Genetics of the interaction between rapeseed and the cabbage stem flea beetle (*Psylloides chrysocephala*)

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Abstract

The cabbage stem flea beetle (CSFB), *Psylliodes chrysocephala*, is a major pest of *Brassicaceae*, in particular, winter oilseed rape (WOSR), *Brassica napus*. After the 2013 EU-wide moratorium on the use of neonicotinoids, CSFB numbers dramatically rose in the United Kingdom (UK). In 2015, resistance to pyrethroids was confirmed in Germany, Denmark and the UK. A near complete ban of the use of neonicotinoids across the EU was declared in April 2018. As a result, farmers have been left with no viable control for CSFB and are turning away from growing WOSR in the future. WOSR is one of the UKs most profitable crops and a reduction in the area grown would be detrimental to the economy and UK farming.

Previous research attempts have been made to reveal resistance and tolerance traits to flea beetles in general, however few have focused specifically on CSFB adults. A large gap in knowledge of the genetics underlying phenotypic responses to CSFB is preventing advancements in development of new resistant/tolerant varieties of WOSR.

Herein this thesis presents:

- Six-way choice chambers identifying variation in adult CSFB feeding damage,
- Two-way and non-choice experiments confirm results from six-way choice chambers,
- Three-way and non-choice experiments of a more resistant *B. napus* variety, a more susceptible variety and F1 cross of these parental lines,
- Field trial data confirms adult feeding preferences seen in lab are retained in the field,
- Single Nucleotide Polymorphisms (SNPs) and Gene Expression Marker (GEM) outputs from an Associative Transcriptomics pipeline for CSFB herbivory data,
- Arabidopsis mutant feeding assays further investigating a candidate gene.

Access Condition and Agreement

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Abbreviations

AHDB – Agricultural and Horticultural Developmental Board

BnaDFFS – *Brassica napus* Diversity Fixed Foundation Set

BPM – Brassica Pod Midge

CSFB – Cabbage Stem Flea Beetle

CSW – Cabbage Stem Weevil

DEFRA - Department for Environment, Food and Rural Affairs

EPF - Entomopathogenic Fungi

EPN - Entomopathogenic Nematodes

GFS – Grey Field Slug

GSS - Glucosinolate Sulfatases

IPM – Implemented Pest Management

JIC – John Innes Centre

kdr – Knock Down Resistance

LLS – Light Leaf Spot

OREGIN – Oilseed RapE Genetic Improvement Network

PCR – Polymerase Chain Reaction

RIPR – Renewable Industrial Products from oilseed Rape

RNAi – RNA interference

RSW – Rape Stem Weevil

SOSR – Spring Oilseed Rape

WOSR – Winter Oilseed Rape

Chapter 1 – Introduction

1.1 Origins of *Brassica napus*

Oilseed rape (*Brassica napus*) is an agricultural crop species that is part of the *Brassicaceae* family and results from a recent hybridisation event (approximately 7,500 years ago) between two diploid parents, *Brassica rapa* (turnips and mustards) and *Brassica oleracea* (vegetable brassicas such as broccoli, cabbage and cauliflower) (Chalhoub et al., 2014). In this hybridisation event, *B. napus* was formed with 10 chromosomes from *B. rapa* (A genome) and 9 from *B. oleracea* (C genome), followed by chromosome doubling, resulting in a 19 chromosome allopolyploid (AACC) (Chalhoub et al., 2014). *Arabidopsis thaliana*, also a member of the *Brassicaceae*, and Brassica are believed to have diverged from a common ancestor approximately 14 to 24 million years ago (Koch et al., 2000). Due to this ancestral relationship, *A. thaliana* has many genes that have the same function in *B. napus,* providing a useful tool for genomic analyses.

1.2 *Brassica napus* as a crop

Brassica napus comprises of multiple crop types, including Winter Oilseed Rape (WOSR), Spring Oilseed rape (SOSR), Siberian kale and swede. From these, oilseed rape is a highly important crop being the second biggest contributor to vegetable oil globally (Commission-Implementing-Regulation, 2018). Oilseed rape is commonly grown as a break crop, in rotation with other crops such as wheat, barley, peas and beans (Alford, 2003).

Oilseed rape is drilled late summer to early autumn, around mid-August to late-September and harvested mid to late summer (https://ahdb.org.uk/). It is sown to produce a density of around 25 to 40 plants/m2, with sowing density ranging from 50 to 100 seeds/m2 dependant on variety, drilling date and other environmental conditions (https://www.frontierag.co.uk/). The Recommended List produced by the Agricultural and Horticultural Developmental Board (AHDB) indicates that oilseed rape has the yield potential for five to six tonnes/hectare (https://ahdb.org.uk/), but the average typical yield achieved is around three to four tonnes/hectares (https://www.gov.uk/).

In the United Kingdom (UK), oilseed rape was the 5th most produced crop in 2022 (in terms of hectares, Department for Environment, Food and Rural Affairs (DEFRA), 2022). The area of oilseed rape was growing in the UK but in recent years has been declining, going from 741,920 ha in 2012 to 365,721 ha in 2022 (DEFRA, 2022) . This is largely due to changes in policy leading to stricter regulations on pesticide usage and increasing threats from invertebrate pests (Scott and Bilsborrow, 2019).

1.3 Pests and diseases of Brassicas

Oilseed rape faces many different pests and diseases. Some of the most problematic diseases are as follows. Light Leaf Spot (LLS), caused by the fungus *Pyrenopeziza brassicae* (Oxley and Walters, 2012), reduces plants photosynthetic ability by due to lesions and death of plant material, thus reducing frost tolerance and yield (Boys et al., 2007). The AHDB considers disease incidence of LLS as high across the UK and suggests management by cultural practises, monitoring and carful timing of fungicides (https://ahdb.org.uk/). Another major disease faced by oilseed rape is stem canker, caused by the fungus *Leptophaeria maculans,* which also results in lesions to plant material including the stems, which can increase lodging and reduce yields (Howlett et al., 2001). The AHDB consider this disease moderate to high in the UK, particularly in the South, and again recommend careful monitoring and fungicide application (https://ahdb.org.uk/). Finally, clubroot represents a challenging disease for oilseed rape, caused by the pathogen *Plasmodiophora brassicae*, which causes galls on the roots to form, resulting in decreased nutrient uptake and thus reduced yield (Hwang et al., 2012). There is currently no recommended treatment for clubroot other than planning rotations and growth of varieties to reduce disease pressure (https://ahdb.org.uk/). Pressures from diseases are also further exacerbated by a range of pest species that feed on oilseed rape.

For Europe, some of the most significant pests are the grey field slug (*Deroceras reticulatum*), the brassica pod midge (*Dasineura brassicae*), rape stem weevil (*Ceutorhynchus napi*), cabbage stem weevil (*Ceutorhynchus picitarsis*), pollen beetle (*Brassicogethes aeneus*) and the cabbage stem flea beetle (*Psylliodes chrysocephala*) (Zheng et al., 2020).

The grey field slug (GFS) is a prolific pest of oilseed rape feeding on leaves and stems. The GFS life cycle means that it has overlapping generations, making its herbivory a problem all year round (https://ahdb.org.uk/). Furthermore, restrictions in use of metaldehyde pellets and expense of use of biological controls, such as nematode *Phasmarhabditis hermaphrodita*, makes GFS a difficult pest to manage (Forbes et al., 2021).

The brassica pod midge (BPM) feeds predominantly on the pods of *B. napus*. Larvae feed inside the pod and can cause significant yield losses through splitting of pods resulting in loss of seed (Meakin and Roberts, 1991). The BPM is an understudied pest of oilseed rape leaving large gaps in the understanding of its biology, thus there are limited effective control measures in place to tackle it (Hausmann, 2021).

The rape stem weevil (RSW) is also a major pest for oilseed rape. Larvae feed within the main stems of plants and can cause significant distortions, leading to stunted growth and lodging of the crop (Juran et al., 2011). The cabbage stem weevil (CSW) causes similar issues as with RSW (Juran et al.,

2011), but with larvae starting by feeding in the petioles before moving into the main stems of the crop (Alford, 2003).

The pollen beetle has received more research attention and continues to become an increasing problem for oilseed rape agriculture as pesticide resistance develops (Zimmer et al., 2014). Adult beetles feed on young, unopened buds which causes the plant to drop them, leaving podless stems (Williams and Free, 1978). Furthermore, if plants make it through this stage of herbivory, beetles will continue to feed on open flowers (Williams and Free, 1978), which can result in severe yield losses. However, the invertebrate pest species which has been highlighted as the most problematic and threatening to oilseed rape production across Northern Europe is the cabbage stem flea beetle (CSFB) (Zheng et al., 2020).

1.4 The cabbage stem flea beetle

The cabbage stem flea beetle (CSFB), a member of the *Chrysomelidae*, is a small black, iridescent beetle about 4mm long with enlarged femurs, allowing them to jump (Figure 1.1). CSFB feed on many *Brassicaceae* species native throughout Northern Europe but are most problematic for winter oilseed rape (WOSR) (Zheng et al., 2020). They have an annual life cycle (Figure 1.2) which aligns to the WOSR cropping cycle, although multiple generations per year are possible in captive populations (personal observations). This indicates that their life cycle is phenotypically plastic to environmental conditions.

Figure 1.1. Photographs of the adult cabbage stem flea beetle, with the right image demonstrating the enlarged femurs on the back pair of legs used for jumping.
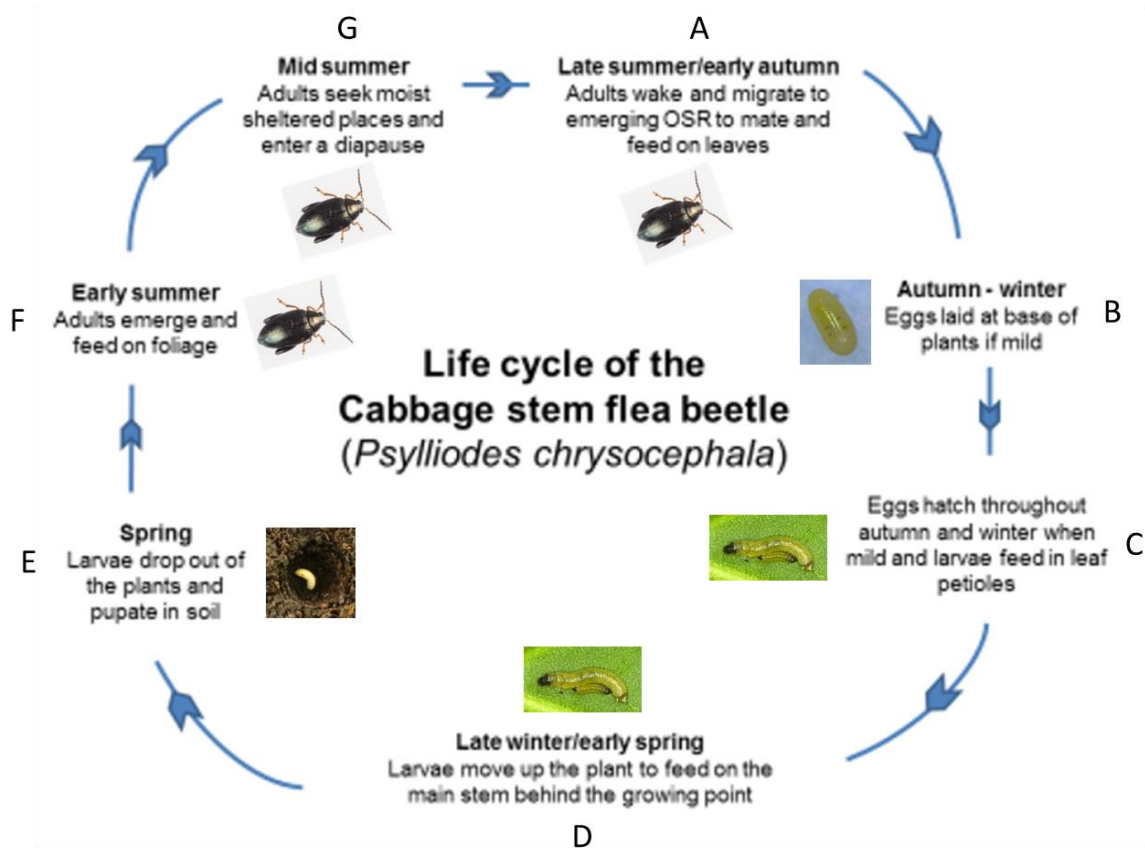
Figure 1.2. The annual life cycle of CSFB in the UK (adapted from (Nicholls, 2016) with letters denoting the key life stages throughout the year.

Adult CSFBs fly during the autumn (in temperatures 16°C and above) into newly sown oilseed rape fields (Figure 1.2A) and are capable of travelling 3-4km during this migration (Williams, 2010). What initiates the start of this migration is not fully understood, but (Tixeront et al., 2023) demonstrate increasing temperatures and decreasing air humidity correlates with numbers of beetles caught during migration events. It is also not clear how CSFB detect crop fields to migrate into. However, (Bartlet et al., 1999) discovered sensilla on CSFB antennae that likely have an olfactory role, indicating the possibility of detecting volatiles of food sources in the air.

Upon arrival in the crop beetles feed on seedlings cotyledons, creating a distinctive ''shot holing'' feeding pattern (Figure 1.3). At this stage the young WOSR crop is particularly vulnerable to high levels of CSFB herbivory and can be completely decimated if the growing points are consumed. Furthermore, if coupled with other non-favourable factors, such as warm and dry weather conditions, the crop can be lost very quickly (as observed personally in our 2019 field trial). During this period of feeding, flight muscles are degraded, female ovaries mature, and mating occurs (Williams, 2010).

Figure 1.3. Examples of CSFB shot holing damage on cotyledons (left) and true leaves (right).

During autumn, female CSFBs lay eggs at on the soil at the base of plants (Figure 1.2B). If conditions remain mild enough oviposition can continue into the winter months (Sáringer, 1984). Eggs are oval and cream coloured, about 0.5mm long (Figure 1.4a). Time taken to hatching is temperature dependent, ranging from about 37 days at 10°C to 70 days or more at less than 6°C (Alford, 1979).

Figure 1.4a: CSFB egg, 1.4b: first instar and third instar CSFB larvae.

Upon hatching throughout autumn and winter, larvae (Figure 1.4b) burrow into petioles and stems of nearby host plants and feed throughout the winter into spring (Figure 1.2C and D). Larvae are susceptible to cold conditions, especially if exposed to temperatures under -5°C for a number of continuous days in a row (Mathiasen et al., 2015). However, the UK rarely experiences prolonged periods of cold under -5°C, particularly in the South-East where CSFB are most prominent. Damage throughout this winter larval period is equally as detrimental as adult herbivory and can lead to complete plant collapse (Figure 1.5).

Figure 1.5. Examples of stem damage where the plant has completely collapsed (left) and the inside of a stem that has been mostly consumed (right).

Larvae go through three instars (Figure 1.4b for an example of a first and third instar larvae) before tunnelling out of the plant and burying themselves in the first few centimetres of soil for pupation (Figure 1.2E and Figure 1.6) (Alford, 2003). Depending on environmental conditions in the field, pupation takes about eight to 12 weeks (Sáringer, 1984). Adult CSFBs begin to emerge in May where they remain in the crop and feed on the leaves (Figure 1.2F) (Ortega-Ramos et al., 2022b). In mid-late summer, during the warmest months, beetles go through a period of aestivation, sheltering under lumps of soil, rocks or in plant matter on the ground (Figure 1.2G) (Sivčev et al., 2016). When conditions begin to cool in autumn beetles wake and become active again, migrating to newly sown crops and starting the life cycle once more.

Figure 1.6. CSFB pupae in soil.

