* subsp. pekinensis (Chinese cabbage) using a cork borer, avoiding the mid-vein, and placing the disc into a 35 x 10 mm Nunc™petri dish (Thermo Fisher Scientific, Inc.) filled with 2 mL 1.5 % water agar. A single female CSFB was starved for 24 hours, then added to each dish, with at least 30 beetles screened per week. The petri dishes were placed into a 20 L, 710 mm x 440 mm x 120 mm clip-lock box (Shelving Plus Ltd) with 3 sheets of water-saturated blue roll to keep humidity high and prevent leaves and agar from drying out [Fig. 6.1]. Beetles were allowed to feed for 24 hours. Damage to leaf discs was measured using the ImageJ pipeline, and if more than 5 % of the leaf area was damaged an adult was considered to be feeding, and therefore to have exited aestivation. It was estimated that three-quarters of the adults would need to be feeding to have a sufficient number to perform the experiment. Initially, 5 % of individuals had exited aestivation on 19/08/21, and this number gradually increased over time to 71 % of individuals by 24/09/22 [Fig. 9.1]. Once the threshold of 71 % of insect feeding was reached, 500 seed from the F2 population was sown immediately to be used for phenotyping 7 days later. 20 seeds were arranged uniformly in a 15 cm black pot and grown in the CER using conditions

described previously.

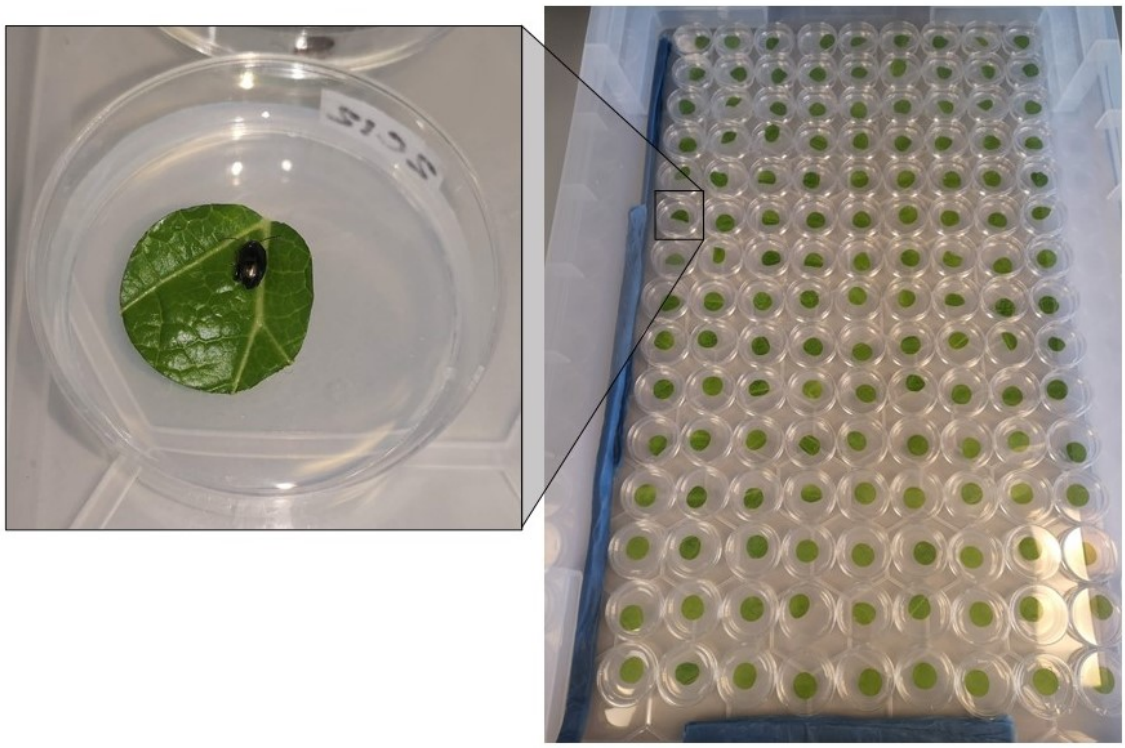

Figure 6.1: Image of the experimental design used to screen CSFB for the quantity of feeding in preparation for phenotyping an F2 population for bulked segregant analysis. A close-up of a one replicate is seen of the left, with one female adult in a petri dish with water agar and one leaf disc of Chinese cabbage (lid removed for the photo). This petri dish was placed with the others in clip-lock boxes containing water-saturated blue-roll to maintain humidity (right). These were stacked and placed in a controlled environment room. This same design was used screening the F2 population, where leaf disc was replaced by a single cut cotyledon for each seedling of the F2 population.

Due to the high work-load the phenotyping screen was performed in two blocks using approximately 500 plants per time. The steps below to select insects were also performed twice. To assess which specific individuals had exited aestivation, all beetles were screened again 48 hours prior to the F2 screen. Approximately 1000 female CSFB were starved for 24 hours by removing food plants from boxes. Petri dishes using leaf discs of Chinese cabbage were prepared as above, and one female was added to each petri dish for all 1000 females. In total, 8 clip-lock boxes of 125 insects each were stacked 4 boxes high, and placed under the benches of the CER [Fig. 6.1]. Boxes did not receive direct overhead lighting, but were exposed on the sides of the boxes to the lighting in the room. Beetles were allowed to feed for 24 hours before scoring. Damage to the leaf discs was estimated by eye, and insects that ate more than 5 % of the leaf disc were moved to boxes lacking food plants to be starved immediately in preparation for use in the F2 population screen 24 hours later. Those insects that did not eat a sufficient amount ($<$ 5 % of the leaf disc)

were placed in boxes with food and maintained using the standard rearing conditions to be screened for feeding one week later for phenotyping the second block.

Phenotyping the F2 population

The F2 plants were phenotyped 7 days after sowing using the same experimental design as above, but the Chinese cabbage leaf disc was replaced with a single cotyledon cut along the lamina base (removing the petiole). Whole plants were not used here as the resources required for this were particularly challenging. The resistance between the two lines was also maintained in cut cotyledons in preliminary experiments, which supports the previous work in the previous chapter which indicated the resistance was constitutive in this case and should not be significantly altered. The other cotyledon from the seedling was placed into a 1.2 mL polypropylene round tube, racked in a 96 well format. The material was frozen by placing the racks into a -80 °C freezer, where they were maintained prior to sampling for DNA. Each petri dish was arranged haphazardly and labelled with a corresponding rack number a cell location. Five clip-lock boxes of 96 petri dishes were assembled, and a single starved female CSFB was added to each dish. Boxes were stacked and placed under the benches of the CER as above, and 24 hours later all leaves were scanned and scored using the ImageJ pipeline.

After repeating both the screen to select feeding adults and subsequent phenotyping of the F2 population, a total of 767 F2 plants were phenotyped for CSFB antixenosis, with 395 in block 1 and 372 in block 2. Not all plants from the 500 seed planted per block were used, as not all seed germinated and only those without malformations were selected for experimentation.

A third screen was performed to compare the parental lines 86 and 91 as above, using insects selected for feeding. 58 replicates for line 86 and 52 replicates of line 91 were screened, and the percent area damage to the two parental lines was compared using an unbalanced ANOVA with a square-root transformation to stabilise variances. This test was performed to demonstrate that this experimental design was able to distinguish between the parents to a magnitude that was previously seen when using whole plants.

Bulking, DNA extraction and sequencing

Due to experimenter error, in the second block of the F2 phenotyping screen approximately 25 % of the insects included were “non-feeding” insects. As some of the low feeding in this week would be due to inclusion of insects that were still aestivating and not feeding, it was not possible to accurately score for a resistant phenotype in this block. Therefore, all individuals in the second block that ate less than 5 % of the cotyledon were filtered out. Consequently, this second run was not used for selection of the resistant pool. Instances where insects died during the experiment were also excluded. From 767 phenotyped plants, 548 were used for selection of the two bulks.

The susceptible bulk (S) consisted of the 5 % of individuals with the highest quantity of leaf area eaten from CSFB feeding from both blocks, consisting of 28 individuals. The resistant bulk (R) consisted of the 5 % of individuals with the lowest quantity of leaf area eaten from CSFB feeding from the first block only, which was 19 individuals.

For DNA extraction of each pool, each cotyledon previously stored at -80 °C was snapped in half, and combined with the rest of the material from the pool. Samples were ground in a pre-cooled pestle and mortar, and prior to DNA extraction two 1.5 mL sorbitol washes were used to remove contaminants (potentially phenols and polysaccharides) (Jones and Schwessinger, 2020). DNA extraction was performed using an Illustra Phytopure DNA extraction kit according to the manufacturers instructions. The two bulks and both parents were subjected to whole-genome resequencing using an Illumina HiSeq (Illumina, Inc., San Diego, CA, USA) platform (Illumina NovaSeq6000), by Novogene Co., Ltd., Hong Kong (https://www.novogene.com), producing $>$ 98 % effective reads per sample. Mean depth of coverage for the bulks was 143x (71 G data), and for the parents was 77x (39 G data). Sample quality control was also performed by Novogene Co., Ltd., HK, including trimming of adapter sequences. FastQC was used to generate a report on the quality of the sequencing, and quality was high for all samples.

Processing and ΔSNP-index calculation

The QTL-seq v. 2.2.3 pipeline was used on a HP cluster to perform alignment, SNP calling, and ΔSNP-index calculation using default parameters (Takagi et al., 2013). Using this tool, high-quality reads from the resistant and susceptible pool, and the susceptible parental line were aligned to the previously established *de novo* assembly of the resistant parent line by BWA-MEM (Li and Durbin, 2009). Haplotypes were identified for chromosomes 2, 5, 10 and 11, but as the resistant allele was assumed to be fixed due to a consistent phenotype, only the haplotypes with a greater number of SNPs were used. Genomic positions were considered genetically identical if no alternative sequence was found in $\geq$ 95 % of line 86 sequence reads. All other SNPs were excluded from further analysis.

SAMtools was used to filter improperly paired reads and PCR duplicates, and to call SNP variants between the two pools. A minimum sequencing depth of 20 reads per base in both bulks was used. The number of individuals in each bulk was specified for calculating the probability threshold for calling the SNP (Li et al., 2009).

To reduce the variance introduced due to sequencing error, SNP index values that were $<$ 0.3 in both bulks were removed (Takagi et al., 2013). A VCF file was generated by BCFtools (Li, 2011), and the ΔSNP-index was calculated by subtracting the SNP-index of one bulk from the other (R-S). As the resistant parent was used as the reference, a ΔSNP-index of 1 indicated all sequencing reads at that position were different between the two bulks, while a ΔSNP-index of 0 indicated they were identical. A ΔSNP-index

below 0 indicates the susceptible bulk was closer to the resistant parent than the resistant bulk in that region, and therefore this region would not be linked to the trait of interest.

Confidence intervals and the ΔSNP-index were smoothed for plotting over 100 bp. For positions where the ΔSNP-index was greater than the 95 % confidence interval, this region was significantly linked to the phenotype ($P \geq 0.05$). A 1 Mbp region was selected either side of the peak, and a list of all genes in this region was generated using the *S. alba* line 91 genome annotation. To annotate the SNP set within the 2 Mbp window, and identify potentially causative SNPs, SnpEff version 5.1 was used to generate a SnpEff binary database file (.bin) using the whole annotated genome reference of *S. alba* line 91, using default parameters (Cingolani et al., 2012).

6.4.3 RNA-seq

Plant Treatments

RNA was extracted from the cotyledons of line 86 and 91 at the same growth stage, where plants were presented to CSFB in feeding screens. Plants were grown in compost in the CER using the standard method described in the general materials and methods, which was also used for plants grown for phenotyping CSFB resistance. When the growing point of the true leaves were just visible (approximately 7 days after sowing), both cotyledons of 3 seedlings were sampled by cutting at the lamina base, placed in tin-foil and immediately snap-frozen in liquid nitrogen. This was repeated 3 times for each line to give 3 pooled replicates. Samples were stored at -80 °C until ready for extraction.

Sequencing and processing

RNA was extracted using an ENZA Plant RNA kit (Omega Bio-tek) according to the manufacturers instructions. Reads were generated using Illumina Novoseq by Genewiz Inc (Leipzig, Germany), generating 150bp paired-end sequences with an mean of 36 million reads of between between 5.9 and 17.0 Mbp per sample. For both *S. alba* line 86 and line 91, raw read data were quality-controlled with FASTQC v. 0.11.8 to examine the GC content, base quality score and the total number of reads sequenced (Andrews, 2010). Paired-end reads were trimmed with Trimmomatic v. 0.38 (Bolger et al., 2014). Then, reads were aligned to the reference genome of *S. alba* line 91 using the splice-aware aligner STAR v. 2.7.2 (Dobin et al., 2013). The mean quality score was 35.76, with 93.17 % of bases ≥ 30.

Transcript quantification

Alignment files obtained from the read mapping step were used for transcript quantification. This quantification step is based on the overlap between the mapping

coordinates of the read and any specified feature in the annotation file. It was performed with featureCounts v. 2.0.1 using the reference annotation in a GFF file, and specific options for paired-end reads (Liao et al., 2014).

Differential gene expression

Differential gene expression was calculated using SARTools v. 1.7.3, an R (v. 3.6.3) package (Varet et al., 2016). Differential analysis was run with DESeq2 v. 1.26.0 (Love et al., 2014), and genes were considered as differentially expressed genes (DEGs) when there was both a significance value of $P < 0.05$, and a Log2FoldChange in expression of $>$ 1.5 for over-expressed genes and $<$ -1.5 for under-expressed genes. Only DEGs were kept for analysis.

Gene set enrichment analysis with Gene Ontology (GO) terms

To evaluate genome-wide changes in gene expression, gene ontology (GO) analysis was performed. Production of the list of significant GO terms and associated genes was performed by the collaborators at Innolea. GO analysis grouped genes into sets corresponding to biological terms. TopGO v. 2.46.0, an R (v. 4.1.1) package was used to compute gene set enrichment analysis with GO terms (Adrian and Jorg, 2022). The list of the genes with GO term annotation obtained with EggNOG, and the list of DEGs were used to assign GO terms. TopGO was run using the weight01 method Fisher statistical test, and genes with a False Discovery Rate (FDR) of $P \leq 0.01$ were considered.

6.5 Results

6.5.1 Bulked Segregant Analysis

Feeding results from F2 phenotyping

In accordance with previous experiments, *S. alba* line 91 was eaten significantly less than line 86 ($F = 17.47$, $P < 0.001$). The predicted mean for line 86 was 10.0 % (± 1.8 %) leaf area eaten, and for line 91 was 3.5 % (± 1.1 %). This was a 2.8x difference between the genotypes, which is comparable to the magnitude difference previously seen during phenotyping of these lines using whole plants.

The mean % area eaten between blocks was slightly different. The mean feeding for block 1 was 9.2 % (± 9.4 % SD), block 2 was 9.9 % (± 12.4 % SD), and the parent block 3 was 10.0 % (± 11.4 % SD). These experiments were performed in subsequent weeks, and so feeding appeared to increase slightly each week. It was expected that if CSFB behaviour was consistent the level of feeding for each block should also be the same. Potentially, the difference between blocks is due to adults increasing their quantity of feeding as they

continue to exit aestivation (see supplementary Fig. 9.1). To account for the variation between blocks, the % area eaten data was normalised in order to compare all three blocks together. For each block, every % area eaten value was divided by the highest % area eaten for that block (i.e. the highest value was set to "1", with every other value falling between "1" and "0"). [Fig. 6.2].