From examining the CSFB life cycle, it is evident that they present a two-level problem for WOSR cropping. Adults attack young, vulnerable seedlings, and if plants manage to grow through this stage, they are then infested with larvae feeding within the petioles and stems. This two-fold damage from CSFB makes this pest particularly important to control but since changes in policy surrounding pesticide usage there has been little viable protection for the agricultural community.

Until 2013, the primary control for CSFB on oilseed rape was the use of neonicotinoid pesticides in the form of a seed treatment, which are systemic pesticides that work by attacking the insect nerve system, resulting in paralysis (Simon-Delso et al., 2015). However, an EU-wide moratorium in December 2013 was imposed for neonicotinoid use on flowering crops. In 2014 some areas of the UK (including Suffolk, Bedfordshire and Cambridgeshire) were so badly affected by CSFB that the ban was temporarily suspended in 2015. Despite the struggles for the oilseed rape growing community, the ban on neonicotinoids was made permanent and extended to other non-flowering crops, such as sugar beet, across the EU (Commission-Implementing-Regulation, 2018).

The main reason for this ban was due to evidence of negative effects on wild bee populations, specifically reducing the growth and reproduction of colonies (Rundlöf et al., 2015). Neonicotinoid pesticides influence also extends to birds, with research demonstrating that birds consuming neonicotinoids either directly through treated seed or indirectly via insects show reduced migration ability (Eng et al., 2017) and significant weight loss (Hallmann et al., 2014). These pesticides are even more problematic due to high levels of persistence in soils and leaching into water systems, thus spreading to other environments (Goulson, 2013).

Instead, growers are advised to use pyrethroids in the form of a foliar spray as an alternative chemical treatment. These also attack the insect nerve system but are non-systemic and require contact with the pest to be effective. However, a target-site mutation known as knockdown-resistance (kdr) has been found in CSFB populations in Germany (Zimmer et al., 2014) and is correlated with resistance levels to pyrethroids (Højland et al., 2015). Højland et al. (2015) further confirm presence of kdr in UK CSFB populations, with an additional metabolic-based resistance. Willis et al. (2020) also confirm kdr in the UK, demonstrating pyrethroid levels remain high in the South-East but have additionally spread further North and West. Højland and Kristensen (2018) investigated pyrethroid resistance in 15 field populations of CSFB in Denmark, demonstrating that populations in southern Denmark have also been shown to have kdr, and some of these CSFB populations showed reduced susceptibility to pyrethroids. Although there was a correlation between pyrethroid susceptibility and kdr in CSFB in Denmark, it wasn't statistically significant. Højland and Kristensen (2018) therefore suggest that a metabolic resistance, such as the one discovered in UK populations, may also be playing a role.

Other researchers also support the notion of an unidentified metabolic resistance also being present among some CSFB populations (Stara and Kocourek, 2019). Using glass vial experiments to expose beetles to pesticides, Stara and Kocourek (2019) reported high susceptibility of CSFB populations from two localities of Czech Republic to six different pyrethroids, indicating they do not possess kdr. However, in the same experiment, these two CSFB populations do appear to be resistant to the neonicotinoid thiacloprid. Therefore, it is important to consider the local population of CSFB before countries implement the use of difference pesticides to not speed up evolution of resistance.

Overall, a combination of neonicotinoid withdrawal and spreading pyrethroid resistance has left oilseed rape growers with no viable control for CSFB (Zhang et al., 2017). Scott and Bilsborrow (2019) address the impacts of neonicotinoid withdrawal in England on oilseed rape production with survey data on WOSR production area, damage from CSFB and management techniques used to control pest damage. Notably, they report large county and yearly variation in crop losses in the 2014/2015 and 2015/2016 growing seasons, making implemented pest management approaches challenging, although incidence of larval CSFB supports crop losses reported here. Additionally, Scott and Bilsborrow (2019) report significant costs of controlling CSFB in the 2014/2015 and 2015/2016 growing seasons, £25.2 million and £23.3 million, respectively (cost of chemicals and applications, crop loss and re-drilling of lost areas). also exemplify changes in UK oilseed rape cropping, reporting a sharp drop in production levels between 2015 to 2016. Furthermore, the area harvesting in 2016 was 10% less than in 2015, with predictions for the area of oilseed rape cultivated predicted to continue dropping (Dewar, 2017). This has led to a deficit in UK oilseed rape production, meaning imports are now necessary and often from countries which still have access to neonicotinoid pesticides (Ortega-Ramos et al., 2022a).

Therefore, it is clear that the cost of controlling CSFB along with the perceived (and real) lack of control for CSFB and substantial temporal and spatial variation in CSFB incidence is severely impacting the viability of growing OSR in the UK. Alternative controls are desperately needed as reliance on environmentally damaging chemicals are no longer a sustainable option. There is encouragement to take up Implemented Pest Management (IPM) strategies to combat CSFB, and the research community is focusing on discovering effective control methods.

1.5 IPM strategies for CSFB as control methods

With no viable controls for CSFB, the research community is looking at alternative methods as part of a IPM approach to agriculture. Some of these include intercropping or trap cropping oilseed rape with other crop species, farming practises such as drill date and straw cover and biological controls, such as entomopathogenic nematodes and parasitoids, all of which are focused on combating the CSFB directly. Here, some of these methods before addressing more plant-focused strategies for CSFB control are examined.

There has been research into companion cropping, or intercropping, where oilseed rape is grown along with another crop species that acts as a ''trap'' with CSFB preferring to feed on that over the focal crop. Recent work has demonstrated the effectiveness of growing cereals (wheat and oat) alongside oilseed rape in four field trials in the UK and Germany (Seimandi-Corda et al., 2023). Seimandi-Corda et al. (2023) observed a significant reduction in adult CSFB to oilseed rape when grown with these companion crops. Additionally, they observed this effect with legumes and mustard, but this was only found in one field trial. They also assessed the effect on larval load but did not observe any consistent effects of companion cropping.

However, other research has had success in identifying an effect of companion cropping on larval loads. Barari et al. (2005a) reported from a UK field trail that oilseed rape sown with the trap crop turnip rape had significantly fewer CSFB larvae than oilseed rape grown on its own. Furthermore, they demonstrated that the turnip rape had significantly higher loads of CSFB larvae compared with oilseed rape when cropped together. More recent work also demonstrates the benefit of companion cropping in reduction of larval CSFB levels in oilseed rape, this time with faba bean and grass pea (Breitenmoser et al., 2022). Therefore, results are promising for companion cropping as part of an IPM strategy, but more research is needed on what species to grow and how best to implement them into agricultural practices.

Other IPM research has focused on a variety of other farming practices, such as adding straw mulch cover to the crop. This has been demonstrated to result in significantly less adult CSFB feeding damage in oilseed rape crops, but does not appear to impact larval load (Seimandi-Corda et al., 2023). Research on reduced or minimal tillage has also been demonstrated to reduce adult CSFB herbivory, particularly if the previous crops stubble is left, in addition to reducing larval infestation (Ortega-Ramos et al., 2022b). Grazing or mowing off has also been demonstrated to effectively reduce larval load, but timing is crucial as if it is done after crop extension there is a negative impact on final yield of the crop (Sacha White, personal communications).

Date of drilling has also been addressed, where earlier sowing dates (from late July to early- mid August) being particularly beneficial for oilseed rape crops to become better established before adult CSFB move in for feeding (Personal communications with Sacha White & Steve Ellis). However, earlier sow dates have also been associated with higher larval loads late in the oilseed rape growing season, potentially due to adults having a longer window for egg oviposition (Conrad et al., 2021). Generally, however, one of the most important factors for sowing date is that there is an adequate amount of soil moisture, otherwise the crop struggles to establish and withstand CSFB herbivory (personal observations).

Location of cropping has also been subject to investigation, and it is generally regarded in the growing community that crops should not be sown nearby to an area that was previously used to grow oilseed rape, due to there being a potential bank of CSFB. Recent research has also indicated that growing next to woodlands can lead to an increase in adult CSFB migration into crops, as numbers of aestivating beetles was significantly higher in these borders compared with others, such as flower strips (Pigot et al., 2023).

Therefore, whilst there is encouraging evidence for manipulation of farming practices to control levels of adult and larval CSFB infestations, further research is required to better understand the mechanisms behind these benefits. Furthermore, more research is needed to refine the timing and combinations of such practises to provide the greatest levels of protection. As well as these sorts of farming practices, another area of IPM research has explored the impact of use of biological controls on CSFB.

Entomopathogenic fungi (EPF) has received limited research attention for combating CSFB in oilseed rape. However, some early research from found one cultivar of *Metarhizium anisopliae* EPF to deliver 100% mortality to adult CSFB in laboratory assays after 14 days of exposure (Butt et al., 1992). Unfortunately, subsequent tests revealed that after repeated exposure to *M. anisopliae* effectiveness dropped significantly and thus would not be suitable to develop as a biocontrol. Butt et al. (1992) go on to discover two other *M. anisopliae* EPF strains that delivered 88% and 73% mortality to CSFB, but did not further test in field conditions.

There has been slightly more research focus on entomopathogenic nematodes (EPN) with some recent promising discoveries. One research group recently identified four species of EPN which caused a 70% or greater mortality in adult CSFB under laboratory conditions, the most successful being *Steinernema carpocapsae* providing 80% mortality after six days (Price et al., 2023). Further tests were performed to determine how addition of adjuvants, designed to protect EPN in field environments, effected their survival. However, addition of some adjuvants did appear to compromise survival of EPNs, thus further research is required to select optimal combinations of adjuvants to protect EPNs and then test their efficacy under field conditions.

Other recent research has highlighted the effectiveness of using EPNs for CSFB larval control. (Godina et al., 2023) used plants sprayed with three different strains of EPNs to discover significantly lower levels of live CSFB larvae compared with non-sprayed control oilseed rape plants. They go on to quantify 82% mortality of CSFB larvae in plants treated with *Steinernema feltiae* and reaffirm the ability of *S. carpocapsae* to infect adult CSFB. Taking their research a step further by testing EPN effectiveness in four fields and recorded the highest reductions of CSFB larvae to be 45% for a cold activated EPN strain, *Heterohabditis bacteriophora*. However, results from the field were highly variable and thus again highlights the need for further research required to test the efficacy of EPNs in agricultural systems.

Natural predators and parasitoids of CSFB have also received some, but limited, research attention as a mode of IPM. Having the ability to jump, adult CSFB face few threats from predators and parasitoids. However, a parasitoid wasp was recently discovered, *Microctonus brassicae*, which infests UK CSFB populations (Jordan et al., 2020). The parasitoid slowly stalks an active adult CSFB before ovipositing an egg into the body through a gap in the beetles elytra. In captivity, an average parasitism rate of 44% was observed and methods demonstrated to rear *M. brassicae* as a potential biocontrol for release in fields. However, raising *M. brassicae* was time-consuming and costly, thus further research is needed to optimise this process and additionally improve understanding of natural populations of this parasitoid.

Other research into parasitoids has focused more on CSFB larvae as these are more common. Barari et al. (2005b) identified *Tersilochus microgaster* for the first time in the UK as an endoparasite of CSFB larvae in the field, but only found low levels of parasitism. Ulber et al. (2010) later discovered 11% levels of parasitism for *T. microgaster* in a UK field trial, which was an encouraging sign that this parasitoid may be spreading naturally. However, research also indicates that certain farming practices are harmful to beneficials, such as tillage (Nilsson, 2010). Furthermore, there are consistent negative effects of pesticide use on these natural populations of beneficials (Geiger et al., 2010). Therefore, farming practices would need to be carefully balanced to be able to fully monopolise on the benefits of parasitoids and predators as modes of biocontrol.

Finally, biocontrols in the form of RNA interference (RNAi) are now being considered. Recent work has demonstrated feeding adult CSFB oilseed rape leaf discs coated with double-stranded RNA targeting the gene sec23 (a gene involved in endoplasmic reticulum-golgi transport), resulted in 76% mortality in pre-aestivated beetles (Cedden et al., 2023). This however dropped to 56% when fed to post-aestivated beetles. Therefore, whilst this is promising research it highlights the importance of timing for potential RNAi foliar sprays.

Overall, as demonstrated above, a combination of these agricultural and biocontrol practices may work as part of an IPM system but require more research to make them practically viable for

growers to implement. Additionally, these practices may not be enough on their own to control CSFB in a post-neonicotinoid system. Other research is taking a more plant focused approach to identify properties giving oilseed rape enhanced resistance or tolerance to CSFB and aid in the development of stronger varieties.

1.6 Phenotyping for resistance/tolerance traits in brassicas

Phenotyping of resistance or tolerance traits in plants is the first step in breeding or developing more robust oilseed rape varieties. Variation in morphological plant traits have been indicated to be involved in brassica defence against CSFB herbivory, some of which are introduced here.

Leaf epicuticular waxes provide protection from herbivory, either by providing physical or chemical barriers to feeding. Bodnaryk (1992b) investigate feeding behaviour of *Phyllotreta cruciferae*, the crucifer flea beetle (a major pest which feeds on canola and other brassicas), on high leaf wax species (>1000mg kg-1), including *Brassica oleracea* and *Brassica napus*, and low leaf wax species (<240mg kg-1), including *Brassica rapa*, *Brassica juncea* and *Sinapis alba*. They demonstrate varieties with waxy leaves receive significantly lower levels of flea beetle feeding compared with low leaf wax species. Notably, the most fed-upon species was *S. alba* but demonstrated high tolerance to flea beetle feeding. Furthermore, all species with high leaf wax had edge feeding only, and all those with low leaf wax showed random feeding patterns across the leaf. This aligns with personal observations where mature *B. napus* leaves receive much less damage from CSFB adults, confined to the edges, compared with less waxy leaves such as *B. rapa*. *P. cruciferae* feeding was also recorded on *B. napus* mutants, with one-two thirds reduced leaf wax levels. Feeding on these mutants was 1.6-2.2 times higher and occurred in a random pattern compared to wildtypes where >96% of damage was found on the edge of leave (Bodnaryk, 1992a). Finally, manually removing wax from leaves of *B. oleracea* and *B. napus* by gentle rubbing with cotton buds also increased *P. cruciferae* herbivory, with initially beetles only feeding at the edges of leaves until wax was removed.

Some research has addressed the influence of leaf waxes on CSFB herbivory in field trials, demonstrating a correlation between increased leaf waxes and reduced adult beetle feeding (Lambdon et al., 1998). Little research has related plant waxes to larval feeding or colonisation, but report states that larvae of *Phyllotreta nemorum*, the turnip flea beetle, struggle to enter leaf petioles with high levels of wax (Nielsen, 1977).

Therefore, leaf epicuticular wax has been identified as a resistance trait against adult flea beetle herbivory and potentially larvae but there has been little recent research into this trait. Leaf waxiness traits have been attributed to the CC genome originating from *B. oleracea* (CC), which is

also present in *B. napus* (AACC) (Bodnaryk, 1992a). It is possible that leaf waxes may be a suitable target for creation of transgenic material or conventional breeding programmes. Based on leaf epicuticular wax research WOSR has high leaf wax so should be relatively more resistant to flea beetles feeding compared with other *Brassicaceae*. However, reports from growers and research since the neonicotinoid ban in 2013 demonstrate that flea beetle damage has drastically increased. Therefore, it is unlikely leaf waxiness traits alone are enough to protect from flea beetle herbivory.

Trichomes help prevent insect herbivory by acting as a physical barrier between the insect and plant (Gavoski et al., 2000). Soroka et al. (2011) use a transgenic line, named ''Hairy1'', to suggest that trichomes act as anti-herbivory traits to the flea beetle *P. cruciferae*. They created Hairy1 by inserting genes from *Arabidopsis thaliana* into Westar, a spring *B. napus* variety. In the laboratory adult beetles fed significantly more on cotyledons and 2nd true leaves of Westar compared to Hairy1. Field results support this result with Hairy1 receiving equal or less feeding damage compared to its parental lines and plants which have grown from insecticide treated seeds, indicating trichomes may be as effective at deterring adult *P. cruciferae* feeding as pesticides.