The mean normalised damage for line 86 was 0.31 (± 0.037 SE), for 91 was 0.11 (± 0.017 SE), and for the F2 population was 0.23 (± 0.0088 SE). The general phenotype of the F2 population was that the mean feeding damage was in between what was quantified for lines 86 and 91; the F2 population was fed upon less than line 86, but more than line 91, and was near the mean between the two parents (0.24). There were very few instances where feeding was slightly higher in the F2 population before normalisation, but it is difficult to know if this is due to variation in the beetles and the very large number of replicates used for the F2s compared to the parents, opposed to transgressive segregation.

The distribution of feeding in Fig. 6.2 shows two main differences between feeding of the two parents. Firstly, line 86 had more cases of higher feeding (feeding beyond 0.570, which was the maximum for line 91). Overall, 8.03 % of the F2 population had feeding greater than 0.570 and can be considered susceptible. As only the top 5 % of most eaten individuals in the F2 population were selected for the susceptible bulk, this bulk is unlikely to have escapes and is likely a robust sample.

Secondly, line 91 had a greater number of individuals that were fed upon a very small amount (clustering at below 0.05). For line 91, 50 % of the individuals had ≤ 0.05 normalised area damage, whereas just 15 % of 86 had ≤ 0.05 normalised area damage. However, the resistance phenotype is not as clear, as a significant proportion of the susceptible genotype still had low levels of feeding (≤ 0.05). If a Mendelian inheritance pattern is assumed for the F2 population, approximately 3.3x more individuals with ≤ 0.05 feeding damage would be resistant than susceptible for feeding at this level, or around 7 out of every 10 individuals would be resistant. Consequently, it would be highly unlikely to assemble a resistant bulk without susceptible escapes, and instead it is likely that around 70 % of the resistant bulk could be resistant, and the other 30 % could be susceptible. Therefore, a SNP-index in the resistant bulk for the SNP of interest would be at most approximately 0.7, as it would not be completely homozygous at the QTL region of interest due to escapes. A ΔSNP-index of 1 was highly unlikely, and instead it was expected that a ΔSNP-index approaching 0.7 would indicate a significant QTL.

The susceptible individuals showing a resistant trait may be because the susceptible phenotype may not be dominant, or because of variable beetle behaviour. Although CSFB were found to be feeding heavily the day before the F2 phenotyping assay, they could be satiated and no longer wish to feed. This result highlights one of the drawbacks of this assay which uses single insects, as controlling the behaviour of individual CSFB is extremely challenging.

Due to the complex phenotype whereby the resistant phenotype is not easily differentiated, it is not possible to determine from these results the dynamics of inheritance, such as if there is Mendelian inheritance.

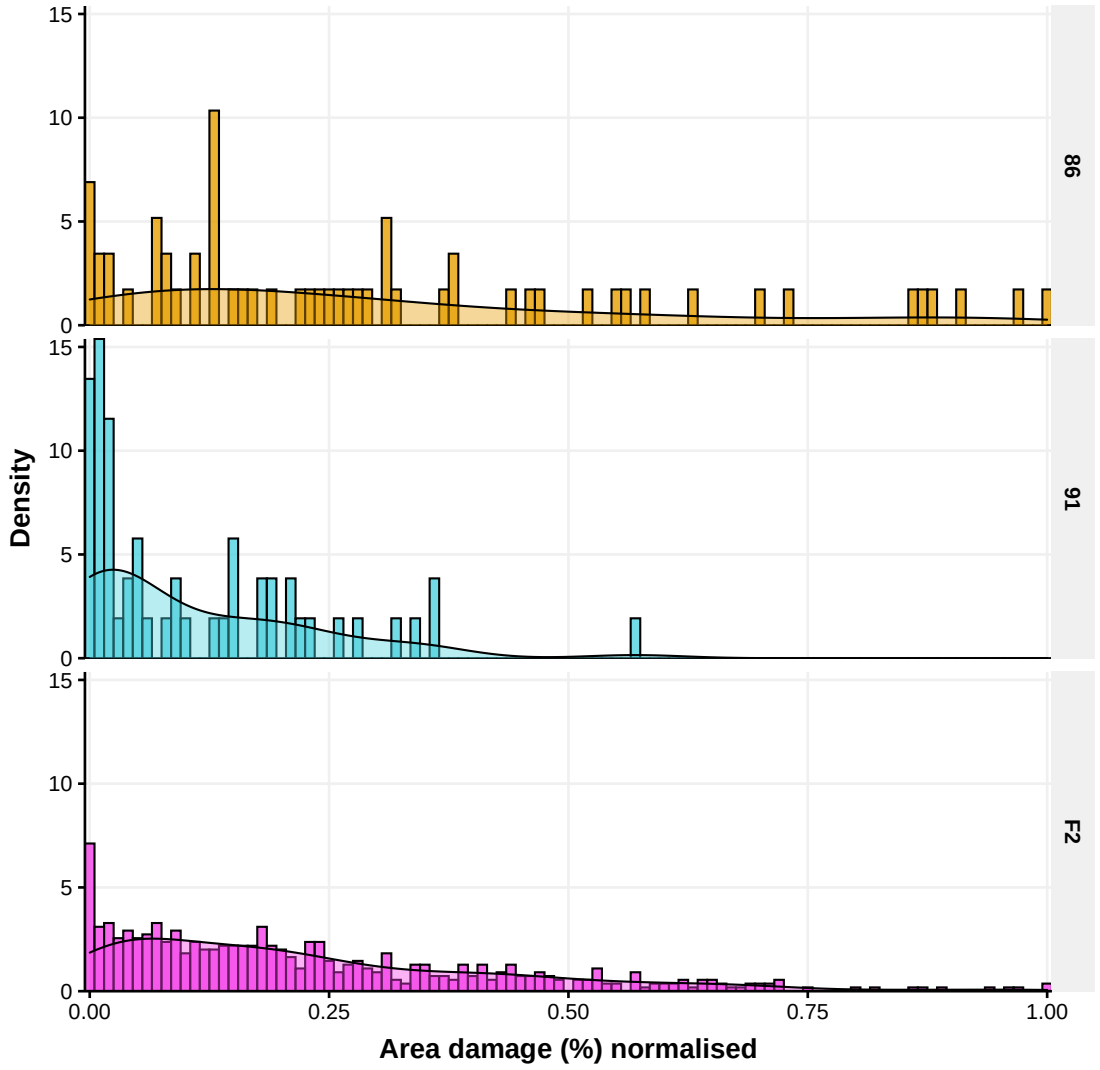

Figure 6.2: Normalised feeding for the screen of the F2 population and the two *S. alba* parents (86, susceptible; 91, resistant) used to generate the population. The cotyledons that had the highest level of damage are closer to "1". The density represents the proportion of individuals within that group showing that level of feeding damage (i.e. the proportion). Each bin is 0.01 unit of normalised area damage. A LOESS-smoothed histogram is overlaid for each plot.

Identification of QTL

As expected, no ΔSNP-index values were calculated at 1. Instead, the highest value was 0.7727 on chromosome 12, at position 30.63 Mbp [Fig. 6.3]. However, this region was not statistically significant because there was also a region close by where the susceptible

bulk closely matched the resistant parent; a ΔSNP-index of -0.7695 on chromosome 12 at position 30.51 Mbp (which was also the lowest ΔSNP-index). Although recombination could have occurred in the 0.1 Mbp region between these peaks, in an F2 population this is statistically unlikely. These juxtaposing peaks were seen across the genome, and they indicate that the particular region does not contain a QTL as there are nearby SNPs in both bulks that match the resistant parent. Instead, a rolling mean was used to find peaks in the ΔSNP-index to detect consistent regions of a high ΔSNP-index. Where the rolling mean was higher than the predicted confidence intervals, it indicated a QTL was significantly linked to the resistance phenotype.

Using a sliding window of 100 bp, at one location in the genome the ΔSNP-index exceeded the 95 % confidence interval, indicating a significant contrast between the SNPs within that region and a potential QTL [Fig. 6.4]. This was detected on chromosome 11, and the position of the peak from the rolling mean is between 16.54 Mb and 17.00 Mb (though this position is shifted off the peak due to the calculation of the sliding window). The 95 % confidence interval in this region had a mean of 0.301, whereas the rolling mean of the ΔSNP-index had a mean of 0.311. This corresponds to a contrasting peak of SNPs at 15.94 Mb which had a ΔSNP-index of 0.5892. No other chromosomes had significant peaks, and no peaks exceeded the 99 % confidence interval.

There is a potential secondary peak on chromosome 11, though it was not statistically significant. At 22.76 Mbp, the rolling mean was 0.2744 whereas the 95 % confidence interval was 0.2827. This region may contain a second loci associated with the trait, or it could be an assembly issue in that region; potentially, an inversion may have occurred during assembly and this region could be associated with the significant peak.

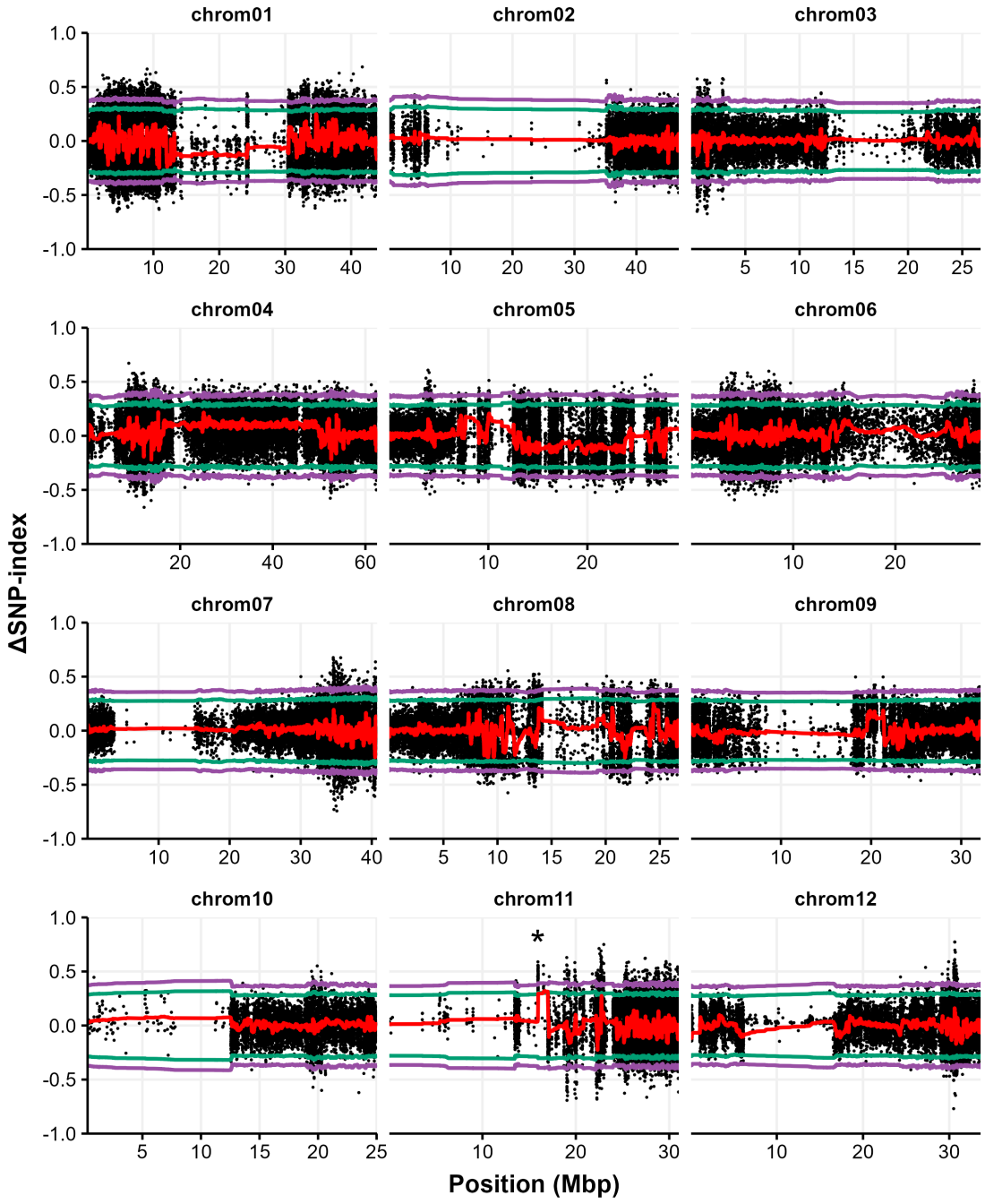

Figure 6.3: The distribution of significant QTL across the 12 *S. alba* chromosomes, identified from phenotyping an F2 population generated from contrasting parents in to cabbage stem flea beetle herbivory in the laboratory. Statistical confidence intervals under the null hypothesis of no QTL (green, $P < 0.05$; purple, $P < 0.01$). Where the rolling mean across 100 bp of the ΔSNP-index (red) crosses the confidence interval, the region contains statistically significant SNPs associated with antixenosis to cabbage stem flea beetle. One significant region was found, shown using an asterisk (*).