Other research reports other *Brassica* species with high trichome density being resistant to *Phyllotreta striolata* damage, the striped flea beetle (Palaniswamy and Bodnaryk, 1994), although this research is on *Brassica villosa*, a species only endemic to Sicily. Furthermore, trichomes are not present on seedling *B. villosa* and thus failed to protect them from *P. striolata* adult feeding at their most vulnerable developmental stage (Gavoski et al., 2000). Trichomes on pods of *S. alba* have been shown to receive insignificant amounts of damage from *P. cruciferae* whilst growing next to plants which have fewer hairs and show pod damage (Lamb, 1980). Furthermore, removal of pod hairs significantly increases feeding damage from flea beetles. However, another study investigating *Barbarea vulgaris* resistance to *Phyllotreta nemorum* didn't find trichomes to be associated with flea beetle resistance (Kuzina et al., 2011).

Overall, research into trichomes indicates that they can reduce feeding in some species of flea beetle and that it's possible to create transgenic varieties with higher levels of trichome expression. However, to my knowledge, no research has been done addressing trichomes as resistant traits to the CSFB or to flea beetle larvae.

Seed size has also been indicated to influence incidence of flea beetle herbivory. Plants grown from small seed for both *S. alba* and *B. napus* suffered a higher proportion of mortality compared to those grown from large seed. In a study examining *P. cruciferae* herbivory, 45% of *S. alba* and 100% *B. napus* seedlings were killed off when they when grown from small seeds, compared to only 9% and 28% killed respectively when grown from large seeds (Bodnaryk and Lamb, 1991). As adult CSFBs attack plants as they emerge from the soil, quick establishment is essential to outgrow damage from pests. Larger seeds have more resources, allowing plants to grow away quicker and

tolerate more damage. Selectively breeding plants for large seed is desired by growers and it may help protect crops from flea beetle herbivory when they are most vulnerable.

Glucosinolates typically act as plant defence compounds to deter herbivores from feeding on plant material and are characteristic to *Brassicaceae*. However, in laboratory experiments, Bartlet and Williams (1991) indicate that glucosinolates act as a feeding stimulant rather than deterrent for adult CSFBs, showing that they only feed on plants with glucosinolates. Furthermore, adding glucosinolates to agar stimulated flea beetle feeding and increasing glucosinolate content increased the amount of feeding (Bartlet et al., 1994). Other research has supported this with field experiments of 28 lines of *B. napus* with altered glucosinolate levels (Giamoustaris and Mithen, 1995). Lines with increased glucosinolate content received more damage from adult CSFB. However, research indicates that it is not just total glucosinolate content which is important to flea beetle herbivory.

There are two groups of glucosinolates which more frequently appear in the literature; Indolic and aliphatic glucosinolates. One particular indolic glucosinolate identified is glucobrassicin, which positively influences CSFB feeding on *B. napus* (Bartlet et al., 1994) but negatively influences *Phyllotreta spp*. feeding on *S. alba* (Bohinc et al., 2013). Many of the glucosinolates identified to increase flea beetle feeding by Bohinc et al. (2013) are aliphatic glucosinolates. Giamoustaris and Mithen (1995) identified in particular *B. napus* lines with reduced levels of butenyl glucosinolates were less susceptible to CSFB feeding. This is supported by reduced herbivory from *Pyllotreta spp.* recorded by Soroka and Grenkow (2013) of canola quality *S. alba,* which has reduced levels of butenyl glucosinolates compared to standard *S. alba*. Additionally, Bohinc et al. (2013) identify 3-butenyl glucosinolates to stimulate feeding of *Pyllotreta spp.* on *B. napus*. Other research focusing on the CSFB doesn't show any significant effect of aliphatic glucosinolates on feeding damage (Bartlet et al., 1996). Therefore, further research is required to better understand how glucosinolate profiles influence flea beetle herbivory.

More recent research has focused on improving understanding as to why and how flea beetles feed on plants with glucosinolates. Glucosinolates themselves are not toxic, however upon herbivory plants hydrolyse glucosinolates using the myrosinase enzyme to create toxic isothiocyanates (Brown and Hampton, 2011). However, *P. striolata* has been demonstrated to selectively accumulate glucosinolates and hydrolyses them to isothiocyanates with their own myrosinase system (Beran et al., 2014). Furthermore, they demonstrate that the major substrates for the insect myrosinase enzyme were aliphatic glucosinolates, rather than indolic or aromatic glucosinolates. Taking this with previous research it appears that flea beetles may be attracted to brassica varieties which have higher levels of aliphatic glucosinolates as they can utilise them for themselves. This

may explain why flea beetles seem to have higher feeding rates on brassicas with higher overall glucosinolate content, even though this is considered a plant defence compound against herbivory.

Unlike *P. striolata*, adult CSFB do not demonstrate myrosinase activity and only around 26% of glucosinolates are sequestered or desulfidised, indicating that isothiocyantes are taken up from the plant when feeding (Beran et al., 2018). Instead, Beran et al. (2018) demonstrated that adult CSFB detoxify isothiocyanates by conjugating them to glutathione, which has previously been recorded in other invertebrates (Jeschke et al., 2016). However, what happens to 40% of ingested glucosinolates still remains unknown, indicating that conjugation of isothiocyanates to glutathione may not be the only method of detoxification. Beran et al. (2018) further demonstrate that CSFB adults, pupae and larvae all have similar glucosinolate profiles, but eggs have profiles more similar to that of the host plant. This suggests that adults are transferring glucosinolates to eggs, indicating that CSFB sequestration of glucosinolates has an ecological purpose. Finally, as *Phyllotreta* and *Psylliodes* have different methods to overcome glucosinolate defences, it is likely that they evolved separately and therefore should be studied at a species-specific level.

There has been a recent advancement on information on glucosinolate sulfatases (GSS) activity in adult CSFB (Ahn et al., 2019). Ahn et al. (2019) demonstrate that GSS activity occurs mainly in the gut membrane and has strongest activity towards sinalbin, a relatively uncommon benzenic glucosinolate which is found in *Sinapis alba* (Agerbirk et al., 2008). Such strong activity towards sinalbin is surprising given that CSFB have a wide source of Brassica food plants and that *S. alba* does not appear to be a preferred food source (personal field observations). GSS activity converts sinalbin into desuflo-sinalbin, allowing 80% to be safely excreted, indicating that this specific glucosinolate is not sequestered by CSFB (Ahn et al., 2019), unlike others previously reported by Beran et al. (2018). It is therefore important to consider that different food plants may require different detoxification mechanisms.

Other recent research has attempted to address performance of CSFB larvae on different plants and the role of glucosinolate profile of plants (Doering and Ulber, 2020). Larval weight and number recovered from plants did not differ between the four oilseed rape (OSR) varieties tested. Significantly fewer larvae were recovered from *S. alba* and larvae in *S. alba* appeared to have slower development compared with OSR varieties. Interestingly, Doering and Ulber (2020) also report no correlation between larval weight and glucosinolate content of plants but do see a positive correlation between larval weight and certain glucosinolates, specifically progoitrin (aliphatic) and 4-hydroxyglucobrassicin (indolic). This collection of research considering glucosinolates highlights the need to consider individual species, life stages within species and target food source to advance our understanding in pest mechanisms for overcoming plant defences.

Overall, research on pre-identified plant traits thought to give flea beetle resistance is promising but requires further understanding of the underlying mechanisms and how best these could be incorporated into oilseed rape farming. Another approach has been to phenotype panels of brassica varieties for levels of CSFB herbivory without obvious morphological differences, then identify underling genes conferring this variation.

Gavloski et al. (2000) also conducted laboratory experiments to identify 11 cultivars of *S. alba* seedlings as consistently resistant to *P. cruciferae* feeding, in contrast to *B. napus*, *B. rapa* and *B. juncea* cultivars which showed no consistent resistance. *Sinapis pubescens* was also tested and shown to be susceptible to flea beetle feeding, indicating that resistance/tolerance is very species specific. Having identified *S. alba* as showing resistant properties they created 308 hybrids of *S. alba* crossed with *B. napus*. Out of these, 34 show some resistance to flea beetle damage; they were damaged significantly less than the control in at least one out of four replicates. However, only one hybrid showed consistent resistance across all four replicates. Despite only one resistant hybrid being identified, it demonstrates that resistant traits can be bred into *B. napus* from relatives, such as *S. alba*, via conventional breeding methods. This indicates that traits such as increased trichomes, leaf wax and seed size could also be bred into *B. napus* to create plants with multiple lines of defence. Gavloski et al. (2000) take their research a step further by demonstrating that the amount of *S. alba* DNA in the hybrids does not appear to correlate with resistance to flea beetle feeding. However, they don't identify any genes or mechanisms by which this resistance is conferred.

Identifying phenotypic traits which confer resistance or tolerance, such as pubescence or glucosinolate profiles, is the first step in combating agricultural pests, but research can be taken a step further by identifying the genes underlying these useful traits. Genetic mapping aims to identify the regions on the genome in which these genes of interest exist causing the differences in plant phenotypic responses. There are different methods used to discover variation of genes within a population of plants, such as genome wide association studies or quantitative trait loci (QTL) mapping in a cross between two parents which segregate for the trait of interest. Little research has focused on identifying candidate genes or genomic regions within flea beetles in general and to my knowledge none focused on the CSFB. However, some research has attempted to uncover underlying genetic variation of *Barbarea vulgaris* resistance to *Phyllotreta nemorum*.

Kuzina et al. (2011) created a F_2 segregating hybrid population from two parental lines of *B. vulgaris* – Glabrous-type, which is hairless and resistant to *P. nemorum* larvae, and Pubescent-type, which is hairy and susceptible to *P. nemorum* larvae. From this F_2 population they create a genetic map and using QTL analysis identified two QTLs for flea beetle resistance and that both alleles are inherited from the Glabrous-type parent. Interestingly, these resistance QTLs colocalised with QTLs for saponins, a group of detergent like chemicals which are naturally found in plants and have a

role in plant defence. Therefore, it's likely that these saponins play a role in G-type *B. vulgaris'* resistance to flea beetle larvae. This research demonstrates that it is possible to identify areas of the genome associated with resistance and traits which may be linked to resistance.

Kuzina et al. (2011) also observed synteny between linkage groups conferring resistance to flea beetles in *B. vulgaris* and areas of the *Arabidopsis thaliana* genome, giving the potential for exact genes to be identified which underlie the resistance and become more comparable to other plant species. To my knowledge there is no published research on underlying genetic basis of resistance to CSFB in *Brassicas*. Furthermore, to my knowledge no published research has utilised transcriptomic approaches of resistant/tolerant phenotypes compared to those with susceptible/intolerant phenotypes.

1.7 Aims and objectives

Overall, the aims of this project were to identify biological and genetic traits which confer resistance to adult and larval cabbage stem flea beetle within *Brassica napus*, to aid development of more targeted pest management approach. More specifically, the objectives were as follows;

1. Phenotype diverse *B. napus* germplasm for differences in adult and CSFB palatability by;
   a. Developing a protocol for reliably recording levels of adult herbivory,
   b. Identifying feeding variation within germplasm,
   c. Confirming if herbivory differences are maintained in the field.
2. Identify genetic variation underlying tolerance or resistance to CSFB by;
   a. Mapping genetic variation linked to tolerance/resistance phenotypes identified in laboratory assays,
   b. Selecting candidate gene(s) for further investigation.
3. Improve understanding of CSFB feeding behaviour and life cycle.

Chapter 2 – Phenotypic differences in adult CSFB herbivory

2.1 Background

Winter oilseed rape, *Brassica napus*, is the United Kingdom's second most profitable crop, regularly used in rotation with the most profitable crop, winter wheat (AHDB). Cabbage stem flea beetle, *Psylliodes chrysocephala*, are a major insect pest species of *B. napus* and are now regarded globally as one of the most important economic pest species threatening oilseed rape farming (Zheng et al., 2020). Specifically, after a period of aestivation in summer, adult CSFB feed heavily on young leaves (cotyledons) of newly emerged *B. napus* crops that are yet to fully establish (Alford, 1979). These young plants are vulnerable and unable to withstand high levels of pest damage, often resulting in large amounts damage, if not total crop loss.

The CSFB was relatively well controlled by neonicotinoid seed treatment pesticides, but these pesticides have been shown to have adverse effects on wildlife, particularly wild bee populations (Rundlof et al., 2015). As a result, neonicotinoids were subject to a temporary ban in 2013 for flowering crops such as *B. napus*, but this ban was extended to include non-flowering crops across the EU in 2018. Since these bans were imposed there has been little to no control over CSFBs. Growers are alternatively advised to use other chemicals treatments such as pyrethroids. However, there is mounting evidence that these are ineffective, and pests are becoming quickly resistant. Particularly concerning are multiple modes of resistance being recorded across multiple countries for CSFB (Zimmer et al., 2014; Højland et al., 2015; Willis et al., 2020) demonstrating the lack of reliance that can be put on chemical controls in the long term. Therefore, it is evident that alternative control measures are needed to maintain the integrity of oilseed rape farming in the UK and across the world.

Efforts are being made to research alternative control methods and tackle CSFB with an integrated pest management approach (Ortega-Ramos et al., 2022). For example, biological control in the form of entomopathogenic fungi, where one study by Price et al. (2023) demonstrated that administration for four species to adult CSFB resulted in mortality rates of 70% and above in laboratory assays. Other means of control look to farming practices, such as inter-cropping or application of straw cover. Seimandi-Corda et al. (2023) demonstrated, in four field trials, the effectiveness of growing cereal companion crops alongside oilseed rape, as well as the application of straw mulch, in significantly reducing adult CSFB herbivory.

However, although there has been research into phenotypic traits that confer some resistance/tolerance to adult CSFB herbivory, to date there is limited exploration into the underlying genetics conferring these traits.

Therefore, the aims and objectives of the following chapter were as below:

1. Develop a suitable assay system for testing a large number of *B. napus* varieties.

2. Generate quantitative, phenotypic data on adult CSFB feeding damage to *B. napus* in controlled laboratory assays, suitable for use further genomic investigation and to identify more and less resistant varieties.

3. Refine laboratory assays to further explore preliminary findings of more and less susceptible *B. napus* varieties.

4. Conduct field trials to further investigate any differences observed in laboratory assays.

2.2 Methods

To investigate how adult CSFB feeding damage varied amongst *B. napus* varieties, a series of experiments were conducted; three laboratory experiments and two years of field trials are demonstrated here, generating phenotypic adult CSFB feeding trait data for *B. napus* varieties.

2.2.1 Beetle culturing

Beetles were collected from the field by sweep-netting or gathering decaying leaf matter in the Summer and Autumn. Additionally, whole oilseed rape plants were collected in Winter and Spring and potted up and contained in a bread bag to allow adults to emerge in the Spring/Summer. Alternatively, these plants were contained in plastic bags and allowed to decay, resulting in larval evacuation. These larvae could then be collected and applied to intact plants. Field collected beetles were kept separately from laboratory cultures until one generation had passed. These offspring were then incorporated into the laboratory population.

All beetles used in experiments were laboratory reared for at least one generation and maintained in a Controlled Environment Room in the John Innes Centre insectary (22°C:22°C and 16h daylength). To continue the laboratory population, adults were collected from bread-bagged whole plants and placed in plastic boxes with ventilation holes and lined with damp blue roll. They were provided with fresh Chinese cabbage leaves weekly. Here beetles would lay eggs down the sides of the blue roll, allowing them to be collected and applied to the base of intact, bagged oilseed rape or Chinese cabbage plants. Larvae would then feed within these plants and adults could be collected upon emergence and moved to boxes for egg laying/collection.

2.2.2 Six-way choice assays

2.2.2.1 Plant material

The *Brassica napus* Diversity Fixed Foundation Set (BnaDFFS) consists of 189 lines of *Brassica napus*, originating from a genetically diverse set of material produced at the university of Warwick developed within the Oilseed RapE Genetic Improvement Network (OREGIN) (Teakle, G., University of Warwick, https://www.brassica.info/resource/plants/diversity sets.php). The RIPR (Renewable Industrial Products from oilseed Rape, https://yorknowledgebase.hosted.york.ac.uk/resources.html) consists of 383 *B. napus lines*, some of which are also part of the BnaDFFS. These diversity sets contain a range of *B. napus* varieties; winter oilseed rape (WOSR), spring oilseed rape (SOSR), Kale, Fodder, Exotic, Synthetic and Swede types, giving them a rich genetic diversity (see Appendix 1 for details of specific varieties, crop types

and origin). From these *B. napus* lines 96 were selected for a baseline initial experiment for exploring adult CSFB feeding preferences.

To generate seedlings for use in CSFB feeding choice assays, ten seeds per *B. napus* line for each chamber were germinated in small petri dishes with damp blue roll (Figure 2.1) in a controlled environment room (CER) (26°C:20°C, 16h day length). After around 16 hours these seeds were potted up into 286 planter trays, cut down to six by five sections containing Levingtons F2 soil, with two seeds per pot (Figure 2.2). These were then grown in the John Innes Centre insectary glassroom (23°C:20°C, 16h day length) for a further six days. These seedlings were checked daily and once the second seedling emerged from a pot it was removed, leaving just one seedling per plot. After these 6 days the seedlings were ready to use in CSFB feeding assays.
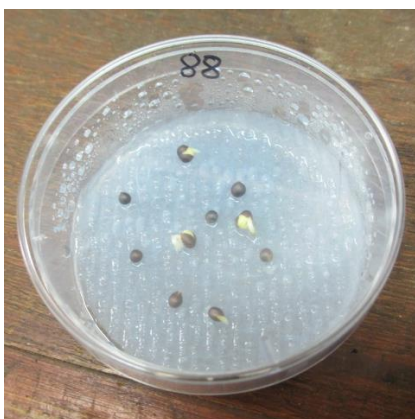
Figure 2.1. Petri dish with damp blue roll for germinating seeds.

Figure 2.2. 286 cell trays cut into six by five sections with seedlings growing in them, each section providing seedlings for one assay.

2.2.2.2 Insect material

Six adult CSFBs were used from a laboratory maintained population per assay, giving a 2:1 ratio of plants per beetle. From a pilot study examining differences in CSFB herbivory between two commercial *B. napus* varieties (Skye and Kielder, obtained from Mark Nightingale, Elsoms Seeds Ltd) under different herbivory pressure (six or 15 beetles), increasing beetle number per assay increased damage amounts observed but also resulted in a reduction of differences between the varieties (Figure 2.3). Therefore, we selected six beetles per assay as the appropriate level of herbivory pressure to examine differences in CSFB damage.
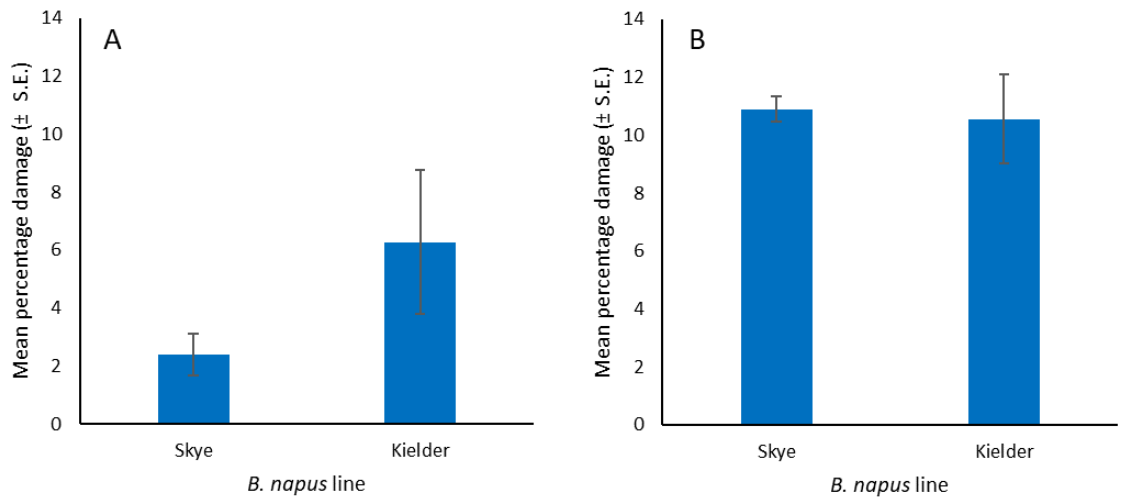
Figure 2.3. Results of a pilot experiment testing different numbers of CSFBs impact on percentage damage to cotyledons for two *B. napus* varieties, Skye and Kielder in two-way choice assays (± standard error). A: damage differences with six beetles per assay and B: differences with 15 beetles per assay. n = 3.

As CSFBs have a four to six week period of aestivation two weeks after eclosion (Alford, 2003 and personal observations), it was decided that beetles would be screened for feeding activity five to seven days prior to inclusion in a feeding assay. The screen involved starving beetles for 24 hours before introducing single beetles to a Chinese cabbage leaf disc on agar and allowing them to feel for 48 hours (Figure 2.4). If the beetle had consumed more than 5% of the leaf disc area it was deemed a ''feeder'' and used in subsequent chambers.
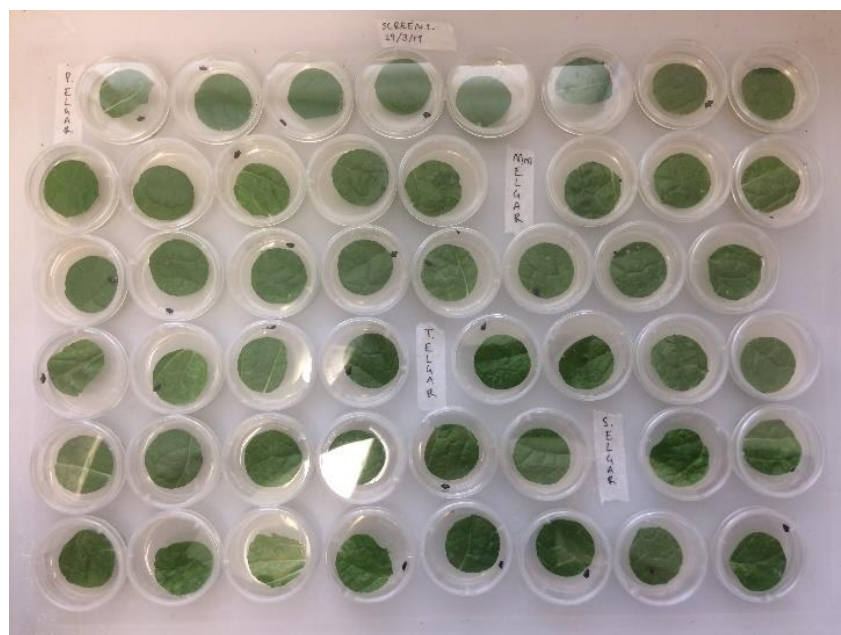
Figure 2.4. Example of setup used to examine whether CSFB were currently active and feeding, with one beetle per petri dish containing a disc of Chinese cabbage leaf on agar.

2.2.2.3 Design and process of running six-way choice assays

A number of adaptations were made to the petri dish assay initially designed by Anna Jordan in the John Innes Centre insectary before developing the final choice-chamber set up displayed in Figure 2.6. Assays ran for 48 hours, the same duration as other researchers have previously selected when running laboratory feeding experiments with flea beetles (Bartlet et al., 1996; Soroka et al., 2011). To aid with seedlings wilting throughout the duration of the assays, pots were covered with cling film to help retain moisture in the soil. Additionally, halfway through the assay (at 24 hours) plants were watered 1.5ml water with a syringe and needle directly into the soil to prevent them from desiccating. Furthermore, the inclusion of agar in the base of the petri dish kept moisture levels higher in the chamber. The agar also prevented beetles from squeezing through gaps to escape as the lid could slot into the agar creating a tight seal.

To set up the six-way choice assays, plant pots containing seedlings were cut up and slotted into grooves in the base of the petri dish and standard water agar as demonstrated in Figure 2.6. Choice chambers consisted of 12 plants of six different *B. napus* lines from the 96 being tested, with replicates opposite each other (Figure 2.5). *B. napus* variety Matador was the control so appeared in every chamber and additionally replaced any missing lines. Each *B. napus* line appeared in three separate chambers according to an alpha design, to account for interactions between accessions within chambers (Figure 2.9). Six beetles were introduced to the chamber after being starved for 24 hours. Chambers ran for 48 hours in a CER at 22°C:22°C and 16h daylength.

Figure 2.5. The layout of different *B. napus* accessions in a six-way choice chamber, with the letters representing two replicate seedlings of each variety opposite each other in an assay, making 12 plants in total.

Figure 2.6. The final choice chamber assay design with six accessions slotted into agar, two seedlings of each. Lid secured with micropore tape and hole in centre to add the beetles, sealed with a foam bung. Clingfilm covers the plant pots to help keep soil damp.

After 48 hours of feeding, beetles were removed, and plants scored for a number CSFB feeding types. Firstly, if the first two true leaves had started growing at the start of the assay, they were scored for herbivory damage; 1) a score of 1 denoting complete, intact true leaves, 2) a score of 0.5 indicating herbivory damage and 3) a score of 0 demonstrating complete consumption of true leaves. Secondly, herbivory damage to stems was scored by assigning a score of 0 if no damage had occurred, or a score of 1 if feeding damage was present.

For the next three feeding traits, cotyledons were removed from seedlings and laid out on white paper, as demonstrated in Figure 2.7. Figure 2.8 displays the three feeding behaviours recorded. Shot holing, where beetles eat completely through the leaf, and grazing, where beetles just consume the surface of the leaf, were visually estimated to the nearest 5% of total area of the cotyledons. A final metric was scored where an estimate of total percentage damage to cotyledons was estimated. If for any of these three feeding traits, shot holing, grazing or total, had less than 5% damage they were given a score of 1%. This was to enable recording of the fact some damage had occurred, even if minimal as this could provide important information about the beetles feeding behaviours.

Table 2.1 summarises the timeline for plant and beetle preparation, setup and running of the assay and shutdown and scoring.
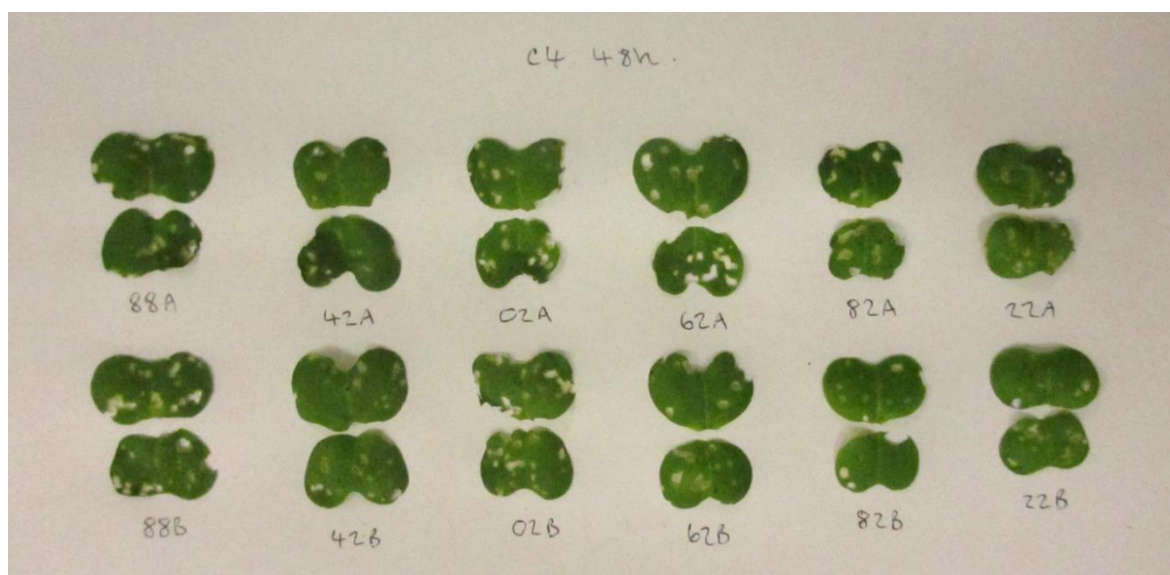
Figure 2.7. Cotyledons removed from seedlings, laid out on white paper for scoring percentage eaten after 48h in choice chamber with six CSFB adults.
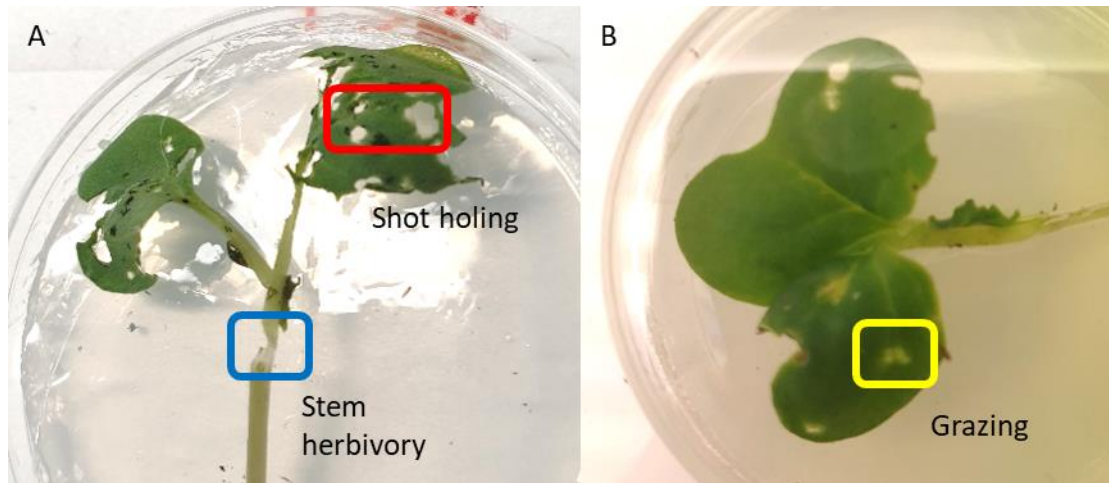
Figure 2.8. Examples of the different types of feeding damage traits scored for *B. napus* seedlings, with total damage to cotyledons comprising of a combined score of shot holing and grazing damage. For total damage, shot holing and grazing, cotyledons were scored visually to the nearest 5%. A: Photograph demonstrating shot holing damage in the red box and stem herbivory in the blue box. B: Photograph demonstrating grazing damage in the yellow box.

Table 2.1. Process for preparing plant and beetle material, running and shutting down a choice chamber assay.

Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9
Germinate seeds	Plant seeds	Screen beetles for feeding	Screen beetles for feeding		Beetles starved for 24 hours	Assay begins	Seedlings watered	Shutdown assay and score

2.2.2.4 Statistical analysis

The experiment ran over the course of three blocks, organised by an alpha design demonstrated in Figure 3.9. 96 *B. napus* lines were screened for CSFB herbivory, each appearing once per three blocks. The alpha matrix was designed to ensure that the same lines would not appear together in subsequent assays (with the exception of Matador which was present in every assay as a control). In total 60 assays were run, giving three replicated per *B. napus* line, except for the control line Matador which appeared in all 60. *B. napus* lines were randomly assigned to numbers shown in Figure 2.9, making the experiment blind.