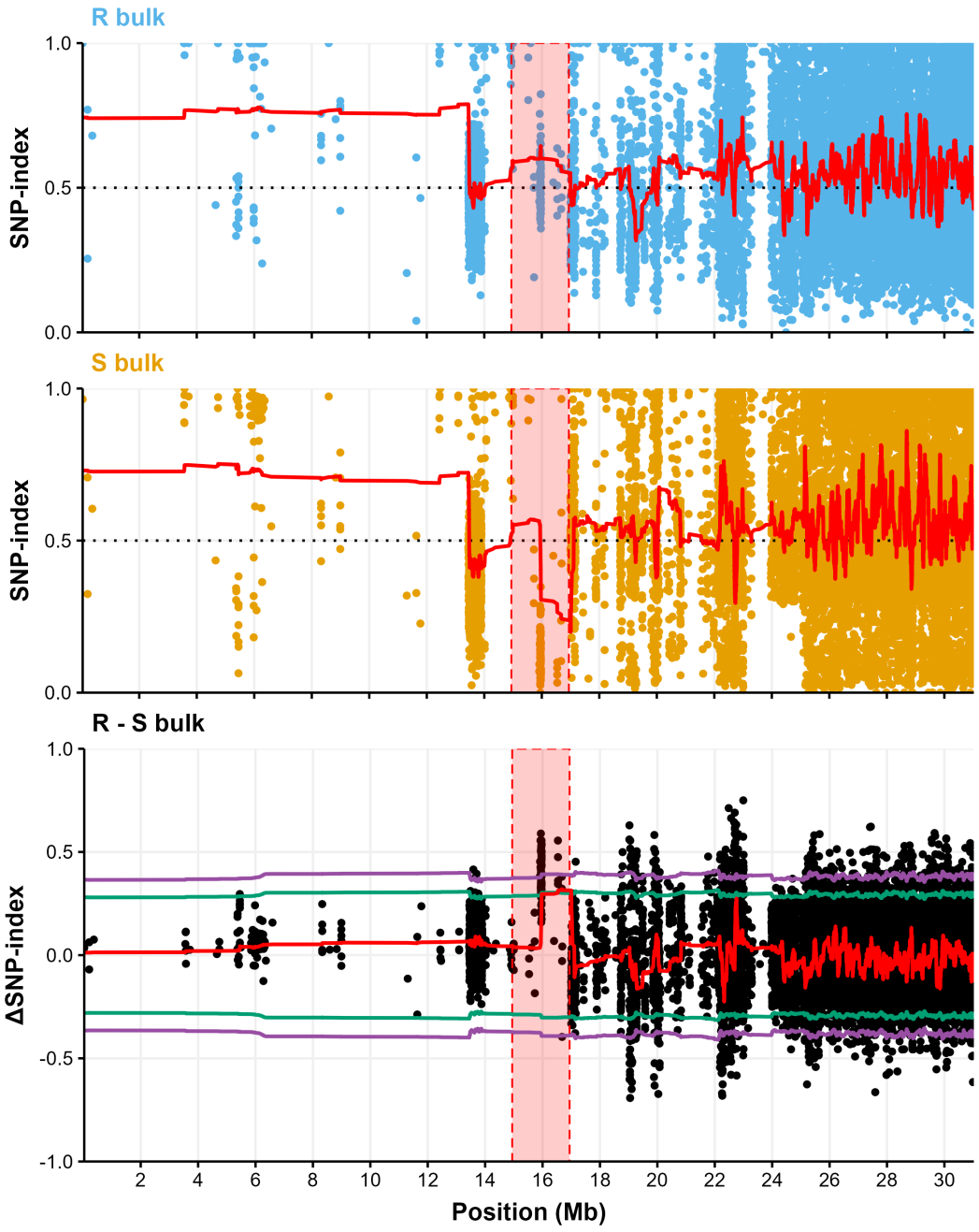

Figure 6.4: For *S. aba* chromosome 11, the SNP-index calculated for the resistant (R) bulk (top) and susceptible (S) bulk (middle), and the ΔSNP-index calculated from subtracting the resistant from the susceptible (R - S; bottom). Regions where the SNP-index is contrasting between the two bulks indicates a QTL region controlling the antixenosis trait. Statistical confidence intervals under the null hypothesis of no QTL (green, $P < 0.05$; purple, $P < 0.01$). Where the rolling mean across 100 b of the ΔSNP-index (red) crosses the confidence interval, the associated region contains statistically significant SNPs.

SnpEff and identification of candidate genes

The 2 Mbp window in the chrm11-QTL spans 56 genes. Of 1300 SNPs in the chrm11-QTL, the majority of the SNPS (58.8 %) were found to be intergenic, totalling 765 SNPs. The other 41.2 % (535) of SNPs were within the gene transcript. The majority (95.5 %) of the SNPs also caused minimal impact to the protein product. Using SnpEff, 36 SNPs had a low impact, 19 had a moderate impact, and 3 were high impact. Of these high-impact SNPs 2 were frame-shift variants including 1 where a premature stop was gained, and 1 was a splice donor variant introducing an intron variant. To further narrow down potential candidate genes, the results of BSA and SnpEff were combined with RNA-seq data to identify those genes that were both within the chrm11-QTL, and expressed.

6.5.2 RNA-seq

In total, 28,095 genes were expressed in at least one sample of 86 or 91, representing about 63.1 % of all *S. alba* genes. Of those detected, 20,655 (73.5 %) were expressed equally in the two lines [Fig. 6.5]. 3174 (11.3 % of total) genes were significantly differentially expressed with at least a 1.5-log2fold difference, which corresponds to a multiplicative factor of at least 2.82 times increased expression. Of these, 2209 (69.6 %) were significant to $P < 0.001$, and 1836 (57.8 %) to $P < 0.0001$.

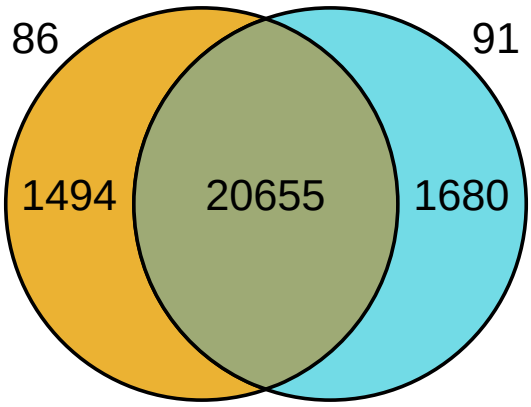

Figure 6.5: Venn diagram showing the number of up-regulated genes in lines 86 and 91, and the overlap of genes that were expressed in both samples. Significant genes were up-regulated with a log2fold change difference of at least 1.5 (or -1.5 for down-regulated genes), and significance value of $P < 0.05$.

To facilitate the global analysis of differential gene expression, all predicted *S. alba* genes were assigned GO functional annotations using topGO. Of 28,095 detected genes, 343 genes were enriched for 47 over-represented GO terms based on sequence homology [Fig. 6.6]. These GO terms were mainly for general processes, such as DNA-binding transcription factor activity (65 genes) and sequence-specific DNA binding (39 genes). In

both cases, these were enriched in line 86. Other terms enriched in line 86 were "response to water deprivation" (31 genes), "response to cold" (28 genes), and "response to wounding" (21 genes). For line 91, the top enriched GO terms were for "antibiotic catabolic process" (6 genes) and "drought recovery" (4 genes). The full list of differentially expressed genes underlying the enriched GO terms can be seen in the supplementary section, Table 9.1 for line 91, and Table 9.2 for line 86.

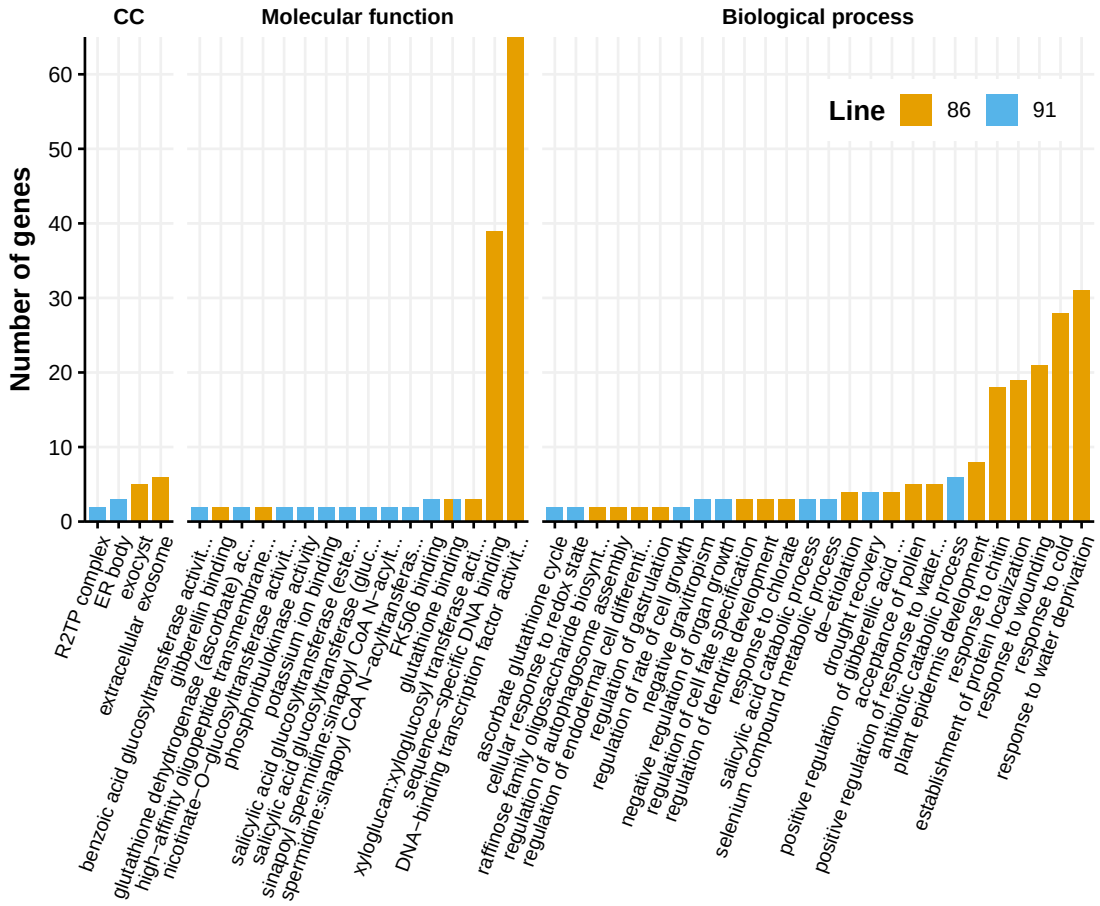

Figure 6.6: The number of genes with significantly enriched gene ontology (GO) classification for *Sinapis alba* lines 91 and 86. GO terms are grouped into three main categories: Cellular component (CC), Molecular function, and Biological process.

6.6 Discussion

The bulked segregant analysis performed here, in conjunction with the RNAseq experiment, present a preliminary exploration of the potential mechanisms underpinning resistance in *S. alba* line 91 compared to line 86. It is not expected that the data produced here would pinpoint a single gene, but instead broadly examine changes to regulatory pathways, linking these to previous evidence of flea beetle/insect resistance. Therefore,

we can gather some potentially useful insights.

6.6.1 Magnitude of feeding in parents and F2 progeny

For *S. alba* lines 86 and 91, the magnitude of resistance to CSFB appears to be unaltered by causing damage to the plants for use of cut cotyledons. Potentially, this screening protocol may be useful as a much higher in throughput phenotyping assay, and could be used for future phenotyping work for populations generated from these two lines instead of using whole plants. However, it would be important to verify that cutting the petiole does not significantly alter CSFB feeding by screening both whole and cut plants together; this experiment did not test this. It is also important to note that this assay may not be representative of the resistance phenotypes of other lines. Damage will alter gene expression, and may alter antixenosis in a way which would not be seen in the whole plants or the natural environment, so caution should be used if the desire is to screen additional plant populations using this method.

6.6.2 Key enriched GO terms

Some enriched GO terms had underlying genes in close proximity to the chrm11-QTL, though no genes fall within the 2 Mbp window. Two close-by genes are involved in root processes; S11_1g0036027 is 1.6 Mbp upstream of the peak and a homolog of *IDD8* (AT5G44160), a transcriptional regulator involved primarily in root development. S11 _1g0036143 is 2.8 Mbp upstream of the peak and a homolog of *maMYB* (AT5G45420). The gene encodes a R2R3-MYB family transcription factor with a role in root hair elongation. A third gene has more broad functions; S11_1g0035866 is 1.7 Mbp downstream of the peak, and a homolog of *CTL06* (AT5G42940), a RING-E3 ligase (Jiménez-López et al., 2018). *CTL06* has been implicated in abiotic defence response networks (Bulley et al., 2021), and other RING-E3 ligases have been found to have involvement in the JA pathways in response to herbivory to the Tobacco cutworm (*Spodoptera litura*) (Ali et al., 2019). However, CTL06 specifically has not been well characterised, and it's potential role in herbivory response is unknown. The next closest gene with an associated enriched GO term is more than 4 Mbp away from chrm11-QTL and so unlikely to be linked to the region.

The most enriched GO terms by far were "DNA-binding transcription factor activity" and "sequence-specific DNA binding", representing 19.0 % and 11.4 % of enriched genes respectively. These enriched transcriptional regulators are involved in a broad arrays of stress responses, which can be seen from the other enriched GO terms for line 86. These were response to water deprivation (31 genes), cold (28 genes), wounding (21 genes) and chitin (18 genes). It is interesting that line 86 is so enriched for stress response genes, and may indicate that the growth conditions were less ideal for this variety. Although no morphological signs of stress were observed (e.g. wilting, yellowing), some conditions

during growth may have triggered these responses. Stress response genes can have significant impacts on defence (Knight and Knight, 2001). Those of particular interest here are the genes involved in responses to chitin and wounding, which have specific relevance to insect herbivory (Bodnaryk, 1994; Le et al., 2014; Libault et al., 2007). Chitin is a protein found in the insect exoskeleton as well as fungi cell walls, so chitin detection by plants may be involved in responses to fungi or insects (Ramonell et al., 2005; Wan et al., 2008). The other stress pathways may also be of interest, as there is generally much cross-talk between stress responsive pathways (Jia et al., 2022; Knight and Knight, 2001).

In line 91, fewer ontologies were enriched. The processes with the best characterised links to insect resistance were "salicylic acid catabolic process", "selenium compound metabolic process", and "response to redox state".

For the significant DEGs underpinning these key GO terms, their functions and potential roles in insect resistance were researched further. Although it is unlikely to implicate the specific roles of these genes in the resistance trait to CSFB owing to their broad functions, understanding their general effects can provide some ideas as to potential differences between the two lines. This may help to guide future research investigating specific pathways linked to CSFB resistance. Genes with highly general functions (such as regulators of general transcription), or those involved in seemingly unrelated pathways (e.g. programmed cell death in response to water stress) were not discussed here, as evidence for their role in insect resistance is lacking.

Response to wounding and chitin

The majority of genes enriched for both responses to wounding and chitin are modulators of transcription. 64.0 % of genes with these two annotations are transcription factors (TFs), and 20.5 % are involved in signalling cascades upstream of transcriptional regulation. This indicates massive reprogramming of line 86 in pathways related to chitin and wounding responses compared to line 91. Some well-characterised genes are involved in both responses to wounding and chitin. S08g0028078, a homolog of *MYC2* (AT1G32640), is a basic helix loop helix (bhlh) subgroup IIIe TF which is a key regulator of diverse jasmonic acid (JA)-dependent functions (Chini et al., 2009). JAs coordinate plant responses to both biotic and abiotic challenges, and is the main regulator of plant defences against chewing insects (Chen and Mao, 2020). For example, JA alters wax composition of *B. napus* (Yuan et al., 2020). It is interesting that the susceptible line is enriched for expression of chitin and wounding response genes, which are usually associated with increased resistance. This may indicate that the defence responses deployed are inappropriate to defend against the CSFB (perhaps due to its role as a specialist), or that these genes are not involved in the resistance mechanism.

Downstream pathways of MYC2 include negative regulation of tryptophan derived secondary metabolites, such as indole glucosinolates, and positive regulation of flavonoid

biosynthesis. The potential importance of glucosinolates and flavonoids for CSFB was discussed previously in the introduction to this thesis. Interestingly, changes to anthocyanin quantity were previously associated with increased flea beetle resistance (Gruber et al., 2018). Gluosinolate composition may also be of importance for CSFB attraction. However, indole glucosinolates do not produce volatile metabolites. Therefore, the emission of CSFB-attractant isothiocyanates is unlikely to be altered (Gruber et al., 2009).

MYC2 modulates downstream genes that are also enriched here, such as S02_2g0006465, or jasmonate-zim-domain protein *JAZ6* (AT1G72450), which was enriched for response to wounding. *jaz6* knock-out (KO) mutants have robust resistance to caterpillars of cabbage looper (*Trichoplusia ni*) (Guo et al., 2018). Therefore, the increased expression of *MYC2* in the susceptible line may have a similar impact in increasing CSFB susceptibility. However, KO mutants of genes upregulated by *MYC2* increased susceptibility to beet armyworm (*Spodoptera exigua*) (Sun et al., 2020). Due it's both positive and negative control over genes involved in potential CSFB resistance it is challenging to pinpoint a specific mode of action for *MYC2*. Mutants of *MYC2* are readily available, and as CSFB feeds upon *Arabidopsis*, these mutants could be assessed for CSFB antixenosis.