Assay Number

Block Number		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
		41	69	95	65	73	84	80	43	87	22	99	46	76	91	92	54	37	38	50	68
		81	49	55	85	53	44	40	63	7	42	79	86	96	71	12	74	97	78	10	48
	1	21	9	15	5	93	4	60	23	27	82	19	66	36	31	72	94	77	18	70	8
		1	89	35	25	33	24	100	83	47	2	59	26	56	11	32	14	17	98	90	88
		101	29	75	45	13	64	20	3	67	62	39	6	16	51	52	34	57	58	30	28
		78	66	97	56	20	31	77	60	64	79	76	33	73	38	39	71	74	25	41	65
		83	91	72	62	70	86	82	54	89	84	50	57	1	68	43	19	48	75	15	13
	2	28	36	100	87	18	101	27	85	34	7	81	63	98	16	69	96	99	80	67	90
		52	40	46	10	95	9	51	30	58	29	26	88	47	93	94	45	24	49	92	59
		6	14	22	32	44	55	5	8	12	53	4	11	23	42	17	21	2	3	37	35
		96	84	49	34	47	58	62	57	41	97	28	40	50	86	21	23	92	52	44	70
		55	71	6	59	88	15	95	65	18	64	101	81	7	45	46	76	51	60	85	83
	3	63	38	77	80	22	99	11	32	82	13	10	35	91	2	74	5	8	93	72	37
		12	100	90	16	4	33	29	98	36	56	94	17	25	20	87	89	26	9	39	42
		30	43	24	67	75	66	54	14	69	31	53	68	78	73	3	48	79	27	1	19

Figure 2.9. Alpha design demonstrating each *B. napus* line of the 96 being tested, appearing in three chambers, once in each block, designed to account for interactions between accessions within

To examine differences in herbivory levels between *B. napus* lines, damage means were analysed for each feeding trait (total, shot holing, grazing and stem) with a linear mixed model fitted via the restricted maximum likelihood method (REML). Percentage data was LOGIT+ transformed for analysis (Equation 2.1).

$$\ln \frac{x + 1.25}{101.25 - x}$$

Equation 2.1. Logit+ transformation equation

First true leaf damage was not analysed due to developmental differences between *B. napus* lines meaning many varieties could not be scored, resulting in a lot of missing data. For the four damage traits successfully scored, Pearsons correlations were run between them to understand how different types of herbivory related to each other. Data was analysed using Genstat software (VSN International, 2015).

2.2.3 Two-way and non-choice assays of Altasweet and Apex-93_5 X Ginyou_3 DH Line

After conducting the baseline experiment assessing adult CSFB feeding differences between 96 *B. napus* lines using six-way choice chamber assays, two were selected for further investigation. Altasweet was selected for demonstrating higher levels and Apex-93_5 X Ginyou_3 DH Line for demonstrating lower levels of CSFB feeding damage.

2.2.3.1 Experimental design, plant and beetle material

From running six-way choice assays, it was discovered that beetles may not be able to distinguish between *B. napus* lines as effectively as aimed for. Therefore, another experiment was conducted with a similar assay set up but with either a two-way choice or no choice of food.

Two assay setups were piloted for two-way choice assays, either alternating seedlings of each variety or a chamber split into 50:50 of each variety, with one in the left half of the petri dish and the other in the right. Commercial varieties Skye and Kielder were used to test these different setups and results are displayed in Figure 2.10. From this, it was observed that a half and half setup gave clear distinctions in CSFB herbivory and thus selected this method (Figure 2.11 demonstrates this setup).
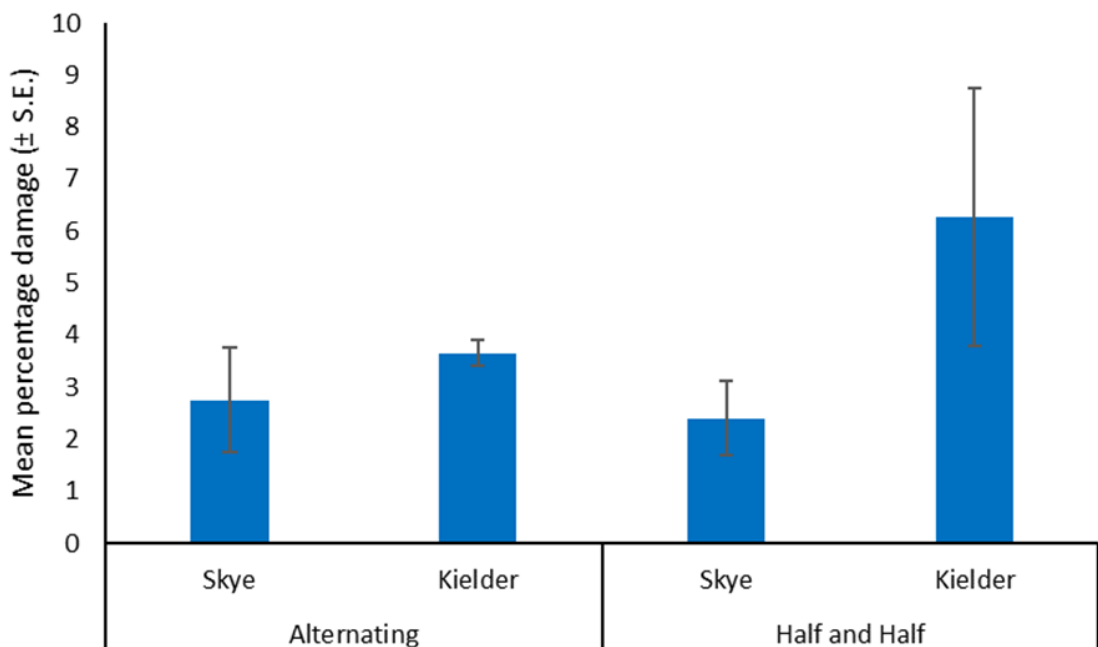
Figure 2.10. Results from two-way choice assays of *B. napus* varieties Skye and Kielder for CSFB herbivory damage (± standard error), in a setup where seedling variety alternates between the two varieties in each slot and where the chamber is split into half of one variety and half of the other.

Figure 2.11 demonstrates how two-way choice assays were laid out, with six seedlings of Altasweet and Apex-93_5 X Ginyou_3 DH Line. In no choice assays, CSFB were offered 12 seedlings of the same *B. napus* variety, either Altasweet or Apex-93_5 X Ginyou_3 DH Line.
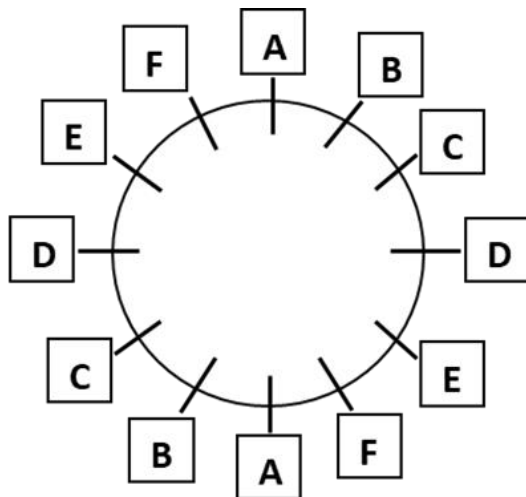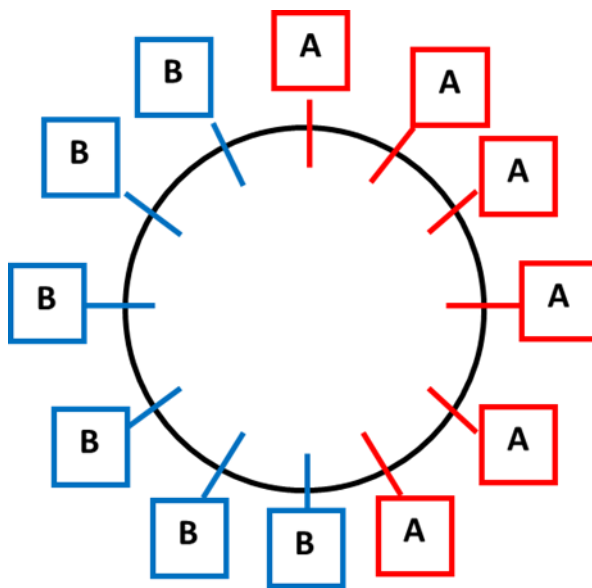

Figure 2.11. The layout of two *B. napus* accessions in a two-way choice chamber, with the letters representing six replicate seedlings of each variety in an assay, making 12 plants in total.

Plants and beetles were prepared in the same way as in six-way choice assays, other than plants being grown in the same CER (22°C:22°C and 16h daylength) where the assays were run. Assays were also conducted and scored in the same way as previously described other than no longer scoring for first true leaf damage (due to differences in development of *B. napus* lines).

2.2.3.2 Statistical analysis

To investigate differences in herbivory levels between Altasweet and Altasweet or Apex-93_5 X Ginyou_3 DH Line, damage means for each feeding trait (total, shot holing, grazing and stem) were compared with a two-way ANOVA. Percentage data was LOGIT+ transformed for analysis (Equation 2.1). Two-way choice and no-choice assays were treated as separate experiments for analysis. Rstudio software was used for analysis (http://www.rstudio.com/).

2.2.4 Three-way choice and no-choice assays of Altasweet, Apex-93_5 X Ginyou_3 DH Line and their F1 cross

Next an experiment was to confirm observed differences between Altasweet and Apex-93_5 X Ginyou_3 DH Line, in addition to testing herbivory levels of their F1 cross. Additionally, a different methodology was examined for scoring cotyledon percentage damage, using image analysis.

2.2.4.1 Plant material

A F1 cross of Altasweet and Apex-93_5 X Ginyou_3 DH Line was generated by growing these parental *B. napus* lines to maturity in a glasshouse. They were then monitored for flowering and first blooms removed. Using a fine paint brush, pollen was taken from the anthers of one parent and applied to the just exposed stigma of the other. This was done for five to ten buds and then contained in a perforated bag and secured with a paper tie to ensure pollinating insects could not access the buds/flowers. These were left to mature and when ready cut from the plant and threshed to obtain the seed.

A subset of these seeds were then grown in a glasshouse to generate F2 crosses (not for use in these experiments). Leaf material from these F1 plants, along with parental lines Altasweet and Apex-93_5 X Ginyou_3 DH Line, was sampled and frozen to obtain DNA from. DNA extraction was done using Edwards DNA extraction protocol (Edwards et al., 1991).

The microsatellite marker CJR534_BRMS-071 amplified different bands for the parental lines (obtained from Rachel Wells, Table 2.2) and QiagenTaq DNA Polymerase (Qiagen) was used to conduct a Polymerase Chain Reaction (PCR). The final reaction volume was 20µl, consisting of; 1µl genomic DNA (20ng), 2µl PCR buffer, 0.25µl dNTPs (10mM), 0.25µl for forward and reverse primers (10µM), 0.2µl QiagenTaq (5u/µl) and 16.05µl water. The PCR programme ran as follows; Initial denaturation at 94°C for 2 minutes, then denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute, for 30 cycles. Then there was a final extension at 72°C for 4 minutes, finishing with a final cooldown at 12°C.

Table 2.2. Primer details used for genotyping F1 plants of Apex-93_5 X Ginyou_3 DH Line and Altasweet cross.

Primer name	Sequence
CJR534_BRMS-071_F	CAAAGCGAGAAAGTGCAGTTGAGAG
CJR534_BRMS-071_R	TCCACGAAACTACTGCAGATTGAAA

Gel electrophoresis was used to visualise the amplified PCR products. To do this, 5µl PCR products were mixed with loading dye and run on a 2% agarose gel (10g agarose, 500ml 10X Tris Borate EDTA (TBE), 5µl ethidium bromide). A 100bp DNA ladder was used as a reference. Resulting DNA bands confirmed that Apex-93_5 X Ginyou_3 DH Line and Altasweet had successfully been crossed to create a F1 line and thus currently growing F1 plants were suitable to take forward for F2 lines and the F1 seed suitable for growing seedlings for adult CSFB feeding assays.

2.2.4.2 Beetle material

Adult CSFBs were obtained from captive populations and screened for feeding activity as previously described. However, differences in herbivory amounts between male and female beetles (observed by Lucy Thursfield) directed us to control the sex ratio of beetles entering assays. Beetles were sexed by examining their tarsal segments under a microscope. Females were differentiated from males by having a triangular shaped tarsal segment and males more broad, heart shaped tarsal segments. Six beetles were used in a 1:1 ratio of males and females.

2.2.4.3 Experimental design

Assays were conducted in the same petri dish setup as previously described, consisting of 12 *B. napus* seedlings. Here there was no choice, with assays consisting of just one *B. napus* line, either Altasweet, Apex-93_5 X Ginyou_3 DH Line or the F1 cross of these parental lines. Additionally, three-way choice assays of these lines were run alongside these, arranged as demonstrated in Figure 2.12.
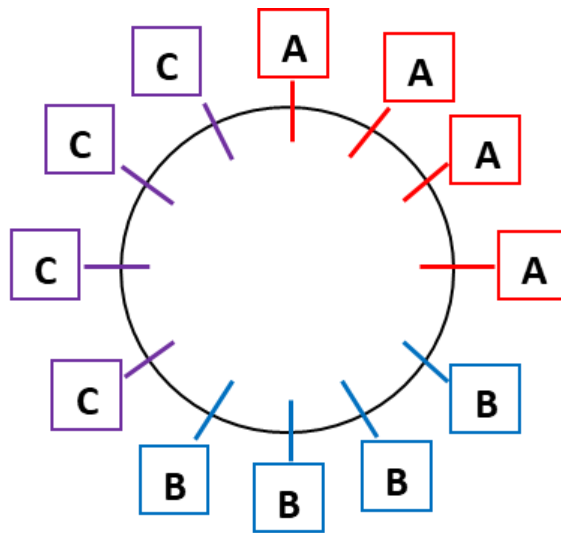
Figure 2.12. The layout of three *B. napus* accessions in a three-way choice chamber, with the letters representing four replicate seedlings of each variety in an assay, making 12 plants in total.

For scoring, grazing and stem damage were not recorded. Additionally, shot holing damage was not scored as this was largely captured by scoring total percentage damage to cotyledons. Total percentage damage to cotyledons was scored again by visual estimates to the nearest 5%. However, ImageJ software was also used to obtain more accurate, computerised scores for percentage damage to cotyledons.

The process involved placing cotyledons removed from seedlings onto a PVC A4 sized plastic board and scanning them. These images were then thresholded and interpolated in ImageJ, before running a pipeline script (developed by Lucy Thursfield) to extract percentage loss of cotyledon area (i.e. percentage damage scores).

2.2.4.4 Statistical analysis

To compare means for total percentage damage to cotyledons for Altasweet, Apex-93_5 X Ginyou_3 Line and an F1 cross of these two *B. napus* lines, data was analysed with a two-way ANOVA. As data was percentage it was LOGIT+ transformed (Equation 2.1). Three-way choice and no-choice assays were treated as separate experiments for analysis. Additionally, visually estimated scores and computerised scores were analysed separately. These were then correlated to see how visual estimates related to scores from image analysis via ImageJ. Analyses were conducted in Rstudio (http://www.rstudio.com/).

2.2.5 Field trials of 2019-2020

Conducting controlled laboratory experiments enabled identification of variation in palatability across different *B. napus* lines. To better understand differences in damage in a more natural environment, two field trials were conducted, the first in 2019, introduced in the following section.

2.2.5.1 Plant material and field treatment

In total eight *B. napus* lines were selected for inclusion in these field trials. Based upon data collect from laboratory adult CSFB feeding assays, six *B. napus* lines from the 96 tested in the laboratory were included. Apex and Apex-93_5 X Ginyou_3 DH Line were included as having lower levels of feeding and York, Altasweet and Shannon X Winner were included as having higher levels of feeding in the laboratory. Cabriolet was included as it had an intermediate to high level of damage recorded in the laboratory. In addition to these *B. napus* lines, two commercial varieties were included, Skye and Kielder (obtained from Mark Nightingale, Elsoms Seeds Ltd).

To increase chances of successful data collection, two field trials were run at two locations: One at The Morley Agricultural Foundation, in Morley St. Botolph (about three miles away from Wymondham, Norfolk) and one at the John Innes Centre field station, in Bawburgh (about two and a half miles away from the main John Innes Centre (JIC) site). Both had the same plot and block layout (Figure 2.13), with same seed densities used (roughly 864 seeds per plot) and a plot size of 4m x 1.5m (6m x 2.7m including border rows). Both trials were ploughed the day prior to drilling and harrowed the morning of drilling. After drilling (drill depth one to two centimetres) the trials were rolled, and no insecticides used throughout the duration of the trials. Fertilisers and herbicides were used to aid the establishment of *B. napus* seedlings (see Appendix 2 for full application schedule).

Block 1	ShannonXWinnner	York	Skye	Kielder	Apex	Cabriolet	ApexXGinyou	Altasweet
Block 2	ApexXGinyou	Apex	Cabriolet	York	Altasweet	ShannonXWinner	Skye	Kielder
Block 3	York	ApexXGinyou	ShannonXWinner	Apex	Cabriolet	Kielder	Altasweet	Skye
Block 4	Cabriolet	Skye	Kielder	Altasweet	ShannonXWinner	Apex	York	ApexXGinyou
Block 5	Kielder	Altasweet	York	Cabriolet	ApexXGinyou	Skye	ShannonXWinner	Apex

Figure 2.13. Schematic of plots of eight *B. napus* lines laid out in complete randomised block design at Bawburgh field station and Morley Agricultural Foundation in 2019.

The main difference between the two trials (aside from location) was the drilling date. Morley trial was drilled on 23/08/2019 and Bawburgh on 29/08/2019. Unfortunately, the weather following drilling at Morley was very warm and dry, resulting in very poor establishment. Given the lack of establishment and damage from bird activity, the Morley trial was not suitable for any data collection and thus abandoned shortly after drilling. Establishment of the Bawburgh field trial was

more successful, likely due to a better soil moisture content and slightly cooler temperatures. Therefore, Bawburgh site became the only focus for collecting field data.

However, feeding pressure from adult CSFB was too great for the plots to fully establish, so the Bawburgh trial was also abandoned 01/11/2019. Nonetheless, the trial survived long enough to allow successful data collection of CSFB herbivory.

2.2.5.2 Scoring methodology

To understand how different *B. napus* lines established in the field, we used a drone image to count number of plants per plot. Drone images were taken on 03/10/2019, 35 days after drilling (taken by Phil Robinson on the following drone model: DJI Phantom 4 Pro V2, picture size: 20MP, picture format: .DNG (RAW), but .tiff used for analysis. Note this model of drone does not record height accurately, but images were taken approximately 20m above the trials). However, establishment was poor for *B. napus* lines Skye and Kielder, uncharacteristically so for commercial varieties, thus these were removed from analyses.

To assess whether there were differences in percentage damage to seedlings in the field, 20 plants were sampled from border runs for each experimental plot and brought into the laboratory in petri dishes lined with damp blue roll to keep them from desiccating, on 18/09/2019, 20 days after drilling. Cotyledons were removed and laid out on white plastic making CSFB shot holes clearer, then visually scored for percentage damage to the nearest 5%. If a seedling had no cotyledons, this was removed from the analysis.

2.2.5.3 Statistical analysis

Means for establishment (number of seedings per plot) and damage (percentage eaten for 20 seedlings per plot) were both analysed, separately, using a two-way ANOVA. Percentage damage data was LOGIT+ transformed for analysis (Equation 2.1). These data were then correlated to see if there was a relationship between establishment and damage. Field herbivory scores were further correlated with scores from six-way choice assays to better understand how laboratory derived damage scores related to in field damage scores. Analyses were conducted using Rstudio software (http://www.rstudio.com/).

2.2.6 Field trials of 2020-2021

After losing the 2019 field trial early due to high levels of pest damage, a second round of field trials was run at Bawburgh field station in 2020 to 2021. These trials consisted of two parts; a non-pesticided sprayed part, treated the same way as the trial in 2019, and a pyrethroid treated part, as ''insurance'' to help ensure *B. napus* plants could make it through the winter where CSFB larvae invade plants.

2.2.6.1 Plant material and field treatments

Nine *B. napus* lines were selected for the 2020 field trials. The same six *B. napus* lines from the 2019 field trial were selected again, based upon laboratory and field damage data; Apex and Apex-93_5 X Ginyou_3 DH Line with lower levels of feeding, Cabriolet with intermediate levels of feeding and York, Altasweet and Shannon X Winner with higher levels of feeding. In addition, three commercial *B. napus* varieties were selected; Skye and Kielder (as with the previous year) and Elgar (obtained from Mark Nightingale, Elsoms Seeds Ltd). Due to limitations in seed numbers for some varieties, Elgar was used to replace these plots (see Figure 2.14).

After observing such high levels of adult CSFB the previous year, we ran two field trials to increase our chances of successful data collection into the larval CSFB season during winter. This included a non-pesticide treated trial, similar to that ran in 2019. The other ran alongside was treated with pyrethroid pesticides. Figure 2.14 demonstrates the layout of both trials, giving an incomplete block design. Both trials were treated the same other than pesticide treatment, with about 864 seeds per plot drilled and a plot size of 4m x 1.5m (6m x 2.7m including border rows). They were ploughed the day prior to drilling, harrowed the morning of drilling and rolled once drilled. Fertilisers and herbicides were used to aid the establishment of *B. napus* seedlings in both insecticide treated and non-treated trials (see Appendix 3 for full application schedule).

Block 1	Block 2	Block 3	Block 4	Block 5			Block 1	Block 2	Block 3	Block 4	Block 5
Kielder	Altasweet	Apex	Cabriolet	Elgar (replaced Skye)			Altasweet	Elgar (replaced Skye)	ApexXGinyou	Apex	ShannonXWinner
Apex	Cabriolet	ApexXGinyou	Elgar (replaced SW)	York			Apex	York	Elgar (replaced SW)	Skye	Kielder
ApexXGinyou	Skye	Cabriolet	York	ShannonXWinner			Cabriolet	ApexXGinyou	Altasweet	ShannonXWinner	York
ShannonXWinner	Kielder	Elgar (replaced Skye)	Altasweet	ApexXGinyou			ApexXGinyou	ShannonXWinner	Apex	Kielder	Altasweet
Elgar (replaced York)	Apex	Kielder	Skye	Cabriolet			Kielder	Cabriolet	Elgar (replaced York)	Altasweet	Elgar (replaced Skye)
Altasweet	York	ShannonXWinner	ApexXGinyou	Kielder			York	Kielder	Skye	Cabriolet	Apex
Skye	Elgar (replaced SW)	Altasweet	Kielder	Apex			Elgar (replaced SW)	Altasweet	Kielder	ApexXGinyou	Cabriolet
Cabriolet	ApexXGinyou	York	Apex	Altasweet			Skye	Apex	Cabriolet	Elgar (replaced York)	ApexXGinyou
		Pesticide treated							Non-pesticide treated		

Figure 2.14. Schematic of plots of nine *B. napus* varieties for pesticide treated (left) and non-pesticide treated (right) trials, organised in an incomplete block design. Yellow plots in between the two trials represent *B. napus* variety Dazzler to serve as a barrier between pesticide treated and non-treated trials. Note ''SW'' represented Shannon X Winner DH Line.

2.2.6.2 Scoring methodology

Drone images were used to obtain seedling counts per plot, on 15/09/2020, 25 days after drilling, to better understand how well different *B. napus* lines established in pesticide treated and non-treated trials (taken by Phil Robinson, same drone details as the 2019 trial). Figure 2.15 provides an example of the types of drone images used for scoring. Establishment was better than in the 2019 field trial and thus all *B. napus* lines could be examined for CSFB damage.

CSFB herbivory was scored in-field by visually estimating percentage damage to ten seedlings cotyledons per plot, on date 22/09/2020, 31 days after drilling. A random area within the plot was selected and then a continuous run of plants scored. If a seedling had no cotyledons present it was not included in the analyses.

Figure 2.15. An example of a drone image used to score for establishment by counting number of seedlings per plot.

2.2.6.3 Statistical analysis

Pesticide treated and non-treated plots were considered as separate trials and thus analysed separately. For both, establishment was analysed by running a two-way ANOVA to compare mean seedling counts per *B. napus* line. For the non-pesticide treated trial, seedling count scores from 2020 were correlated with 2019 scores to compare establishment between years. This comparison was not appropriate for pesticide treated plots and the field trial in 2019 did not include pesticide treatments.

Mean percentage damage scores for *B. napus* lines were analysed by running a two-way ANOVA, investigating pesticide treated and non-treated separately. As with other percentage damage data, it was LOGIT+ transformed for analysis (Equation 2.1). For both treated and non-treated trials, CSFB herbivory scores were correlated with establishment to understand if there was a relationship between them. The non-pesticide treated damage data was additionally correlated with laboratory and field 2019 scores to examine the consistency of herbivory to specific *B. napus* lines.

For the pesticide treated field trial, it was not appropriate to compare damage scores to those obtained from the laboratory assays or the 2019 field trial as they had not received insecticide treatments. However, we could compare them to the non-pesticide treated 2020 field trial as they were grown side by side and wanted to better understand how insecticide treatment may have influenced CSFB herbivory. All analyses were conducted in RStudio (http://www.rstudio.com/).

2.3 Results

2.3.1 Variation recorded in feeding damage to *B. napus* seedlings in six-way choice chambers

Using 96 *Brassica napus* lines from the Diversity Fixed Foundation Set (originating from a genetically diverse set of material produced at the university of Warwick developed within the Oilseed Rape Genetic Improvement Network, (Teakle, G., University of Wawrick, https://www.brassica.info/resource/plants/diversity_sets.php)) and RIPR (Renewable Industrial Products from oilseed Rape, https://yorknowledgebase.hosted.york.ac.uk/resources.html) diversity set, the aim was to test differences in palatability to adult cabbage stem flea beetle (CSFB), with the hypothesis that there will be differences in CSFB feeding damage across the accessions. To test the hypothesis that there are differences in palatability between varieties, six-way choice assays were conducted using *B. napus* seedlings according to an alpha design (generated by Rachel Wells and James Brown), with a control accession (Matador), present in every assay (see 2.2 Methods for further details on choice chamber development and design).

The experiment involved scoring for the following five CSFB feeding traits; (1) Total damage (an overall seedling percentage damage score), (2) Shot holing damage (a percentage damage score referring only to holes that go entirely through the cotyledon), (3) Grazing damage (a percentage damage score referring to surface cotyledon damage only), (4) Stem damage (a 0 or 1 score referring to the stem being chewed or intact, respectively, with mean scores varying between 0 and 1) and (5) True Leaf damage (a 0, 0.5 or 1 score according to 0 true leaves, partially damaged true leaves or intact true leaves). See 2.2.2.3 Methods Figure 2.8 for photographic examples of the types of damage recorded. Due to developmental differences between *B. napus* varieties, the trait true leaf damage was missing too many data points to be analysed any further. Raw data for total, shot holing and grazing traits has been LOGIT+ transformed (see Methods 2.2 Equation 1) for analysis of percentage data.

Data were analysed using a linear mixed model (fit by REML), with the fixed effect of Line (*B. napus* variety) as our main effect of interest. Random effects are Block (as per the alpha design), Block.Date (accounting for variation between dates within a block) and Block.Date.Assay (accounting for variation between assays (individual choice-chambers) within dates within a block), summarised in Equation 2.2. The same model was used to assessing differences in damage between *B. napus* crop types, where instead of ''Line'', ''Crop type'' is used. Due to restrictions of this REML variance components analysis, we do not generate F statistics or *p* values for random effects.

$$\mathrm{lmer}(\mathrm{formula} = \mathrm{Trait} \sim 1 + \mathrm{Line} + (1|\mathrm{Block} + \mathrm{Block.Date} + \mathrm{Block.Date.Assay})$$

Equation 2.2. Linear mixed model used to analyse six-way choice assays of 96 *B. napus* varieties. Fixed effects = *B. napus* line (or when specified, crop type). Random effects = Block (three blocks from an alpha design), Block.Date (for variation between dates within blocks) and Block.Date.Assay (for variation between assays within dates within blocks). Trait = measurable variable of interest, indicating total, shot holing, grazing, stem and true leaf damage.

A two-way ANOVA was run, to assess whether total damage for control variety Matador varies significantly between blocks, assays and whether there is an interaction between the two. The results of this ANOVA are summarised in Table 2.3 and revealed that there was a significant variation in total damage score for Matador between blocks, but not between assays and there was no interaction between blocks and assays. Therefore, the term assay was removed from the model and was re-run as a one-way ANOVA, summarised in Table 2.4.

Table 2.3 Output summary from a two-way ANOVA assessing variation of total feeding damage on Matador between blocks, assays, and their interaction.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Block	2	8.44	4.219	6.922	0.00157
Assay	19	19.20	1.011	1.6858	0.05813
Block:Assay	38	32.51	0.855	1.404	0.09524
Residuals	94	57.29	0.610		

Table 2.4. Output summary from a one-way ANOVA assessing variation of total feeding damage on Matador between blocks, with assay and block:assay interaction removed.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Block	2	8.44	4.219	6.922	0.00157
Residuals	151	109.00	0.722		

Due to the significant effect of block, data collected in this experiment has been adjusted for between block variation. Adjusted mean percentage scores were back-transformed using the EXPIT+ function (Equation 2.3) with these results displayed in the following sections.

$$x = \frac{(101.25e^y - 1.25)}{(1 + e^y)}$$

Equation 2.3. EXPIT+ back-transformation equation

2.3.1.1 Total feeding damage

Total percentage damage was scored with the aim of identifying overall herbivory differences for *B. napus* accessions. Equation 2.4 displays the linear mixed model used to analyse total percentage damage data. Adjusted mean total damage scores vary across the accessions, ranging from 0.89% to 9.03% for accessions Jaune A Collet Vert and York, respectively (Figure 2.15). However, this variation was over a limited range and no statistically significant difference was observed for *B. napus* line (Table 2.5). Additionally, all random factors were kept in the model as they explained a large amount of variation observed in total percentage damage (Table 2.6).

$$\text{lmer(formula} = \text{Total percentage damage} \sim 1 + \text{Line} + (1|\text{Block} + \text{Block. Date} + \text{Block. Date. Assay})$$

Equation 2.4. Linear mixed model used to assess the effect of *B. napus* line on total percentage damage.

Table 2.5. Output from linear mixed model demonstrating a statistically non-significant effect of *B. napus* line on total percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Line	96	112.21	1.17	0.145

Table 2.6. Output from linear mixed model, summarised in Figure 3.18, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0677	0.0815
Block.Date	0.0189	0.0482
Block.Date.Assay	0.1856	0.0612
Residual	0.530	0.0316

We also were interested in the effect of crop type on total percentage damage to different *B. napus* lines. Running the model defined in Equation 2.5, no significant differences between crop types for total percentage damage were observed (Table 2.7). Again, there was a large component of variation attributable to random factors of the model, and thus are kept in the analysis (Table 2.8).

$$\text{lmer(formula} = \text{Total percentage damage} \sim 1 + \text{Crop type} + (1|\text{Block} + \text{Block. Data} + \text{Block. Date. Assay)}$$

Equation 2.5. Linear mixed model used to assess the effect of crop type on total percentage damage.

Table 2.7. Output from linear mixed model demonstrating a statistically non-significant effect of *B. napus* crop type on total percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Crop type	5	3.24	0.65	0.664

Table 2.8. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0682	0.0818
Block.Date	0.0294	0.0419
Block.Date.Assay	0.1580	0.0502
Residual	0.548	0.0303

Therefore, from this experimental design it could not be concluded whether there were differences between *B. napus* varieties for total percentage CSFB feeding damage. However, these results provided preliminary limited evidence for variation in total damage to *B. napus* cotyledons from CSFB which prompted further investigation of selected lines.