Further enriched TFs for both wounding and chitin response are also involved in secondary metabolite biosynthesis. S02_2g0006164 is a homolog of WRKY40 (*AT1G80840*), which plays a role in glucosinolate and phenylpropanoid biosynthesis (Schweizer et al., 2013). Phenylpropanoids can be used to synthesise insecticidal metabolites such as flavonoids, which may also alter CSFB resistance (Gruber et al., 2018; Hallett et al., 2005). However, the specific metabolites altered by increased *WRKY40* expression is unknown. S09g0031779, homologous for *ZAT6* (AT5G04340), is a zinc finger TF and also an up-regulator of flavonoid biosynthesis (Shi et al., 2018).

Further TFs regulating both wounding and chitin responses include two *S. alba* copies (S03g0008435 and S09g0030514), homologous for *ZAT12* (AT5G59820), a C2H2-type zinc finger family protein. *ZAT12* is involved in broad stress responses including responses to salt, water, oxidative stress, high light, and heat stresses, (Davletova et al., 2005), chitin (Ramonell et al., 2005), and aphid infestation (Xu et al., 2021). Another C2H2-type zinc finger, S08g0026712 *ZAT10* (AT1G27730), was also enriched and induces expression of ROS-responsive genes (Mittler et al., 2006). Reactive oxygen species (ROS) production can be triggered by many stresses, including to insect herbivores (Kerchev et al., 2012). Also, *ZAT10* is up-regulated by herbivory by chewing insects (Jia et al., 2022; Lawrence et al., 2014). Expression of both *ZAT10* and *ZAT12* was found to increase oviposition success and herbivory by the specialist of *Brassicaeceae*, the large white (*Pieris brassicae*) (Little et al., 2007). Feeding by the generalist chewing herbivore, the Egyptian cotton leafworm *Spodoptera littoralis*, also increased expression of *ZAT10* and *ZAT12* (Schweizer et al., 2013). In addition, the leafworm larvae feeding on T-DNA mutants of *ZAT10* and

ZAT12 gained more weight than on the wildtype *Arabidopsis*, Col-0. Therefore increased expression of *ZAT10* and *ZAT12* could indicate resistance to the CSFB.

Several downstream genes involved in wounding response are not TFs, but leucine-rich repeat transmembrane protein kinase (LRR-RLK) proteins. LRR-RLKs are known to play critical roles in plant innate immunity by recognising pathogen associated molecular patterns (PAMPs), and endogenous damage associated molecular patterns (DAMPs) generated from pathogen infection or insect feeding respectively. S12g0041593, homologous for *LIK1* (AT3G14840), encodes an LRR-RLK which negatively regulates chitin-induced plant defence responses (Le et al., 2014), which may dampen the ability of line 86 to respond to chitin and potentially insects such as CSFB. However, *LIK1* also positively regulates jasmonic acid and ethylene signalling, and has found to be over-expressed when (*P. brassicae*) oviposit onto plants (Little et al., 2007). Therefore it is unclear if *LIK1* could actually increase CSFB resistance, which would juxtapose the susceptible phenotype seen in 86. Two additional LRR-RLKs are also enriched for wounding response. S02_1g0004715 or *GSO1* (AT4G20140), and S10_1g0033157 or *GSO2* ((AT5G44700; and homolog of *GSO1*) are two LRR-RLKs involved in epidermal patterning at the seedling stage, and knock-out (KO) mutants have thicker cotyledons (Tsuwamoto et al., 2008). This may explain the small significant difference seen in leaf thickness here, as line 86 was slightly thicker. KO mutants also have a defective cuticle and are abnormally permeable (Zhang et al., 2022a). Potential changes to cuticle patterning could change how CSFB perceive the palatability at the cotyledon surface.

It is surprising that so many genes previously implicated in insect defence pathways are up-regulated in the susceptible line 86. However, these genes can have a multitude of down-stream effects that can be antagonistic, such as the activity of *LIK1* down-regulating chitin responses while increases JA signalling. Although JA is generally seen as the primary phytohormone for controlling chewing herbivore defence, both SA and JA can influence chewing herbivore resistance, and their interaction seems to be specific to the herbivore (Ederli et al., 2020; Lortzing et al., 2019). Clearly, there is a network of interactions between pathways which are not well defined here. In addition, the specific physiological parameters determining CSFB preference are seemingly complex. Though some of the regulators over-expressed in line 86 can increase resistance against some insects, this may not extrapolate to CSFB. The preferences of specialist herbivores such as the CSFB can be in opposition to generalists, such as the attractant nature of isothiocyanates for CSFB which are repellent to generalists (Giamoustaris and Mithen, 1995; Pivnick et al., 1992). Potentially then, the downstream effects of these immune responses in line 86 are in fact attractive to CSFB, perhaps owing to its specialist nature. It would be interesting to see how generalist herbivores perform on these two lines of *S. alba*, as line 86 may be more resistant against generalists if it is really the case that the immune response in line 86 is greater as suggested by GO-enrichment analysis. An alternative hypothesis is that line 86 may be desensitized to CSFB due to the already-high expression of defence

pathways. A recurrent stimulus can reduce the intensity of defence responses, known as "familiarization" (Galviz et al., 2022). Potentially, line 86 is "familiarized" to wound and chitin responses, and therefore does not induce as strong a defensive response to CSFB as line 91.

All of the enriched chitin-responsive genes are involved in transcriptional regulation, but for wounding responses there are some genes involved in specific pathways. S11_1g0037801 is homologous for *LDOX* (AT4G22880), an enzyme directly involved in anthocyanin biosynthesis (Pelletier et al., 1997). S09g0030621 is homologous for *FAR1* (AT5G22500), which is an enzyme involved in generating C22:0 primary fatty alcohols for synthesis of waxes (Yang et al., 2022), and is hypothesised to be a main gene driving cuticular wax production (Kurschner, 2022). Waxes are one of the few metabolites to have been previously linked to Brassicaceae-specialist flea beetle resistance (Bodnaryk, 1992b; Bohinc et al., 2014; Isidoro et al., 1998). A dominance of C22 primary alcohols in epicuticular wax is characterised by waxes forming films on the cuticle, such as in lettuce (*Lactuca sativa*) (Bakker et al., 1998). Both *S. alba* lines were seen to have films in the previous chapter. Although no structural differences in epicuticular waxes were observed by cryo-SEM, the composition or quantity of the wax profile could still be altered as these were not assessed. Changes to *LDOX* may indicate a difference in the quantity of wax on the cuticle, which could be studied further through solvent washes of the leaf surface and subsequent gas-chromatography mass spectrometry (GC-MS) for identification, and flame ionisation detection for quantification.

Salicylic acid catabolic process

Salicylic acid (SA) is a key regulatory signal in plants which activates defence pathways in response to pathogens and sap-sucking insects (Zarate et al., 2007). Pathogen immune responses are typically seen as antagonistic to JA responses, which are correlated with chewing herbivore defences (Costarelli et al., 2020). Thus increased catabolism of SA may lead to lower expression of SA-mediated signalling pathways in *S. alba* line 91.

Two of the genes are suppressors of pathogen-mediated immune responses. S11_1g0037145, homologous for *DMR6* (AT5G24530), and S03g0010263, the partially redundant DMR6-LIKE OXYGENASE 1 *DLO1* (AT4G10500), both encode salicyclic acid (SA) oxygenases. When over-expressed they can increase disease susceptibility (Zeilmaker et al., 2015), and they are significantly up-regulated by treatment with SA (Budimir et al., 2021). The down-regulation of an important pathway mediating immune responses could indicate that SA-induced pathways could make line 86 more susceptible to CSFB, as activity of those pathways may be suppressed in line 91 due to SA catabolism. Alternatively, the SA pathways suppressed here may be involved in regulation of processes unrelated to CSFB antixenosis, such as growth and development which SA also mediates (Rivas-San Vicente and Plasencia, 2011).

The third SA-catabolism involved gene is S03g0009320, or *PAL1* (AT2G37040), which encodes a phenylalanine ammonia-lyase involved in lignin biosynthesis (Raes et al., 2003). Increased lignin composition can serve to fortify plant tissues and make them more challenging to consume by chewing insects (Santiago et al., 2013). Although lignin composition for these two lines was not measured, a lower lignin content may have contributed to the lodging of line 86 seen in the field trial. Only one study to date has evidence in lignin and flea beetle resistance. A crucifer flea beetle resistant transgenic line of *B. napus* had significantly reduced levels of lignin compared to the susceptible wild type. suggesting that lignin may reduce resistance, which is in opposition to what the results here may indicate (Gruber et al., 2018). However, the change in lignin between the lines found by Gruber et al. (2018) was slight, and lignin content was one of may traits to change due to the genetic modification. Therefore, it is not clear if lignin influences flea beetle resistance. It would be interesting to quantify the lignin content of the two *S. alba* lines identified here. The lignin content of the whole panel of 15 *S. alba* could also be quantified through techniques such as pyrolysis gas-chromatography mass-spectrometry (py-GC-MS) (Van Erven et al., 2017), and compared to CSFB feeding to see if there is a positive correlation between the two.

It does not seem likely that increased lignin could be the defining trait controlling the high levels of resistance generally seen in *S. alba* compared to *B. napus*, as there is not an obvious difference in leaf toughness between the species. However, differences in lignin content could be the causative reason behind the differences between the two contrasting *S. alba* lines, and could explain why the rate of feeding was consistently slower on line 91; it may have taken more time to chew through the potentially tougher leaf tissue.

Cellular response to redox state

Reactive oxygen species are key signalling molecules for stress response pathways, and have also been linked to insect defence pathways. For example, in tomato attack by the cotton bollworm induces accumulation of H_2O_2 (Peng et al., 2004). The gene *PGL3* (AT5G24400) is significantly up-regulated in line 91. KO mutants for *PGL3* had increased ROS accumulation (Ye et al., 2018) and increased pathogen resistance (Xiong et al., 2009), suggesting that the higher level of expression in line 91 would increase pathogen susceptibility. As pathogen susceptibility is considered antagonistic to herbivore defence, this may suggest increased insect resistance (Costarelli et al., 2020). An additional function is control of chlorophyll synthesis (Ye et al., 2018). Another enriched gene, S02_2g0007030 *SIGA* (AT1G64860) is also related to the chloroplast, and controls chloroplast biogenesis (Börner et al., 2015). Therefore the role of these two genes may be more relevant for chloroplast roles opposed to immune functions.

Selenium compound metabolic process

Although Selenium (Se) contributes to overall growth and development of plants under both stressed and non-stressed conditions (Fu et al., 2002; Hasanuzzaman and Fujita, 2011), the three Se-related genes that show higher up-regulation in the resistance line are involved specifically in Se sequestration. For S12g0041888 and S11_2g0038790, the closest homologs in *Brassica napus* var. Darmor and *Arabidopsis thaliana* are ATP sulfurylase 1 (*APS1*) and ATP sulfurylase 4 (*ASP4*) respectively. These two transporters have been shown to be important in *Brassica juncea* (brown mustard), a close relative of *S. alba*, for accumulation of Se (Pilon-Smits et al., 1999; Schiavon et al., 2015). For S10_2g0035240, the closest homolog in *B. napus* and *A. thaliana* is S-methyltransferase (*MMT*; AT5G49810), which when over-expressed increases Se accumulation in *B. jucea* and *A. thaliana* (LeDuc et al., 2004).

Sequestering of Se can be defensive against insect pests. Se can be toxic in high quantities, as it is chemically similar to sulphur, which leads to non-specific binding of Se instead of sulphur (Stadtman, 1990). High levels of Se in *B. juncea* provides defence against insects, including the caterpillars of the *Brassica*-specialist chewing insect, the cabbage white *Pieris rapae* (Hanson et al., 2003, 2004). Research showed that *B. juncea* leaves with 0.1 % dry-weight Se were repellent to caterpillars that tasted them, and lethal if the caterpillars fed for at least 2 days. Potentially then, the up-regulation of genes involved in Se metabolism in line 91 could be due to sequestering of Se for defence against CSFB. It would be relatively simple to test if Se accumulation is important for defence in *S. alba* against CSFB. Firstly, Se sequestration could be tested by increasing selenate levels in the growing media, and quantifying elemental Se such as in (Hanson et al., 2003). If line 91 contains more Se than line 86, it indicates that these DEGs are playing a role in Se sequestering. These high-Se and low-Se plants of line 91 could then be fed to CSFB and correlated to resistance. The effect of Se on antixenosis against CSFB could also be tested directly by coating cotyledons of the susceptible *S. alba* line 86, or *B. napus*, with Se and quantifying changes to feeding damage. This would minimise other physiological changes triggered by Se uptake into plant tissues. However it is interesting to note that if high levels of Se were the cause CSFB resistance, it may be expected that as *B. juncea* should also show resistance due to it's ability to hyper-accumulate Se. However, in previous studies *B. juncea* is as susceptible as *B. napus* to flea beetle feeding (Brandt and Lamb, 1993; Brown et al., 2004; Soroka and Grenkow, 2013). Furthermore, in field trials Se sequestering increased damage to broccoli (*Brassica oleracea* var italica) leaves by *Phyllotreta* flea beetles (Mechora et al., 2017). It therefore seems unlikely that Se sequestering is the causative CSFB defence mechanism of *S. alba* line 91.

6.6.3 Association of candidate genes with resistance

In total, 56 genes were present in the chrm11-QTL window 1 Mb either side of the peak. To select those genes with a higher likelihood of impacting the resistance phenotype, the genes in the chrm11-QTL window were assessed for predicted functional variation to the protein product using SnpEff, and/or expression from the RNA-seq experiment. Table 6.1 presents all of the genes that were identified in chrm11-QTL which also possess these additional evidence of a functional role. The genes of greatest interest are those that were both expressed in the RNA-seq experiment in at least one genotype, in addition to possessing a non-synonymous SNP from SnpEff which may therefore alter protein function (top of the table). It should be noted that SnpEff did not indicate if a SNP was predicted to be present in a promoter. Genes that did not show expression during the RNA-seq experiment, but do also have a non-synonymous prediction are also of interest; the single time-point used for RNA-seq would not have captured expression profiles which occurred earlier in development. Finally, those genes in the chrm11-QTL region with SNPs predicted to have minimal impact on the gene product, but possess differential gene expression, are also shown in the bottom of the table. These regions may still be of interest as they may be involved in regulatory elements. Note that only genes with more than 1 transcript in total for that line were considered as expressed, due to the potential for misalignments. No genes with over-represented GO terms were within chrm11-QTL.

26 genes (46.4 %) in the chrm11-QTL were studied further due to additional evidence from SnpEff and/or RNA-seq data to implicate a change in function between the lines. 16 genes (29 %) in the chrm11-QTL showed expression from the RNA-seq data, and 12 of these were predicted to possess SNPs which would cause a change to the protein product. 5 genes were DEGs with greater than 1.5 log2fold difference in expression between the two lines, and 2 of these DEGs were predicted to have non-synonymous SNPs. 4 genes lacked *Arabidopsis* homologs, and for 2 of these genes eggNOG failed to predict gene function. It is possible that susceptible species such as *Arabidopsis* and OSR lack the resistance genes within *S. alba*, so these particular genes may be important, though some other candidates have functions that make their role in CSFB resistance more promising.