Figure 2.15. Variation in the adjusted mean total percentage damage to cotyledons from adult CSFB herbivory for 96 *B. napus* accessions (± standard error). Colour equates to crop type groupings (Blue = WOSR, Green = SOSR, Red = Swede, Orange = Kale/Forage/Leaf, Yellow = Synthetic, Purple = Semiwinter OSR). See Appendix 1 for specific crop type for each accession. n = 3 except for Matador, where n = 60.

2.3.1.2 Shot holing feeding damage

Shot holing damage was scored with the aim to see if there were different types of feeding behaviour exhibited by CSFB. Adjusted mean shot holing damage scores ranged from 0.31% to 8.01%, for accessions Amber X Commanche DH Line and Altasweet, respectively (Figure 2.16) and largely followed a similar pattern to total damage, which is expected as shot holing is CSFB's main feeding behaviour (see Results 2.3.1.5 for a correlation between these damage traits). Similarly, although there was variation in shot holing damage across the varieties, running the model defined in Equation 2.6 demonstrated that there were no statistically significant effect of *B. napus* variety (Table 2.9). Likewise, strong levels of variation attributable to random effects were observed, hence they are again retained in the model (Table 2.10).

$$\text{lmer(formula} = \text{Shot holing percentage damage} \sim 1 + \text{Line} + (1|\text{Block} + \text{Block.Date} + \text{Block.Date.Assay})$$

Equation 2.6. Linear mixed model used to assess the effect of *B. napus* line on shot holing percentage damage.

Table 2.9. Output from linear mixed model demonstrating a statistically non-significant effect of *B. napus* variety on shot holing percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Line	96	118.54	1.23	0.077

Table 2.10. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0822	0.0977
Block.Date	0.0240	0.0528
Block.Date.Assay	0.1986	0.0673
Residual	0.650	0.0387

When assessing for differences in shot holing damage for crop type, running the model displayed in Equation 2.7 demonstrated no significant difference (Table 2.11). Following a similar pattern to total percentage damage, unsurprisingly large components of variation explainable by random factors of the model (Table 2.12) were observed.

$$\text{lmer(formula} = \text{Shot holing percentage damage} \sim 1 + \text{Crop type} + (1|\text{Block} + \text{Block. Date} + \text{Block. Date. Assay})$$

Equation 2.7. Linear mixed model used to assess the effect of crop type on shot holing percentage damage.

Table 2.11. Output from linear mixed model demonstrating a statistically non-significant effect of *B. napus* crop type on shot holing percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Crop type	5	4.52	0.90	0.477

Table 2.12. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0822	0.0974
Block.Date	0.0370	0.0451
Block.Date.Assay	0.1611	0.0531
Residual	0.678	0.0375

Therefore, shot holing CSFB percentage damage demonstrated limited variability attributable to *B. napus* variety or crop type and were similar to total percentage damage scores.

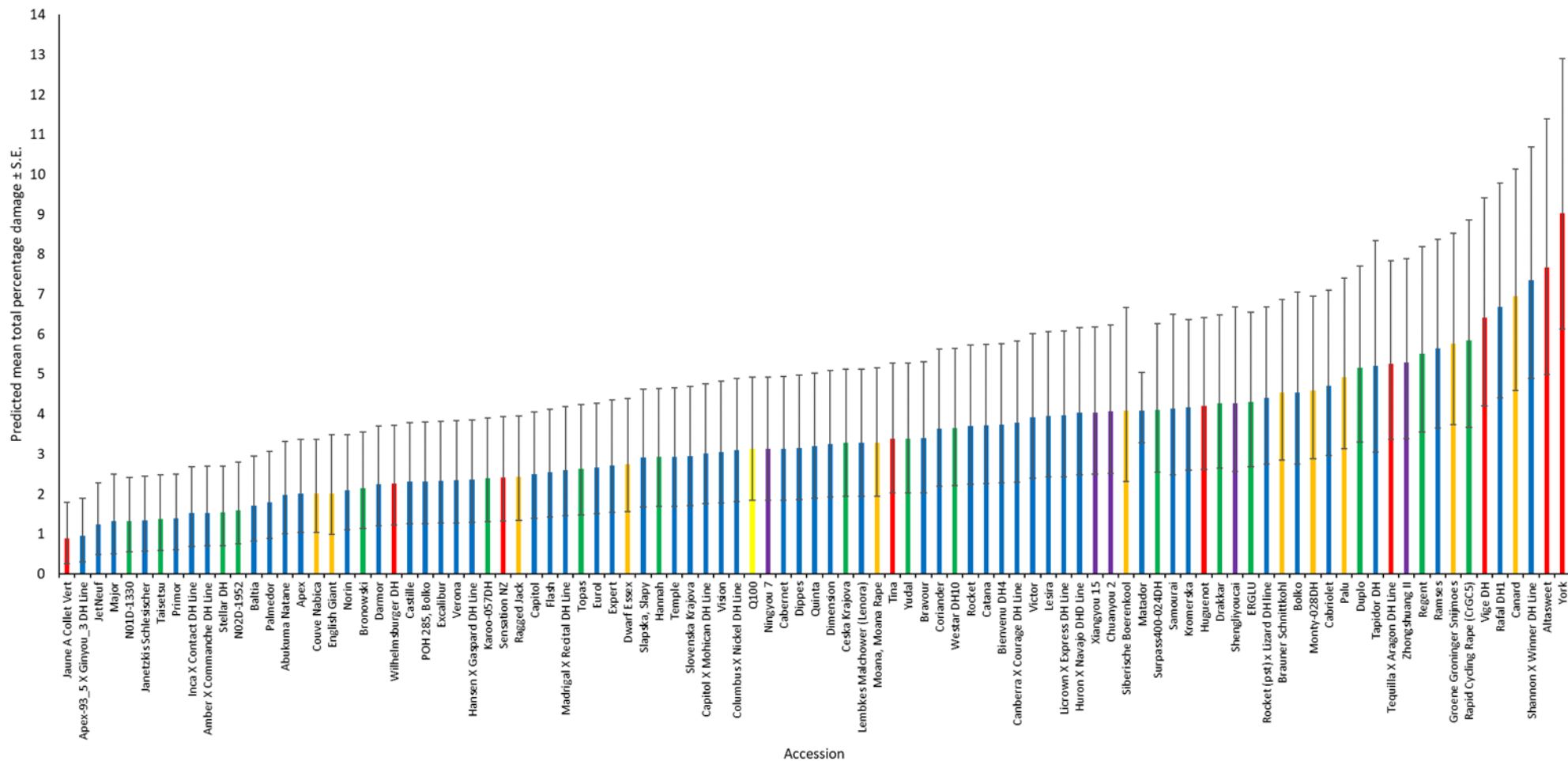Figure 2.16. Variation in the adjusted mean shot holing percentage damage to cotyledons from adult CSFB herbivory for 96 *B. napus* accessions (± standard error). Colour equates to crop type groupings (Blue = WOSR, Green = SOSR, Red = Swede, Orange = Kale/Forage/Leaf, Yellow = Synthetic, Purple = Semiwinter OSR). See Appendix 1 for specific crop type for each accession. n = 3 except for Matador, where n = 60.

2.3.1.3 Grazing feeding damage

For grazing damage, where just the surface of the cotyledon has been eaten, plants were also scored visually to the nearest 5%. Grazing damage was scored with the aim to record different types of feeding behaviour, particularly if CSFB seem to just taste something and then leave it rather than engaging in usual shot holing feeding behaviour.

Adjusted mean grazing damage scores ranged from 0.37% to 2.90% for varieties Altasweet and Dippes, respectively (Figure 2.17). The narrow range of grazing damage was expected as grazing is not the main feeding method observed by CSFB to cotyledons. Despite this limited range in mean scores, running a linear mixed model (summarised in Equation 2.8) revealed grazing damage statistically significantly differed overall between *B. napus* varieties (Table 2.13). However, as with previous feeding trait results presented in this section, a large amount of variation is attributable to the random factors summarised in Table 2.14, thus it was appropriate to retain these within the analysis.

$$\text{lmer(formula} = \text{Grazing percentage damage} \sim 1 + \text{Line} + (1|\text{Block} + \text{Block. Date} + \text{Block. Date. Assay})$$

Equation 2.8. Linear mixed model used to assess the effect of *B. napus* variety on grazing percentage damage.

Table 2.13. Output from linear mixed model demonstrating a statistically significant effect of *B. napus* variety on grazing percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Line	96	131.22	1.36	0.018

Table 2.14. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0028	0.0038
Block.Date	-0.0104	0.0055
Block.Date.Assay	0.0318	0.0116
Residual	0.149	0.0088

Running a Fisher's multiple comparisons test revealed a gradual change significant differences between *B. napus* varieties for grazing damage. Notably, Altasweet, the variety that received the lowest levels of grazing damage, significantly differs from all other *B. napus* lines beyond and including ERGLU. See Appendix 4 for a full table of significant differences between lines.

Additionally investigated was whether there were differences between crop types for percentage grazing damage via analysis with the linear mixed model displayed in Equation 2.9. This revealed a statistically significant effect of *B. napus* crop type on CSFB grazing damage (Table 2.15). Despite this significance, variance components for random factors remained large and were therefore retained in the model (Table 2.16).

$$\text{lmer(formula} = \text{Grazing percentage damage} \sim 1 + \text{Crop type} + (1|\text{Block} + \text{Block. Date} + \text{Block. Date. Assay})$$

Equation 2.9. Linear mixed model used to assess the effect of *B. napus* crop type on grazing percentage damage.

Table 2.15. Output from linear mixed model demonstrating a statistically significant effect of *B. napus* crop type on grazing percentage CSFB feeding damage.

	df	Wald statistic	*F* value	*p* value
Crop type	5	14.15	2.83	0.015

Table 2.16. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0032	0.0043
Block.Date	-0.0053	0.0043
Block.Date.Assay	0.0216	0.0083
Residual	0.155	0.0086

Having observed a significant difference of crop type on grazing damage, a Fisher's multiple comparisons test was run, summarised in Table 2.17. Notably, this highlights swede types differing significantly for grazing damage compared to both WOSR and semi-winter OSR types. Additionally, SOSR types are observed to be significantly different compared to WOSR types.

Table 2.17. Summary of output from a Fisher's multiple comparisons test demonstrating differences between crop types for CSFB grazing damage.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Swede – SOSR	-0.055	-0.192	0.082	0.4342
Swede – Synthetic	-0.098	-0.446	0.251	0.5827
Swede – KLF	-0.132	-0.286	0.022	0.0917
Swede – WOSR	-0.172	-0.290	-0.054	0.0042
Swede – Semi-winter OSR	-0.208	-0.400	-0.020	0.0298
SOSR – Synthetic	-0.043	-0.383	0.297	0.8039
SOSR – KFL	-0.078	-0.210	0.055	0.2499
SOSR – WOSR	-0.117	-0.206	-0.029	0.0091
SOSR – Semi-winter OSR	-0.154	-0.324	0.016	0.0763
Synthetic – KFL	-0.035	-0.380	0.311	0.8436
Synthetic – WOSR	-0.074	-0.407	0.258	0.6606
Synthetic – Semi-winter OSR	-0.111	-0.475	0.253	0.5510
KFL – WOSR	-0.040	-0.152	0.072	0.4878
KFL – Semi-winter OSR	-0.076	-0.260	0.108	0.4180
WOSR – Semi-winter OSR	-0.036	-0.191	0.119	0.6458

Abbreviations: SOSR = Spring oilseed rape, KLF = Kale/leaf/forage, Semi-winter OSR = Semi-winter oilseed rape and WOSR = Winter oilseed rape.

Therefore, variation in grazing damage was observed between *B. napus* varieties and crop types. This provided interesting insight and preliminary data on one of the more subtle adult CSFB feeding traits, grazing herbivory.
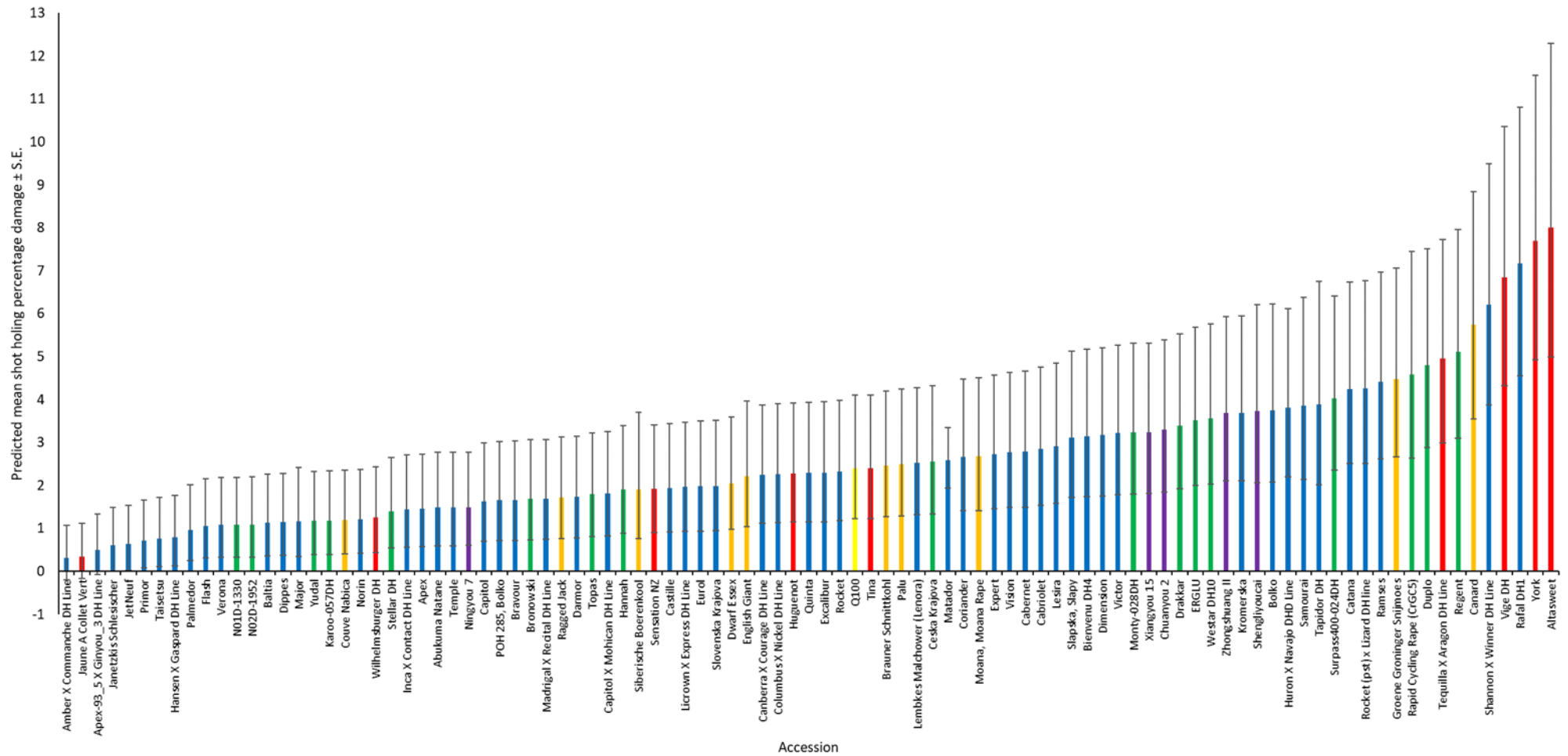
Figure 2.17. Variation in the adjusted mean grazing percentage damage to cotyledons from adult CSFB herbivory for 96 *B. napus* accessions (± standard error). Colour equates to crop type groupings (Blue = WOSR, Green = SOSR, Red = Swede, Orange = Kale/Forage/Leaf, Yellow = Synthetic, Purple = Semiwinter OSR). See Appendix 1 for specific crop type for each accession. n = 3 except for 88 Matador, where n = 60.