S. alba Gene ID	Position (bp)	SNP Impact			Expression			Gene symbol	Gene description and AGI
		High	Mod.	Low	86	91	log2fold change		
Expressed genes within chrm11-QTL, non-synonymous									
S11_1g0035900	14968230	0	0	1	7	257	5.17	AT5G43010	Regulatory particle triple-A ATPase 4A (RPT4A)
S11_1g0035908	15583446	0	3	1	21	19	0.627	AT5G43020	Leucine-rich repeat protein kinase family protein
S11_1g0035919	15992903	0	0	2	239	186	-0.365	AT5G43130	TBP-associated factor 4 (TAF4)
S11_1g0035920	16013507	0	0	3	44	54	0.326	AT5G43140	Peroxisomal membrane 22 kDa (Mpv17/PMP22) family protein
S11_1g0035921	16043339	0	1	0	103	71	-0.577	AT5G43150	Elongation factor
S11_1g0035924	16060589	0	1	1	0	7	5.416	AT1G27730	Salt tolerance zinc finger (ZAT10)
S11_1g0035929	16162960	0	2	7	137	200	0.981	AT1G56145	Leucine-rich repeat transmembrane protein kinase (CORK1)
S11_1g0035931	16177528	0	0	3	47	48	0.574	AT5G43270	Squamosa promoter binding protein-like 2 (SPL2)
S11_1g0035932	16186202	0	4	5	113	119	0.385	AT5G43310	COP1-interacting protein-related
S11_1g0035933	16188778	0	1	0	234	348	0.834	AT5G43320	Casein kinase I-like 8 (CKL8)
S11_1g0035934	16200234	2	0	0	0	6	4.359	None	Belongs to the nucleosome assembly protein (NAP) family**
S11_1g0035950	16664299	0	1	1	165	121	-0.434	AT5G43420	RING/U-box superfamily protein (ATL16)
Non-expressed genes within chrm11-QTL, non-synonymous									
S11_1g0035904	15006008	1	0	2				AT2G31920	Cortical microtubule disordering 7 (CORD7)
S11_1g0035915	15915557	0	1	1				AT5G43110	Pumilio 14 (PUM14)
S11_1g0035916	15919964	0	0	1				AT3G59650	Mitochondrial ribosomal protein L51/S25/CI-B8 family protein
S11_1g0035918	15946389	0	1	0				AT5G43120	ARM-repeat/Tetratricopeptide repeat
S11_1g0035927	16110983	0	1	0				None	MULE transposase domain**
S11_1g0035935	16208885	0	1	0				None	NA
S11_1g0035936	16214311	0	1	0				None	NA
S11_1g0035938	16262836	0	0	3				AT5G43360	Phosphate transporter 1;3 (PHT1;3)
S11_1g0035939	16363193	0	0	5				AT5G43360	Phosphate transporter 1;3 (PHT1;3)
S11_1g0035951	16674589	0	1	0				AT5G40230	Nodulin MtN21-like transporter family protein (UMAMIT37)
Expressed genes within chrm11-QTL, synonymous									
S11_1g0035912	15676274				4236	3476	-0.307	AT5G43060	Granulin repeat cysteine protease family protein (RD21B)
S11_1g0035917	15926877				134	122	-0.133	AT5G43100	Eukaryotic aspartyl protease family protein
S11_1g0035928	16134418				0	18	6.75	AT5G43260	Chaperone protein dnaJ-related

**predicted from EGGNOG

Table 6.1: Combined data from bulked segregant analysis (BSA) and RNA-seq, highlighting potential gene candidates for resistance against cabbage stem flea beetle. All genes are located in the chrm11-QTL window from BSA. The number of non-synonymous SNPs related to the gene are shown with their putative impact on the protein product. Mean normalised expression for lines 86 and 91 from transcriptomics immediately prior to the start of feeding experiments is shown. log2fold change is relative to the resistant line 91. Genes lacking expression or non-synonymous SNPs have irrelevant data greyed out. For genes without an *Arabidopsis* homolog, the predicted function from EGGNOG using the *Sinapis alba* line 91 reference is shown (**). *Arabidopsis* Genome Initiative (AGI) gene code shown when available. *S. alba* gene names are arbitrary, and denote the chromosome number (S) and gene number ordered from the start of chromosome 1.

Genes with non-synonymous SNPs, expressed immediately prior to feeding

The genes with higher levels of expression in line 91 and that were located in the QTL region are orthologous to *Arabidopsis* genes involved in fairly broad functions. These genes could influence CSFB resistance in a large number of ways, and their potential resistance mechanisms cannot be described further here without additional evidence. They may indicate that the specific defence genes are not altered in protein structure to cause the resistance phenotype, but instead differential regulation between the lines could alter the resistance phenotype which are being controlled by the generalist genes highlighted above. Potentially then, closer focus on the RNA-seq data is warranted.

Four genes were both significant DEGs and predicted to have altered protein products. The salt tolerance zinc finger TF *ZAT10* (AT1G27730) was discussed above, as a copy on chromosome 8 was enriched for chitin and wounding GO terms in line 86. Another copy was flagged here on chromosome 11, S11_1g0035924. Due to the roles of *ZAT10* in insect defence, and it's presence in both the RNA-seq and BSA experiment, this gene is of clear interest to study further. Although susceptible *Brassicacae* such as *Arabidopsis* and *B. napus* also possess copies of *ZAT10*, the identity and regulation of downstream targets may be significantly altered in *S. alba* and modulate CSFB resistance.

The other three DEGs in this region control broad functions and may target many pathways. Regulatory particle triple-A ATPase 4A, *RPT4A*) is involved in targeted protein degradation, the eggNOG-predicted nucleosome assembly protein (NAP) alters DNA structure, and the chaperone protein dnaJ-related AT5G43260 regulates other proteins and is potentially involved in stress responses (Park and Luger, 2006; Pulido and Leister, 2018; Vierstra, 2009).

In addition to *ZAT10*, two additional TFs are localised to this region including S11_1g0035919, transcription binding protein-associated factor 4 *TAF4* (AT5G43130), and S11_1g0035931 squamosa promoter binding protein-like 2 *SPL2* (AT5G43270). These two TFs are not well characterised for relevance in insect defence, but their broad roles in transcriptional regulation means they may play a role in regulating important pathways. The predicted impact of SNPs on the structure of TFs may have implications for downstream regulation of many pathways. For *ZAT10* in particular, if the active site of the TF is altered then binding and regulation of transcription may be impacted, and therefore the downstream genes relating the defence could be impacted.

Within the list of the most interesting gene candidates, there are two leucine-rich repeat(LRR) –receptor-like protein(RLP), or LRR-RLPs. Like LRR-RLKs, LRR-RLPs are similarly implicated in a broad range of plant immunological processes, though they lack a cytoplasmic kinase domain. These are S11_1g0035908, homologous for AT5G43020, and S11_1g0035929, homologous for AT1G56145. The particular role of these two LRR-RLPs has not been investigated, but LRR-RLPs have previously been found to play critical roles in plant innate immunity by recognising endogenous Damage Associated

Molecular Patterns (DAMPs) generated from insect feeding. Schmelz et al. (2007) found that an amino-acid fragment, termed inceptin, was released from gut of the fall armyworm (*Spodoptera frugiperda*). The armyworm regurgitates stomach contents instead of using saliva, and inceptin was a fragment of plant chloroplastic adenosine triphosphate (ATP) synthase, i.e. a DAMP. Inceptin triggered induction of immune responses in cowpea (*Vigna unguiculata*), including increases in jasmonic acid, ethylene, salicylic acid, which are all plant hormones involved in stress responses, and synthesis of volatiles to attract parasotoid wasps of the armyworm. The inceptin receptor was a transmembrane LRR-RLP, which was also found to mediate immune responses to another chewing insect pest, the velvetbean caterpillar (*Anticarsia gemmatalis*) in Phaseolinae legumes (Sambade et al., 2014). The expression of these two genes is similar within the two genotypes, suggesting that the abundance of these LRR-RLPs is similar in the two lines. However, there are three moderate and 1 low impact SNPs for S11_1g0035908, and 2 moderate and 7 low impact SNPs for S11_1g0035908AT5G43020. The abundance of impactful SNPs implies significant modification of the LRR-RLP protein structure, and this could influence the activity significantly. For example, by modulating receptor binding.

Although potentially interesting candidates, there is questionable evidence for the role of the two LRR-RLPs identified here on CSFB resistance. Both LRR-RLPs are embedded within the chloroplast membrane, which is an unlikely location for DAMP binding unless the molecular patterns are first transported internally into the cell before detection. Also, previous results suggest that the resistance mechanism is not induced but is consititive, because palatability is not altered during the course of 48 hours of CSFB feeding. LRR-RLPs are in of themselves not the cause of resistance, but instead act on downstream pathways to induce immune responses in response to the herbivore. In addition, induced immune responses generally work through a cascade of reactive oxygen species (ROS). Several experiments were trialled here for ROS induction to various extracts of whole CSFB adults and their frass (faeces) in both *Sinapis alba* and *Brassica napus*, and though plants were responsive to the bacterial ellicitor flg-22, no induction could be generated to CSFB extracts.

Genes with non-synonymous SNPs, not expressed immediately prior to feeding

Cortical microtubule diordering 7, *CORD7* (AT2G31920) has a high-impact SNP; an insertion in the alternative genome (line 86) causes a frame-shift mutation, and two additional low-impact SNPs are also predicted to alter the structure of the protein product. Cortical microtubules act as a spatial template for cellulose deposition at the secondary cell wall which provides mechanical strength (Paredez et al., 2006). Altering the organisation of the microtubules can therefore impact cell wall strength (Santiago et al., 2013; Sasaki et al., 2017). Although *CORD7* had not been assessed for insect resistance, the role of the cell wall structure in insect defence is reviewed thoroughly by Santiago et al. (2013). Both the quantity and arrangement of fibres such as cellulose can reduce feeding by chewing

insect herbivores, potentially by reducing the effective cutting action of the mandibles. Microtubule genes have also been implicated in transcriptomic studies of insect defence. For example, in response to feeding by the cotton boll worm (Helicoverpa armigera), microtubule assembly genes were upregulated in a resistant wild relative of pigeonpea (*Cajanus platcarpus*) (Rathinam et al., 2020). Genes involved in modification of cell wall fibres (hemi-cellulose) were also up-regulated by flea beetle feeding (Gruber et al., 2012). Genes such as *CORD7* may increase physical resistance by altering the composition of the secondary cell wall. Microtubules can also play a role in trichome branching (Sambade et al., 2014), though no differences in trichome structure were found.

6.7 Chapter summary

Here, the genetic variation underlying CSFB resistance in *S. alba* was investigated by combining BSA and RNA-seq, which identified a single QTL linked to CSFB resistance on *S. alba* chromosome 11. Many genes in both experiments were flagged for potential roles in CSFB resistance, such as those involved in accumulation of indole glucosinolates, flavonoids, waxes, lignin, cellulose, and selenium, in addition to a host of other transcriptional regulators with broad roles that may also influence resistance.

These results should be seen as only the first step towards identification of resistance genes to CSFB. Currently, there is an absence of a connection between the identified genes and their proposed roles in CSFB antixenosis. Therefore, this thesis cannot determine which gene, if any of those identified here, may actually alter the level of CSFB resistance between these two lines of *S. alba*. However, using the evidence collected here two speculative hypotheses can be drawn.

Firstly, a single gene localised to chrm11-QTL may have a significant role in the biosynthesis of some CSFB-defensive trait. *LDOX* (anthocyanin biosynthesis) and *FAR1* (waxes biosynthesis) are potential candidates for susceptibility genes, but *CORD7* is the most promising resistance gene candidate; it can significantly alter the structure of the cell wall, and cotyledon thickness was significantly different between the two lines. Conversely, no significant differences were observed from the limited wax study, and anthocyanins also did not appear different from observation.

Secondly, there may be a gene in the chrm11-QTL region altering many pathways at once, such as a TF. As TFs generally act on whole pathways, observing significant changes to resistance pathways would support this hypothesis, and this was seen here from GO-term analysis where genes involved in chitin and wound-responsive pathways were enriched in the susceptible *S. alba* line 86. As previously discussed, the response of generalist and specialist herbivores (such as the CSFB) to defence traits can be contradictory. For example, the attractiveness of glucosinolates to CSFB (Bodnaryk, 1997; Carroll and Hoffman, 1980; Giamoustaris and Mithen, 1995; Pivnick et al., 1992).

Therefore, although enrichment for responses associated with defence in the susceptible line may appear contradictory, they may contribute to the susceptibility to CSFB specifically. Large changes to transcriptional programming may therefore control the resistance phenotype through many small additive effects on the phenotype. As herbivore resistance is a complex trait, it is likely that many pathways are involved in the resistance seen here. *ZAT10* is a TF of particular interest due to its well-recorded role in modulating defence against chewing insect herbivores, and because two copies were flagged as having significant differential expression in the RNA-seq experiment, and one copy was localised at the chrm11-QTL which was also predicted to have a protein product with significantly altered structure.

Before specific genes such as *ZAT10* are tested further for their connection to the resistance phenotype, it would be best to confirm and narrow down the list of candidate genes through further experimentation. Additional mapping may also identify additional minor QTL, which could well have been missed from the relatively noisy BSA results generated here. An F3 population generated from the 86x91 cross is being phenotyped to identify DNA markers linked to the QTL (i.e. QTL mapping) by collaborators Innolea. The results of this independent experiment will provide additional evidence to the importance of the chrm11-QTL, and may identify additional QTL.

If the chrm11-QTL region is also flagged during QTL-mapping, the size of the QTL could be more precisely identified using fine mapping. The methodology developed for screening the F2 population for resistance and subsequent BSA was successful in distinguishing the two parents. Therefore, the method may be useful for phenotyping resistance in additional populations. If so, the candidate QTL could be narrowed down to a smaller region. If the presence of a QTL is confirmed with subsequent replication, future work could focus on pinpointing the region through use of near-isogenic lines (NILs). NILs are generated by repeated back-crossing of progeny from the bi-parental population to the susceptible parent, with repeated phenotyping of the offspring to track the resistance trait. This technique accumulates many extra recombination events, and increases the resolution of a QTL. However, this approach would take a long time; for example, previous work has taken 10 years to clone a gene associated with a QTL (Fan et al., 2016). Alternatively, the F3 population could be used. If repeated work can validate which SNPs are associated with the a QTL, individuals could then be selected on the basis of possessing these SNPs by genotyping the F2 parents. This can be much faster to start with, and can later be combined with the back-crossing approach once the QTL interval is well defined. As only a few hundred plants would need to be screened, this experiment could feasibly use the the laboratory-reared population of CSFB to better control individuals and decrease the variance in the phenotyping due to variability in the insects.

After fine mapping, the remaining candidate genes with functions previously linked to insect resistance, such as *ZAT10*, could be studied further for a causative role. A targeted proteomic approach could be used for individuals of the F3 population possessing SNPs

in the candidate genes to quantify changes in expression, in addition to post-translational modifications of these gene products which was lacking from the transcriptomics approach taken here. Individuals with differential expression or gene products could be phenotyped for CSFB resistance, and the level of resistance could be correlated to the protein expression or structure to provide further evidence of the genes involvement.

The candidate gene could also be tested by altering expression through the use of precision genome editing techniques such as CRISPR/Cas9, though this would be much more challenging. No molecular tools currently exist for generating transgenic *S. alba*, and transformation of *S. alba* may be particularly demanding as it is a species highly resistant to both pests and pathogens, and so may not be amenable to *Agrobacterium*-mediated transformation. It would also be difficult to self transgenic plants to generate homozygous offspring for phenotyping, as *S. alba* is self-infertile. Instead, it may be worthwhile attempting transient expression to over-express the candidate gene temporarily and phenotype these same plants. Transient expression for *Brassica napus* has been demonstrated (Mooney and Graciet, 2020), so there there may be potential to use a similar technique for *S. alba*. However, it may be challenging to induce expression for long enough to maintain CSFB resistance. Alternatively, candidate gene mutants in other *Brassicaceae* such as *B. napus* or *Arabidopsis* could be phenotyped for changes in CSFB resistance, though these species may lack important downstream pathways.

After assessing which pathways are controlled by the gene candidate, the altered biochemistry of these plants could also be compared using techniques such as MS or microscopy. For example, synthesis of defensive compounds may be reduced, or patterning of cell walls may be altered.

An alternative approach is to compare the biochemical differences between lines 86 and 91 first. These techniques are much simpler in comparison to 'omic approaches. Although many metabolites were flagged for potential involvement, both lignin and cellulose seem of particular interest as they were identified in the BSA and RNA-seq experiments, in previous RNA-seq experiments with flea beetles (Gruber et al., 2018), and could also explain the slower feeding behaviour observed for line 91. The quantity of lignin could be measured by analytical pyrolysis, and the morphology of cellulose microfibrils could be compared using transmission electron microscopy. Wax composition would also certainly be of interest to study further as they only metabolite to have been well correlated to flea beetle resistance, and a key wax synthesis gene was up-regulated in line 86.

7. Thesis summary and conclusions

The main aim of this thesis was to advance the understanding of CSFB resistance mechanisms within *Brassicaceae*.

There was an aim to describe the dynamics of a captive CSFB colony. This was necessary both to have a constant supply of CSFB, and to know when these insects could be used in experimentation, as the behaviour of the insect varies considerably with age. This was a particularly challenging organism to rear due to its annual life cycle, including a period of aestivation. No literature for rearing the CSFB was previously available, so how the insects life cycle would respond to a laboratory environment was unknown, though it was expected that the aestivation period would not be altered. This study was able to define when the key life stages occurred, and demonstrated that CSFB feeding changes substantially over a period of 100 days. This research clearly illustrates the need to control cabbage stem flea beetle age for use in experimentation, which has not previously been noted or attempted.

It was hypothesised that altering rearing temperature could improve survival and fecundity. Rearing at a cool temperature did increase survival, reflecting previous results using wild-caught insects. An attempt was made to increase fecundity using heat treatments to better represent conditions in the wild, but impacts to fecundity were not replicated. The number of replicates was not sufficient for these experiments trialling rearing optimisation due to limited insect availability. At this time, the dynamics of the colony were not well understood and so it was more challenging to plan experiments and to produce enough insects. It would be possible to repeat this experiment now that the dynamics are now well described. However, although CSFB rearing can certainly be optimised further, future researchers should consider if the time investment is justified. The duration of fecundity experiments will be equal to the CSFB lifespan, which is approximately 12 months. As the method described here is sufficient to obtain CSFB for phenotyping experiments, the recommendation here is instead to focus on utilisation of these insects for identification of resistance genes.

The rearing methodology was successfully transferred to the collaborating company Innolea, and an independent colony has been utilised for phenotyping resistance. Therefore, this protocol can be successfully transferred to other laboratories and can facilitate other researchers undertaking experiments with the CSFB. As well as increasing the accuracy of antixenosis screens by controlling CSFB, using a reared colony and also opens the door to screening antibiosis.

In this project, experiments utilising CSFB preferentially sourced insects from the colony. This is because the age and health of these insects could be better controlled than for insects collected from the field, improving consistency between experiments. Use of the reared colony also allowed experiments with adult insects to be performed during the entire year. For the 15 different experiments performed using adult CSFB, 10 utilised

insects from the colony and five experiments utilised adults from the field. The first experiment conducted with CSFB in the laboratory used wild-caught adults to test the conditions required for rearing CSFB; a colony could only be established once a suitable rearing temperature was identified for these founding insects. An experiment for testing adult fecundity also used insects from the field, again because a colony had not yet been established. Specifically, this fecundity assay used individuals reared from larvae collected from the field. To clarify, an adult collection and larvae collection were undertaken to obtain a sufficient number of individuals to begin the colony, and also to stagger the age of the individuals to maintain it. The laboratory assay which provided insects a choice of two lines purposefully utilised insects from field to ensure that differences in resistance observed in the laboratory would be relevant to wild CSFB, and were not due to effects caused by sourcing the CSFB from a reared colony. The experiment comparing the preference of male and female CSFB towards either *S. alba* line 86 and 91 utilised field-caught adults as this was the last experiment conducted; the colony was no longer maintained and so adults from the field were used instead. At this point, it had been established that the behaviour of the colony was not significantly different to insects from the field in regards to these two lines. Finally, the experiment for bulked segregant analysis required many thousands of insects (> 2000 individuals), and it was not possible with the resources available to rear such a large number of insects; instead, they were sourced from the field.

The rearing method developed here facilitated experiments with the adult CSFB year-round. However, maintaining the colony required a significant input of both time and resources; this is especially true where only one individual is both performing experiments and colony rearing, as was the case here. For this reason, it is recommended that if work is performed in the future assessing a large population of Brassicaceae for antixenosis that the adults for this are collected from a infested field site during the harvest of WOSR (for convenience, from a seed store). This provides many thousands of adults which can be used for initial assays. The experiments to identify contrasting accessions took approximately 6 months to undertake here. This is because the availability of adults form the CSFB colony was initially low and so few individuals were obtained each week, which necessitated an incomplete block design over many weeks. This also does not account for time spent rearing prior to adult collection. Although greater variability is expected in wild-caught CSFB adults due to differences in factors such as their age and health, a wild population may still be successful in identification of significant contrasts when using a well designed experiment which can quantify tissue eaten. Potential contrasting lines can then be assessed further using laboratory CSFB cultures in smaller experiments. To conclude, laboratory rearing of the CSFB is viable and relatively simple in cases where a colony no greater than several hundred individuals is required. Beyond this, it is likely that assistance will be required to maintain the colony, or experiments such be planned for use of wild-caught adults.

There was also an aim to find intra-specific differences in CSFB resistance. A semi-automated pipeline was developed to improve the precision of phenotyping while not compromising throughput. This technique was successful in providing consistent results in repeated testing, which has previously been a problem in finding CSFB resistance. Although throughput was increased, the time taken to perform this protocol is still the limiting step in phenotyping a large number of accessions. Future research should therefore consider further automating this pipeline.

The phenotyping design itself could also be altered to improve throughput. A design which allowed for vertically-orientated seedlings was selected to minimise space requirements, and was successful in this endeavour. However, it may not be necessary to include so many seedlings per replicate. This was done here because the *S. alba* lines were not genetically fixed, but transplanting so many seedlings was quite time consuming. Ideally, individual seedlings would be grown in individual containers, then the most uniform would be selected and would already be assembled for experimentation. Interpolation of leaf boundaries is also not ideal as it is subjective. The best way to tackle this would be to have images of the seedlings both before and after feeding experiments. To increase consistency and throughput, the ideal screening method would use video recording and automated scoring to measure herbivory. This method would also score feeding rate which may be useful to identify induced resistance.

This project attempted to identify resistance within both *B. napus* and *S. alba*. It was expected that no resistant *B. napus* lines would be identified, but it was attempted as nuanced levels of resistance may have been missed in previous research, as these did not use controlled insects in combination with a high-precision experimental design. No *B. napus* accessions from the 49 assessed demonstrated contrasting levels of CSFB resistance. This result indicates that continuing investigations within *B. napus* is unlikely to identify resistance. Conversely, variable levels of resistance within *S. alba* were expected because contrasts had previously been identified in screens with another insect, and there is greater genetic diversity in this species. It was interesting that the majority of the *S. alba* accessions were highly resistant and only one was significantly more susceptible. This result suggests a breakdown in the typical resistance mechanisms within *S. alba*. No completely resistant varieties were identified, which supports previous findings.

This thesis also aimed to identify the resistance mechanism by characterising traits previously hypothesised to be involved in CSFB resistance. However, none of the resistance mechanisms explored appeared to be causative as they were the same between the susceptible and resistant lines. It was interesting that there were no differences in trichome density, as this is the best characterised flea beetle resistance mechanism. As *S. alba* is a hairy species, it was initially expected that the susceptible variety may be lacking in trichomes which would have explained the difference in resistance. Metabolites including waxes need to be studied further to understand their role fully. The correlation of waxes and glucosinolates to resistance was, but data was not received from collaborators

to complete this work. There are many additional mechanisms to explore, but further investigations were not performed here as during this stage of the project there were too many avenues. Instead, the focus was shifted to narrow down candidate mechanisms and genes for future research.

Monitoring of flea beetle behaviour suggested that the resistance mechanism was not volatile, but instead was detected upon feeding. It was also found that the resistance trait was not induced. Therefore, assessing changes to the transcriptome before and after feeding would likely not aid in identification of the resistance mechanisms or genes. In future research with other insects, monitoring the rate of insect feeding may be a simple method for understanding if resistance is induced before completing transcriptome work with damaged and undamaged plants, which is quite typical during work investigating genes involved in insect resistance.

For investigation of genetics, it was necessary to construct molecular resources for *S. alba*. This was successfully performed by Innolea, and the whole genome sequence and annotation created here could certainly be used for future research into CSFB resistance.

The RNAseq experiment identified gene candidates and pathways which could be characterised further in the two *S. alba* lines. The most enriched GO terms were for "DNA-binding transcription factor activity" and "sequence-specific DNA binding", also suggesting that broad changes in the metabolome of the two accessions could be responsible for CSFB resistance. A polygenic trait may explain by CSFB resistance has previously been so challenging to identify. However, many candidate genes were flagged and it is recommended that these are narrowed down before further investigations are performed into specific traits or genes.

The BSA experimental design identified a preliminary QTL linked to CSFB resistance. Further replication of the BSA experiment could lend further evidence to this region being linked to CSFB resistance, which is recommended before specific genes are studied. The phenotyping experiment could be used for additional mapping of CSFB resistance, or for mapping resistance using other insect and plant species. Although a QTL was identified, the methodology was limited in its resolving power and there may be other QTLs of interest. The BSA experiment was also was not able to define the size of the QTL. The QTL identified in this thesis contains many potential candidate genes associated with insect resistance. The logical next step is to repeat the BSA experiment to confirm the results, and to narrow down the list of candidate genes further (and therefore candidate mechanisms) using fine-mapping techniques.

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9. Supplementary

ImageJ scripts for quantification of leaf area eaten by CSFB. Four scripts are presented, and the data obtained can be either in units area (e.g. mm; with use of a scale and setting a scale in ImageJ) or as a percent of leaf area. Scripts require leaves to be scanned into a good quality flat-bed scanner with a white background.

−− > Manual cropping of individual seedlings is required first.

1. Images are thresholded to black and white, the required format for ImageJ to score damage.

−− > Manual interpolation of the leaf boundaries should be performed at this point.

2. Macro to quantify the area of holes eaten.

3. Macro to quantify the estimated area of the leaf before it was eaten.

// 1. THRESHOLDING

```
open()
outputDir = getDirectory("Choose a folder to save your images");
max=getNumber("How many images do you expect?", 5);
for(i=1; i<=max; i++){
name=getTitle();
run("8-bit");
run("Auto Threshold...", "method=Otsu ignore_black white");
run("Options...", "iterations=1 count=1 black");
run("Invert");
selectWindow(name);
saveAs("Tiff", outputDir + name+"-thresholded");
run("Open Next");
}
```

// 2. QUANTIFICATION OF AREA HOLES

```
open();
max = getNumber("How many images do you expect to analyse?", 5);
// Select how many images to analyse.
for(i=1; i<=max; i++){
// This allows the code to loop for as many images as were selected
window1 = getTitle();
// Gets the name of that image, to be able to save it later
selectWindow(window1);
run("Duplicate...", " ");
run("Options...", "iterations=1 count=1 black");
run("Fill Holes");
window2 = getTitle();
```

```
imageCalculator("Subtract create", window2, window1);
window3 = getTitle();
run("Analyze Particles...", "size=50-Infinity pixel
show=Overlay display clear include summarize");
window4 = getTitle();
selectWindow(window1);
run("Open Next");
close(window1);
close(window2);
close(window3);
close(window4);
```

// 3. QUANTIFICATION OF AREA LEAVES

```
open();
max = getNumber("How many images do you expect to analyse?", 5);
// Select how many images to analyse.
for(i=1; i<=max; i++){
// This allows the code to loop for as many images as were selected
window1 = getTitle();
// Gets the name of that image, to be able to save it later
selectWindow(window1);
run("Duplicate...", " ");
run("Invert");
run("Options...", "iterations=1 count=1 white");
run("Fill Holes");
window2 = getTitle();
run("Analyze Particles...", "size=50-Infinity pixel
show=Overlay display clear include summarize");
selectWindow(window1);
run("Open Next");
close(window1);
close(window2);
}
```

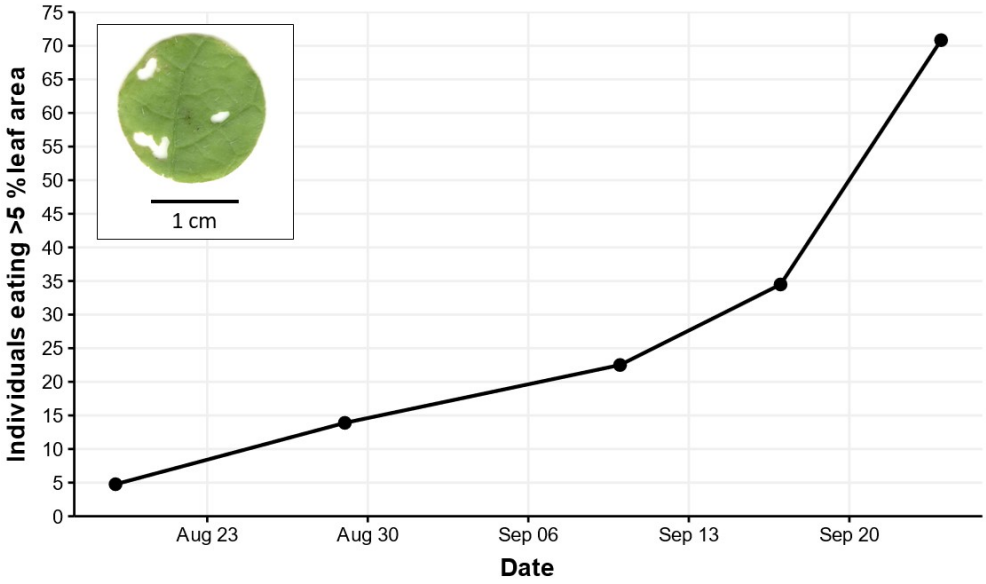

Figure 9.1: Percent of CSFB to have exited aestivation over time during maintenance in the laboratory, from 7 days post-collection from the wild in 2021 ($n > 30$). Adults were considered to have exited aestivation once more than 5 % leaf area of a leaf disc was eaten. Insect picture shows a leaf disc with 5 % damage, with a scale bar of 1 cm.

Line 91 *S. alba* Gene ID	Gene Symbol	Gene description and AGI
Biological Process		
Antibiotic catabolic process		
S03g0010189	AT4G09010	Ascorbate peroxidase 4 (TL29)
S03g0009320	AT2G37040	PHE ammonia lyase 1 (PAL9)
S03g0010263	AT4G10500	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (DLO1)
S07g0023084	AT1G19570	Dehydroascorbate reductase (DHAR1)
S08g0026416	AT1G19570	Dehydroascorbate reductase (DHAR1)
S11_1g0037145	AT5G24530	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein(DMR6)
Ascorbate glutathione cycle		
S08g0026416	AT1G19570	Dehydroascorbate reductase (DHAR1)
S07g0023084	AT1G19570	Dehydroascorbate reductase (DHAR1)
Salicylic acid catabolic process		
S03g0010263	AT4G10500	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein (DLO1)
S03g0009320	AT2G37040	PHE ammonia lyase 1 (PAL1)
S11_1g0037145	AT5G24530	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein(DMR6)
Selenium compound metabolic process		
S11_2g0038790	AT5G43780	Pseudouridine synthase/archaeosine transglycosylase-like family protein (APS4)
S10_2g0035240	AT5G49810	Methionine S-methyltransferase (MMT)
S12g0041888	AT3G22890	ATP sulfurylase 1 (APS1)
Cellular response to redox state		
S02_2g0007428	AT5G24400	NagB/RpiA/CoA transferase-like superfamily protein (EMB2024)
S02_2g0007030	AT1G64860	Sigma factor A (SIGA)
Regulation of rate of cell growth		
S12g0041991	AT2G04550	Indole-3-butyric acid response 5 (IBR5)
S12g0041990	AT2G04550	Indole-3-butyric acid response 5 (IBR5)
Negative regulation of organ growth		
S08g0026405	AT1G19270	Ubiquitin-activated peptidase (DA1)
S05_2g0017500	AT1G45180	RING/U-box superfamily protein (CTL05)
S11_1g0035866	AT5G42940	RING/U-box superfamily protein (CTL06)
Negative gravitropism		
S10_1g0033379	AT2G01940	C2H2-like zinc finger protein (SGR5)
S03g0008041	AT5G14090	Modifier of shoot system architecture (LAZY1)
S11_2g0039026	AT5G46860	Syntaxin/t-SNARE family protein (VAM3)
Drought recovery		
S03g0009320	AT2G37040	PHE ammonia lyase 1 (PAL1)
Sal91V1S01g0000918	AT1G70670	Caleosin-related family protein (CLO4)
S05_1g0014534	AT1G07260	UDP-glucosyl transferase 71C3 (UGT71C3)
S10_1g0034492	AT5G61420	Myb domain protein 28 (MYB28)
Molecular Function		
Sinapoyl spermidine:sinapoyl CoA N-acyltransferase activity		
S07g0023719	AT2G23510	Spermidine disinapoyl acyltransferase (SDT)
S07g0023720	AT2G23510	Spermidine disinapoyl acyltransferase (SDT)
Phosphoribulokinase activity		
S05_1g0016186	AT1G32060	Phosphoribulokinase (PRK)
S05_1g0016187	AT1G32060	Phosphoribulokinase (PRK)
Glutathione dehydrogenase (ascorbate) activity		
S07g0023084	AT1G19570	Dehydroascorbate reductase (DHAR1)
S08g0026416	AT1G19570	Dehydroascorbate reductase (DHAR1)

(Table continues on following page).

(Continued from previous page).

FK506 binding		
S11_1g0036846	AT5G48570	FKBP-type peptidyl-prolyl cis-trans isomerase family protein (ROF2)
S12g0041971	AT3G25220	FK506-binding protein 15 kD-1 (FKBP15-1)
S12g0041972	AT3G25230	FK506 binding protein (ROF1)
Glutathione binding		
S07g0023084	AT1G19570	Dehydroascorbate reductase (DHAR1)
S08g0026416	AT1G19570	Dehydroascorbate reductase (DHAR1)
S11_2g0039519	AT5G27380	Glutathione synthetase 2 (GSH2)
Salicylic acid glucosyltransferase (glucoside-forming) activity		
S03g0008958	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2)
S07g0025422	AT2G43840	UDP-glycosyltransferase 74 F1 (UGT74F1)
Benzoic acid glucosyltransferase activity		
S03g0008958	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2)
S07g0025422	AT2G43840	UDP-glycosyltransferase 74 F1 (UGT74F1)
Salicylic acid glucosyltransferase (ester-forming) activity		
S03g0008958	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2)
S07g0025422	AT2G43840	UDP-glycosyltransferase 74 F1 (UGT74F1)
Potassium ion binding		
S10_2g0035453	AT5G52920	Plastidic pyruvate kinase beta subunit 1 (PKP-BETA1)
Super-Scaf 100028g0043739	AT3G22960	Pyruvate kinase family protein (PKP-ALPHA)
Nicotinate-O-glucosyltransferase activity		
S03g0008958	AT2G43820	UDP-glucosyltransferase 74F2 (UGT74F2)
S07g0025422	AT2G43840	UDP-glycosyltransferase 74 F1 (UGT74F1)
Spermidine:sinapoyl CoA N-acyltransferase activity		
S07g0023719	AT2G23510	Spermidine disinapoyl acyltransferase (SFT)
Cellular Component		
R2TP complex		
S04g0011762	AT1G56440	Tetratricopeptide repeat (TPR)-like superfamily protein (TPR5)
Super-Scaf 1065g0044379	AT1G56440	Tetratricopeptide repeat (TPR)-like superfamily protein (TPR5)
ER body		
S05_2g0017855	AT1G54030	GDSL-like Lipase/Acylhydrolase superfamily protein (MVP1)
S05_2g0017856	AT1G54030	GDSL-like Lipase/Acylhydrolase superfamily protein (MVP1)
S08g0028773	AT1G54030	GDSL-like Lipase/Acylhydrolase superfamily protein (MVP1)

Table 9.1: List of enriched gene ontolog (GO) terms for *S. alba*, line 91 and the associated up-regulated genes. *Arabidopsis thaliana* homologs and gene descriptions are provided. Expression was assessed for 7 day old cotyledons with no damage, i.e. the plants were in the state it would be if it were to be presented to flea beetles in feeding experiments. AGI, *Arabidopsis* Genome Initiative gene code. *S. alba* gene names are arbitrary, and denote the chromosome (S) and gene number ordered from the start of chromosome 1.

Line 86 *S. alba* Gene ID	Gene symbol	Gene description and AGI
		Biological Process
Acceptance of pollen		
S01g0002495	AT3G10380	Exocyst complex component 8 (SEC8)
S05_1g0016702	AT1G47550	Exocyst complex component 3A (SEC3A)
S10_2g0034881	AT4G02350	Exocyst complex component 15B (SEC15B)
S10_2g0034882	AT4G02350	Exocyst complex component 15B (SEC15B)
S10_2g0034883	AT4G02350	Exocyst complex component 15B (SEC15B)
Response to chitin		
S02_1g0003978	AT3G46600	GRAS family transcription factor
S02_1g0005317	AT4G28140	Integrase-type DNA-binding superfamily protein (ERF54)
S02_2g0006164	AT1G80840	WRKY DNA-binding protein 40 (WRKY40)
S03g0008435	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S04g0010836	AT5G62020	Heat shock transcription factor B2A (HSFB2A)
S04g0012043	AT5G18610	Protein kinase superfamily protein (PBL27)
S07g0025158	AT2G38470	WRKY DNA-binding protein 33 (WRKY33)
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S08g0027272	AT4G30370	RING/U-box superfamily protein (IDF1)
S08g0028078	AT1G32640	Basic helix-loop-helix (bHLH) DNA-binding family protein (MYC2)
S09g0029839	AT5G51190	Integrase-type DNA-binding superfamily protein (ERF105)
S09g0030514	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S09g0031779	AT5G04340	Zinc finger of *Arabidopsis thaliana* 6 (ZAT6)
S11_1g0036980	AT5G27420	Carbon/nitrogen insensitive 1 (CNI1)
S12g0041089	AT3G19380	Plant U-box 25 (PUB25)
S12g0041540	AT3G50060	Myb domain protein 77 (MYB77)
Super-Scaf_100028g0043644	AT3G50260	Cooperatively regulated by ethylene and jasmonate 1 (CEJ1)
Super-Scaf 100028g0043708	AT3G15210	Ethylene responsive element binding factor 4 (ERF4)
Response to chlorate		
S11_2g0039237	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039238	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039239	AT2G04030	Chaperone protein htpG family protein (CR88)
Positive regulation of gibberellin biosynthetic process		
S05_1g0014547	AT1G07430	Highly ABA-induced PP2C gene 2 (HAI2)
S07g0024626	AT3G63010	Alpha/beta-Hydrolases superfamily protein (GID1B)
S11_1g0036968	AT5G27320	Alpha/beta-Hydrolases superfamily protein (GID1C)
S12g0041418	None	CDPK-related kinase**
Response to wounding		
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S02_2g0006164	AT1G80840	WRKY DNA-binding protein 40 (WRKY40)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S02_2g0006465	AT1G72450	Jasmonate-zim-domain protein 6 (JAZ6)
S03g0008435	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S06g0019304	AT1G76650	Calmodulin-like 38 (CML38)
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S08g0027087	AT4G24220	NAD(P)-binding Rossmann-fold superfamily protein (VEP1)
S08g0027893	AT4G14550	Indole-3-acetic acid inducible 14 (IAA14)
S08g0028078	AT1G32640	Basic helix-loop-helix (bHLH) DNA-binding family protein (MYC2)
S09g0030514	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S09g0030621	AT5G22500	Fatty acid reductase 1 (FAR1)
S09g0031779	AT5G04340	Zinc finger of *Arabidopsis thaliana* 6 (ZAT6)
S10_1g0033153	AT5G48620	CC-NBS-LRR class disease resistance protein
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
S10_2g0035483	AT5G22500	Fatty acid reductase 1 (FAR1)
S11_1g0036752	AT3G29035	NAC domain containing protein 3 (NAC3)
S11_1g0037180	AT5G24090	Chitinase A (CHIA)
S11_1g0037801	AT4G22880	Leucoanthocyanidin dioxygenase (LDOX)

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S12g0040765	AT1G61340	F-box family protein (FBS1)
S12g0041593	AT3G14840	Leucine-rich repeat transmembrane protein kinase (LIK1)
Positive regulation of response to water deprivation		
S01g0000264	AT3G05500	Rubber elongation factor protein (LDAP3)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S07g0025495	AT2G42620	RNI-like superfamily protein (MAX2)
S09g0029827	AT5G51070	Clp ATPase (ERD1)
S12g0042782	AT3G13672	TRAF-like superfamily protein (SINA2)
Establishment of protein localization		
S01g0002495	AT3G10380	Subunit of exocyst complex 8 (SEC8)
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S02_2g0006460	AT1G72560	Karyopherin, specifically the Arabidopsis ortholog of LOS1/XPOT (PSD)
S02_2g0007023	None	Proteasome assembly**
S05_1g0015979	AT1G29310	SecY protein transport family protein (Sec. 61-alpha1)
S05_1g0016780	AT1G48650	DEA(D/H)-box RNA helicase family protein
S05_2g0016872	AT1G27970	Nuclear transport factor 2B (NTF2B)
S05_2g0017056	AT1G32130	Transcription elongation factor (TFIIS) family protein (IWS1)
S05_2g0017073	AT5G46860	Syntaxin/t-SNARE family protein (VAM3)
S07g0023086	AT1G19510	RAD-like 5 (RL5)
S07g0023767	AT2G33240	Myosin-like protein (XID)
S07g0025301	AT2G45660	AGAMOUS-like 20 (AGL20)
S09g0029952	AT5G52580	RabGAP/TBC domain-containing protein
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
S11_2g0039237	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039238	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039239	AT2G04030	Chaperone protein htpG family protein (CR88)
S12g0041047	AT3G20000	Translocase of the outer mitochondrial membrane 40 (TOM40)
Super-Scaf_100028g0043331	AT3G20920	Translocation protein-related (ATSEC62)
Regulation of endodermal cell differentiation		
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
Raffinose family oligosaccharide biosynthetic process		
S07g0024626	AT3G63010	Alpha/beta-Hydrolases superfamily protein (GID1B)
S11_1g0036968	AT5G27320	Alpha/beta-Hydrolases superfamily protein (GID1C)
Regulation of gastrulation		
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
De-etiolation		
S02_2g0006616	AT1G69930	Glutathione S-transferase TAU 11 (GSTU11)
S11_2g0039237	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039238	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039239	AT2G04030	Chaperone protein htpG family protein (CR88)
Response to cold		
S02_1g0004010	AT3G47470	Light-harvesting chlorophyll-protein complex I subunit A4 (LHCA4)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S02_2g0007015	AT1G64990	GPCR-type G protein 1 (GTG1)
S03g0008190	AT5G17690	Like heterochromatin protein (LHP1)
S03g0008435	AT5G59820	C2H2-type zinc finger family protein (TFL2)
S03g0008680	AT5G54770	Thiazole biosynthetic enzyme, chloroplast (THI1)
S04g0013495	AT2G36530	Enolase (LOS2)
S05_1g0015577	AT1G21910	Integrase-type DNA-binding superfamily protein (DREB26)
S05_1g0015841	AT1G24620	EF hand calcium-binding protein family (CML25)
S05_1g0015867	AT1G27320	Histidine kinase 3 (HK3)
S05_2g0017047	AT1G32060	Phosphoribulokinase (PRK)
S06g0018099	AT1G01120	3-ketoacyl-CoA synthase 1 (KCS1)
S07g0025158	AT2G38470	WRKY DNA-binding protein 33 (WRKY33)

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S07g0025301	AT2G45660	AGAMOUS-like 20 (AGL20)
S08g0025863	AT1G05700	Leucine-rich repeat transmembrane protein kinase protein
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S09g0029266	AT4G04020	Fibrillin (FIB)
S09g0029368	AT4G11880	Fibrilin precursor protein (AGL14)
S09g0030514	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S09g0031779	AT5G04340	Zinc finger of Arabidopsis thaliana 6 (ZAT6)
S10_1g0032386	AT5G57380	Fibronectin type III domain-containing protein (VIN3)
S10_1g0032395	AT5G57560	Xyloglucan endotransglucosylase/hydrolase family protein (TCH4)
S11_1g0036599	AT3G26744	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein (ICE1)
S11_1g0036673	AT3G27690	Light-harvesting chlorophyll a/b-binding (LHC) protein (DEG13)
S11_1g0037180	AT5G24090	Chitinase A (CHIA)
S11_1g0037929	AT4G24960	HVA22 homologue D (HVA22D)
Super-Scaf_100028g0043517	AT3G17390	S-adenosylmethionine synthetase family protein (MTO3)
Super-Scaf 100028g0043644	AT3G50260	Cooperatively regulated by ethylene and jasmonate 1 (CEJ1)
Response to water deprivation		
S01g0000264	AT3G05500	Rubber elongation factor protein (LDAP3)
S01g0001607	AT1G15690	Inorganic H pyrophosphatase family protein (AVP1)
S02_1g0005317	AT4G28140	Integrase-type DNA-binding superfamily protein (ERF54)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S02_2g0006616	AT1G69930	Glutathione S-transferase TAU 11 (GSTU11)
S05_1g0014214	AT1G02820	Late embryogenesis abundant 3 (LEA3) family protein (LEA3)
S05_1g0015642	AT1G22810	Integrase-type DNA-binding superfamily protein (ATERF019)
S05_1g0015867	AT1G27320	Histidine kinase 3 (HK3)
S05_2g0017913	AT1G52890	NAC domain containing protein 19 (NAC019)
S06g0018359	AT1G08920	ERD (early response to dehydration) six-like 1 (ESL1)
S06g0020298	AT3G15500	NAC domain containing protein 3 (NAC3)
S07g0023909	AT3G63060	EID1-like 3 (EDL3)
S07g0024087	AT3G54820	Plasma membrane intrinsic protein 2;5 (PIP2;5)
S07g0025158	AT2G38470	WRKY DNA-binding protein 33 (WRKY33)
S07g0025495	AT2G42620	RNI-like superfamily protein (MAX2)
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S08g0027893	AT4G14550	Indole-3-acetic acid inducible 14 (IAA14)
S08g0028078	AT1G32640	Basic helix-loop-helix (bHLH) DNA-binding family protein (MYC2)
S09g0029266	AT4G04020	Fibrillin (FIB)
S09g0029827	AT5G51070	Clp ATPase (ERD1)
S09g0031779	AT5G04340	Zinc finger of Arabidopsis thaliana 6 (ZAT6)
S10_1g0034375	AT5G26340	Major facilitator superfamily protein (MSS1)
S11_1g0036673	AT3G27690	Light-harvesting chlorophyll a/b-binding (LHC) protein (DEG13)
S11_1g0037180	AT5G24090	Chitinase A (CHIA)
S11_1g0037929	AT4G24960	HVA22 homologue D (HVA22D)
S11_2g0039237	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039238	AT2G04030	Chaperone protein htpG family protein (CR88)
S11_2g0039239	AT2G04030	Chaperone protein htpG family protein (CR88)
S12g0041093	AT3G19290	ABA-responsive element binding protein 2 (ABF4)
S12g0041540	AT3G50060	Myb domain protein 77 (MYB77)
S12g0042782	AT3G13672	TRAF-like superfamily protein (SINA2)
Regulation of cell fate specification		
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
S11_1g0037630	AT4G18170	WRKY DNA-binding protein 28 (WRKY28)
Plant epidermis development		
S02_1g0004715	AT4G20140	Leucine-rich repeat transmembrane protein kinase (GSO1)
S03g0007775	AT5G07870	HXXXD-type acyl-transferase family protein
S03g0008559	AT5G57320	Villin, putative (VLN5)
S05_1g0015841	AT1G24620	EF hand calcium-binding protein family (CML25)

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S08g0027966	AT5G55480	SHV3-like 1 (SLV1)
S10_1g0033157	AT5G44700	Leucine-rich repeat transmembrane protein kinase (GSO2)
S11_1g0036143	AT5G45420	Membrane anchored MYB (maMYB)
S11_1g0036599	AT3G26744	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein (ICE1)
Regulation of dendrite development		
S06g0020800	AT3G23310	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase (NDR3)
S07g0023767	AT2G33240	Myosin-like protein (XID)
S12g0040873	AT3G23310	AGC (cAMP-dependent, cGMP-dependent and protein kinase C) kinase (NDR3)
Regulation of autophagosome assembly		
S04g0013798	AT2G41980	Protein with RING/U-box and TRAF-like domains (SINAT1)
S12g0042782	AT3G13672	TRAF-like superfamily protein (SINA2)
	Molecular function	
Sequence-specific DNA binding		
S01g0001119	AT1G66140	Zinc finger protein 4 (ZFP4)
S01g0002421	AT3G11580	AP2/B3-like transcriptional factor family protein (NGAL2)
S02_1g0003977	AT3G46580	Methyl-CPG-binding domain protein 5 (MBD5)
S02_1g0003978	AT3G46600	GRAS family transcription factor
S02_1g0005317	AT4G28140	Integrase-type DNA-binding superfamily protein (ERF54)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S03g0008023	AT5G13820	Telomeric DNA binding protein 1 (TBP1)
S03g0008190	AT5G17690	Like heterochromatin protein (TLF2)
S03g0008435	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S03g0008582	AT5G56860	GATA type zinc finger transcription factor family protein (GNC)
S04g0013487	AT2G36400	Growth-regulating factor 3 (GRF3)
S05_1g0015577	AT1G21910	Integrase-type DNA-binding superfamily protein (DREB26)
S05_1g0016780	AT1G48650	DEA(D/H)-box RNA helicase family protein
S05_2g0017057	AT1G32150	Basic region/leucine zipper transcription factor 68 (bZIP68)
S05_2g0017403	AT1G43700	VIRE2-interacting protein 1 (VIP1)
S07g0023492	AT5G66940	Dof-type zinc finger DNA-binding family protein (ATDOF5.8)
S07g0025158	AT2G38470	WRKY DNA-binding protein 33 (WRKY33)
S08g0026423	AT1G19700	BEL1-like homeodomain 10 (BEL10)
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S08g0028078	AT1G32640	Basic helix-loop-helix (bHLH) DNA-binding family protein (MYC2)
S09g0028958	AT4G00990	Transcription factor jumonji (jmjC) domain-containing protein (JMJ27)
S09g0029194	AT4G05100	Myb domain protein 74 (MYB74)
S09g0030514	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S09g0031779	AT5G04340	Zinc finger of Arabidopsis thaliana 6 (ZAT6)
S10_1g0032451	AT1G26370	RNA helicase family protein (RID1)
S10_1g0034289	AT5G49450	Basic leucine-zipper 1 (bZIP1)
S11_1g0036475	AT2G03340	WRKY DNA-binding protein 3 (WRKY3)
S11_1g0036615	AT3G27010	Teosinte branched 1, cycloidea, PCF (TCP)-domain family protein 20
S11_1g0036752	AT3G29035	NAC domain containing protein 3 (NAC3)
S11_1g0036944	AT5G28300	Duplicated homeodomain-like superfamily protein (GT2L)
S11_1g0037630	AT4G18170	WRKY DNA-binding protein 28 (WRKY28)
S11_2g0039377	AT3G27550	Mitochondrial protein involved in RNA splicing (CFM9)
S11_2g0039479	AT5G28770	bZIP transcription factor family protein (BZO2H3)
S12g0041093	AT3G19290	ABA-responsive element binding protein 2
S12g0041540	AT3G50060	Myb domain protein 77(MYB77)
Super-Scaf_100028g0043642	AT3G50240	Kinesin-related protein
Super-Scaf_100028g0043643	AT3G50240	Kinesin-related protein
Super-Scaf_100028g0043663	AT3G50650	GRAS family transcription factor
Super-Scaf_100028g0043708	AT3G15210	Ethylene responsive element binding factor 4 (ERF4)
Gibberellin binding		
S07g0024626	AT3G63010	Alpha/beta-Hydrolases superfamily protein (GID1B)
S11_1g0036968	AT5G27320	Alpha/beta-Hydrolases superfamily protein (GID1C)
DNA-binding transcription factor activity		

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S01g0000969	AT1G69570	Dof-type zinc finger DNA-binding family protein (CDF5)
S01g0001119	AT1G66140	Zinc finger protein 4 (ZFP4)
S01g0001366	AT1G69490	NAC-like, activated by AP3/PI (NAP)
S01g0002421	AT3G11580	AP2/B3-like transcriptional factor family protein (NGAL2)
S02_1g0003941	AT3G45260	BALDIBIS member of the BIRD family of zinc finger proteins
S02_1g0003978	AT3G46600	GRAS family transcription factor
S02_1g0005317	AT4G28140	Integrase-type DNA-binding superfamily protein (ERF54)
S02_2g0006164	AT1G80840	WRKY DNA-binding protein 40 (WRKY40)
S02_2g0006249	AT1G77450	NAC domain containing protein 32 (NAC032)
S03g0008435	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S03g0008582	AT5G56860	GATA type zinc finger transcription factor family protein (GNC)
S03g0008845	AT2G46530	Auxin response factor 11 (ARF11)
S03g0009694	AT5G50670	Squamosa promoter binding protein like 13, involved in floral transition
S04g0010732	AT5G64750	Integrase-type DNA-binding superfamily protein (ABR1)
S04g0010836	AT5G62020	Heat shock transcription factor B2A (HSFB2A)
S04g0010954	AT5G25150	TBP-associated factor 5 (TAF5)
S04g0013139	AT2G30470	High-level expression of sugar-inducible gene 2 (HSI2)
S04g0014071	AT2G47520	Integrase-type DNA-binding superfamily protein (ERF71)
S05_1g0015577	AT1G21910	Integrase-type DNA-binding superfamily protein (DREB26)
S05_1g0015642	AT1G22810	Integrase-type DNA-binding superfamily protein (ATERF019)
S05_2g0017013	AT1G30210	TEOSINTE BRANCHED 1, cycloidea, and PCF family 24 (TCP24)
S05_2g0017057	AT1G32150	Basic region/leucine zipper transcription factor 68 (bZIP68)
S05_2g0017403	AT1G43700	VIRE2-interacting protein 1 (VIP1)
S05_2g0017516	AT1G46264	Heat shock transcription factor B4 (HSFB4)
S05_2g0017913	AT1G52890	NAC domain containing protein 19 (NAC019)
S06g0020126	AT3G18010	WUSCHEL related homeobox 1 (WOX1)
S06g0020298	AT3G15500	NAC domain containing protein 3 (NAC3)
S07g0021809	AT1G51950	Indole-3-acetic acid inducible 18 (IAA18)
S07g0023093	AT1G19350	Brassinosteroid signalling positive regulator (BZR1) family protein (BES1)
S07g0023492	AT5G66940	Dof-type zinc finger DNA-binding family protein (ATDOF5.8)
S07g0025158	AT2G38470	WRKY DNA-binding protein 33 (WRKY33)
S07g0025301	AT2G45660	AGAMOUS-like 20 (AGL20)
S08g0026423	AT1G19700	BEL1-like homeodomain 10 (BEL10)
S08g0026648	AT1G25440	B-box type zinc finger protein with CCT domain (BBX15)
S08g0026712	AT1G27730	Salt tolerance zinc finger (ZAT10)
S08g0027782	AT4G16610	C2H2-like zinc finger protein
S08g0027893	AT4G14550	Indole-3-acetic acid inducible 14 (IAA14)
S08g0028078	AT1G32640	Basic helix-loop-helix (bHLH) DNA-binding family protein (MYC2)
S08g0028732	AT1G53170	Ethylene response factor 8 (ERF8)
S09g0029194	AT4G05100	Myb domain protein 74 (MYC74)
S09g0029368	AT4G11880	Agamous-like 14
S09g0029839	AT5G51190	Integrase-type DNA-binding superfamily protein (ERF105)
S09g0030480	AT5G59340	WUSCHEL related homeobox 2 (WOX2)
S09g0030514	AT5G59820	C2H2-type zinc finger family protein (RHL41)
S09g0031350	AT5G15850	CONSTANS-like 1 (COL1)
S09g0031779	AT5G04340	Zinc finger of Arabidopsis thaliana 6 (ZAT6)
S10_1g0034289	AT5G49450	Basic leucine-zipper 1 (bZIP1)
S10_1g0034388	AT5G25810	Integrase-type DNA-binding superfamily protein (TNY)
S10_2g0034865	AT4G01550	NAC domain containing protein 69 (NAC069)
S11_1g0036027	AT5G44160	Indeterminate domain 8, member of the BIRD transcriptional regulators
S11_1g0036475	AT2G03340	WRKY DNA-binding protein 3 (WRKY3)
S11_1g0036599	AT3G26744	Basic helix-loop-helix (bHLH) DNA-binding superfamily protein (ICE1)
S11_1g0036615	AT3G27010	Teosinte branched 1, cycloidea, PCF (TCP)-domain family protein 20
S11_1g0036752	AT3G29035	NAC domain containing protein 3 (NAC3)
S11_1g0036944	AT5G28300	Duplicated homeodomain-like superfamily protein (GT2L)
S11_1g0037320	AT5G61600	Ethylene response factor 104 (ERF104)
S11_1g0037630	AT4G18170	WRKY DNA-binding protein 28 (WRKY28)

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S11_2g0039377	AT3G27550	Mitochondrial protein involved in RNA splicing (CFM9)
S11_2g0039479	AT5G28770	bZIP transcription factor family protein (BZO2H3)
S12g0041090	AT3G19360	Zinc finger (CCCH-type) family protein (ATC3H39)
S12g0041093	AT3G19290	ABA responsive element binding protein 2
S12g0041540	AT3G50060	Myb domain protein 77 (MYB77)
Super-Scaf_100028g0043644	AT3G50260	Cooperatively regulated by ethylene and jasmonate 1 (CEJ1)
Super-Scaf_100028g0043663	AT3G50650	GRAS family transcription factor
Super-Scaf_100028g0043708	AT3G15210	Ethylene responsive element binding factor 4 (ERF4)
Xyloglucan:xyloglucosyl transferase activity		
S02_1g0005482	AT5G48070	Xyloglucan endotransglucosylase/hydrolase 20 (XTH20)
S10_1g0032395	AT5G57560	Xyloglucan endotransglucosylase/hydrolase family protein (TCH4)
S12g0042026	AT2G06850	Xyloglucan endotransglucosylase/hydrolase 4 (XTH20)
Glutathione binding		
S01g0002022	AT2G29450	Glutathione S-transferase tau 5 (GSTU5)
S07g0023193	AT1G17170	Glutathione S-transferase TAU 24 (GSTU24)
S11_1g0036975	AT5G27380	Glutathione synthetase 2 (GSH2)
High-affinity oligopeptide transmembrane transporter activity		
S10_1g0033383	AT2G02020	Major facilitator superfamily protein (NPF8.4)
S10_1g0034076	AT1G62200	Major facilitator superfamily protein (NPF8.5)
	Cellular component	
Extracellular exosome		
S05_1g0016702	AT1G47550	Exocyst complex component sec3A (SEC3A)
S05_2g0017480	AT1G44820	Peptidase M20/M25/M40 family protein
S10_2g0034881	AT4G02350	Exocyst complex component SEC15B
S10_2g0034882	AT4G02350	Exocyst complex component SEC15B
S10_2g0034883	AT4G02350	Exocyst complex component SEC15B
S01g0002495	AT3G10380	Subunit of exocyst complex 8 (SEC8)
Exocyst		
S05_1g0016702	AT1G47550	Exocyst complex component sec3A (SEC3A)
S10_2g0034881	AT4G02350	Exocyst complex component SEC15B
S10_2g0034882	AT4G02350	Exocyst complex component SEC15B
S10_2g0034883	AT4G02350	Exocyst complex component SEC15B
S01g0002495	AT3G10380	Subunit of exocyst complex 8 (SEC8)

**predicted from eggNOG

Table 9.2: Over-represented GO terms for *S. alba* line 86 and the associated up-regulated genes with *Arabidopsis thaliana* homologs and functions. (**) Where no *Arabidopsis* homolog is known, the predicted function is shown from using eggNOG. *S. alba* gene names are arbitrary, and denote the chromosome (S) and gene number ordered from the start of chromosome 1.