2.3.1.4 Stem feeding damage

Stem damage was scored with the aim to further record CSFB feeding behaviour as it was observed that beetles sometimes chew through stems of seedlings resulting in plant death. Plants were given either a score of 0, indicating no damage to the stem, or a score of 1, indicating herbivory damage to the stem. Adjusted mean stem damage scores range from -0.002 to 0.910, for varieties English Giant and Chuanyou 2, respectively (Figure 2.18). Data was analysed with the linear mixed model displayed in Equation 2.10, which revealed a non-significant effect of *B. napus* variety on stem damage (Table 2.18). As with analysis of other CSFB feeding traits scored in this experiment, large amounts of variation and standard error were observed from random factors (Table 2.19).

$$\text{lmer(formula = Stem damage} \sim 1 + \text{Line} + (1|\text{Block} + \text{Block.Date} + \text{Block.Date.Assay)}$$

Equation 2.10. Linear mixed model used to assess the effect of *B. napus* line on stem damage.

Table 2.18. Output from linear mixed model demonstrating a non-significant effect of *B. napus* line on CSFB stem feeding damage.

	df	Wald statistic	*F* value	*p* value
Line	96	32.48	0.96	0.581

Table 2.19. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0173	0.0208
Block.Date	0.0144	0.0114
Block.Date.Assay	0.0204	0.0108
Residual	0.194	0.0116

Finally, reported are results from a linear mixed model which assessed the effect of *B. napus* crop type on CSFB stem damage (Equation 2.11). No statistical difference was observed for crop type on stem damage scores (Table 2.20). Inspection of the variance components and standard errors of random factors indicated that they contributed to much of the differences observed and were thus maintained within the model (Table 2.21).

$$\text{lmer}(\text{formula} = \text{Stem damage} \sim 1 + \text{Crop type} + (1|\text{Block} + \text{Block.Date} + \text{Block.Date.Assay})$$

Equation 2.11. Linear mixed model used to assess the effect of *B. napus* crop type on stem damage.

Table 2.20. Output from linear mixed model demonstrating a non-significant effect of *B. napus* crop type on CSFB stem feeding damage.

	df	Wald statistic	*F* value	*p* value
Crop type	4.54	5	0.91	0.475

Table 2.21. Output from linear mixed model, demonstrating large variance components and standard errors for random terms of the model, Block, Block.Date and Block.Date.Assay.

	Variance component	Standard error
Block	0.0190	0.0215
Block.Date	0.0046	0.0072
Block.Date.Assay	0.0212	0.0091
Residual	0.195	0.0108

Therefore, although the results of *B. napus* variety and crop type demonstrated limited differences, they provided preliminary data on adult CSFB stem damage.
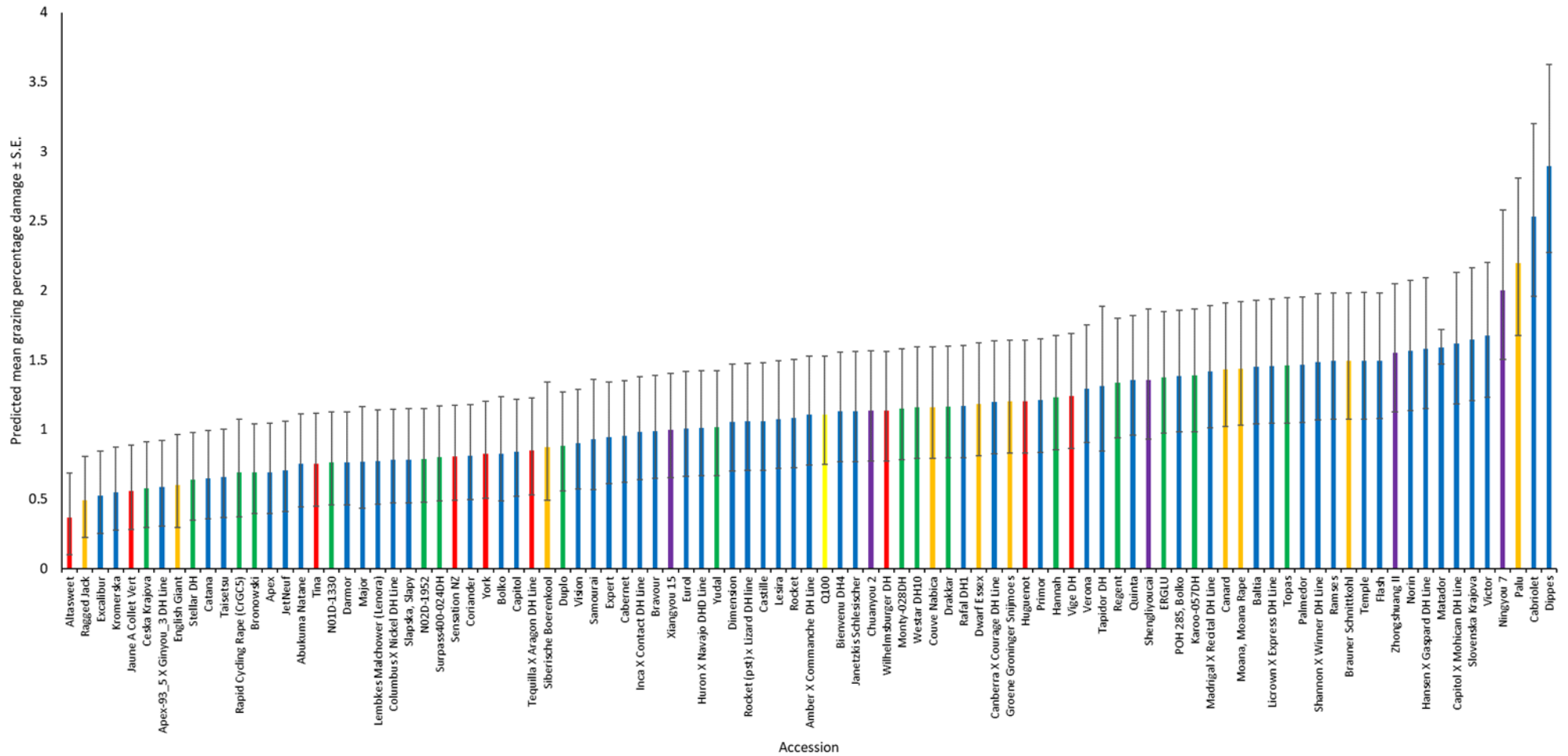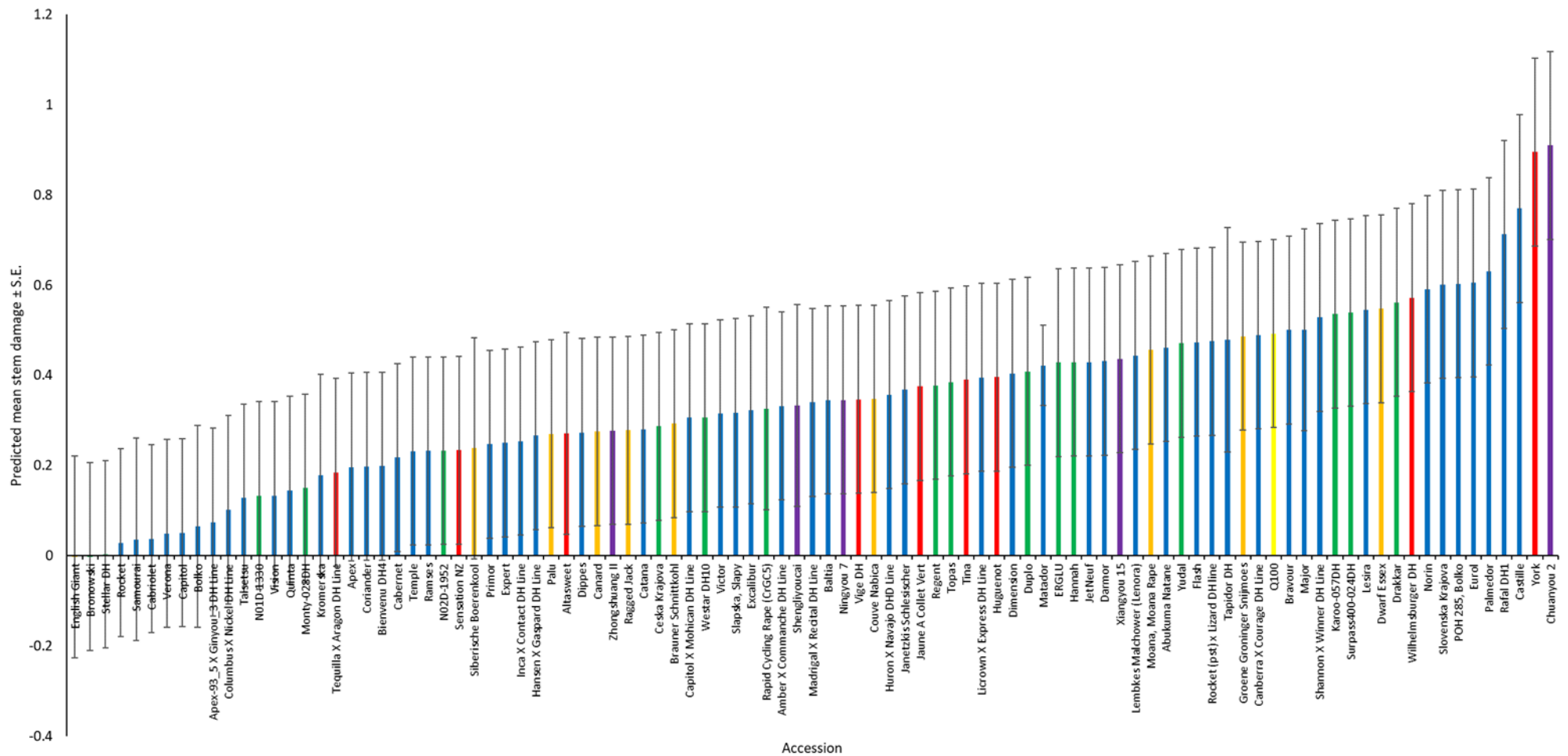
Figure 2.18. Variation in the adjusted mean stem damage to seedlings from adult CSFB herbivory for *96 B. napus* accessions (± standard error). Colour equates to crop type groupings (Blue = WOSR, Green = SOSR, Red = Swede, Orange = Kale/Forage/Leaf, Yellow = Synthetic, Purple = Semiwinter OSR). See Appendix 1 for specific crop type for each accession. n = 3, except for Matador, where n = 60.

2.3.1.5 Correlations between adult CSFB feeding traits

For the four successfully scored CSFB feeding traits (total, shot holing, grazing and stem damage), a Pearson's correlation test was conducted between all pairs to observe whether there were any interactions between the traits. Total and shot holing damage were strongly positively correlated (r (94) = 0.944, p < 0.00001) with strong statistical significance. This was expected as shot holing is the main component of damage a seedling receives from CSFB and is thus encompassed within total damage.

Also observed was a statistically significant but weak positive correlation between shot holing and stem damage (r (94) = 0.210, p < 0.04). Furthermore, stem damage positively correlated with total damage but was a weak interaction and of borderline statistical significance at the 95% confidence interval (r (94) = 0.200, p = 0.051). The rest of the correlations between feeding traits were not statistically significant (p values ranging from 0.550 to 0.214) and none had a correlation stronger than r = 0.082 or r = -0.128.

Therefore, these results indicated a strong positive, predictable relationship between total and shot holing damage. They also suggested a potential positive relationship between these two feeding traits and stem damage, indicating that the different types of CSFB herbivory could be linked.

In conclusion, variation was observed for different adult CSFB feeding traits across the 96 lines but these differences were over a limited range. In all of the linear mixed model analyses, large amounts of variation were attributable to random factors block, block.date and block.date.assay. Therefore, it may be that there were differences between *B. napus* varieties but the experimental design was not appropriate and replication level too small to detect these in this experiment. Nonetheless, the experiment provided preliminary data on differences in CSFB herbivory across a *B. napus* diversity set and was taken forward for GWAS (see Chapter 3). Furthermore, the dataset allowed for selection of *B. napus* varieties displaying more resistant/tolerant and more susceptible traits for further investigation (see next section, Chapter 2.3.2).

2.3.2 Altasweet recorded to receive higher levels of feeding damage than Apex-93_5 X Ginyou_3 DH Line in two-way assays

Focusing on two *B. napus* varieties demonstrating higher and lower levels of adult CSFB feeding in six-way choice assays, it was hypothesised that the line Altasweet would receive significantly higher levels of total and shot holing damage compared with Apex-93_5 X Ginyou_3 DH Line. An additional hypothesis was that there would be no difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line in levels of grazing and stem damage.

To better understand CSFB feeding differences between Altasweet and Apex-93_5 X Ginyou_3 DH Line, two-way choice chambers were run consisting of six seedlings of each variety, presenting beetles with a choice between these two accessions only rather than six (see Methods section 2.2.2.1). As in the six-way choice chambers described in the previous section, seedlings were visually estimated for percentage total, shot holing and grazing damage, as well as scored for stem damage (0 indicating no damage and 1 indicating herbivory of the stem) (see Figure 2.8 in Methods 2.2). Percentage data was LOGIT+ transformed for analysis (Equation 2.1). Data was analysed by a two-way ANOVA of *B. napus* Line and Block (date of assay), with an interaction term between the two to check that individual *B. napus* lines were not behaving differently to each other on different dates.

2.3.2.1 Total feeding damage

For total damage, the trait aiming to capture CSFBs total herbivory to the seedling cotyledons, it was observed that Altasweet received more damage than Apex-93_5 X Ginyou_3 DH Line, with percentage damage scores of 11.80% and 4.73%, respectively (Figure 2.19). Running a two-way ANOVA revealed the difference in feeding damage to be strongly statistically significant between the two *B. napus* lines in two-way choice assays (Table 2.22). Additionally, there was a significant difference between blocks but no interaction between *B. napus* line and block (Table 2.20), allowing this term to be removed from the model. Re-running the model without the interaction term maintained a significant difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line and between blocks (Table 2.23). A Tukey's HSD multiple comparisons test highlighted statistically significant differences between block two with both blocks three and four (Table 2.24), indicating that block two was behaving differently compared to the rest.

Whilst the effect of block can not be ruled out, it was concluded from these two-way choice chambers that Altasweet is more palatable than Apex-93_5 X Ginyou_3 DH Line, as demonstrated by higher levels of total feeding damage.

Table 2.22. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for total percentage feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	10.80	10.796	14.800	0.00034
Block	4	10.88	2.721	3.730	0.00985
Line:Block	4	0.83	0.208	0.286	0.88593
Residuals	50	36.47	0.729		

Table 2.23. Summary output of a two-way ANOVA, with the interaction term removed, highlighting significant differences in total percentage feeding damage between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	10.80	10.796	14.800	0.00023
Block	4	10.88	2.721	3.730	0.00707
Residuals	54	37.31	0.691		

Table 2.24. Summary of pairwise differences between blocks from a Tukeys multiple comparisons test.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Two – One	0.348	-0.610	1.306	0.84234
Three – One	-0.700	-1.658	0.257	0.25100
Four – One	-0.689	-1.647	0.269	0.26571
Five – One	-0.599	-1.557	0.358	0.40353
Three – Two	-1.048	-2.006	-0.091	0.02527
Four – Two	-1.037	-1.995	-0.079	0.02755
Five – Two	-0.947	-1.905	0.010	0.05381
Four – Three	0.011	-0.947	0.969	0.99999
Five – Three	0.102	-0.857	1.058	0.99825
Five - Four	0.090	-0.868	1.047	0.99889
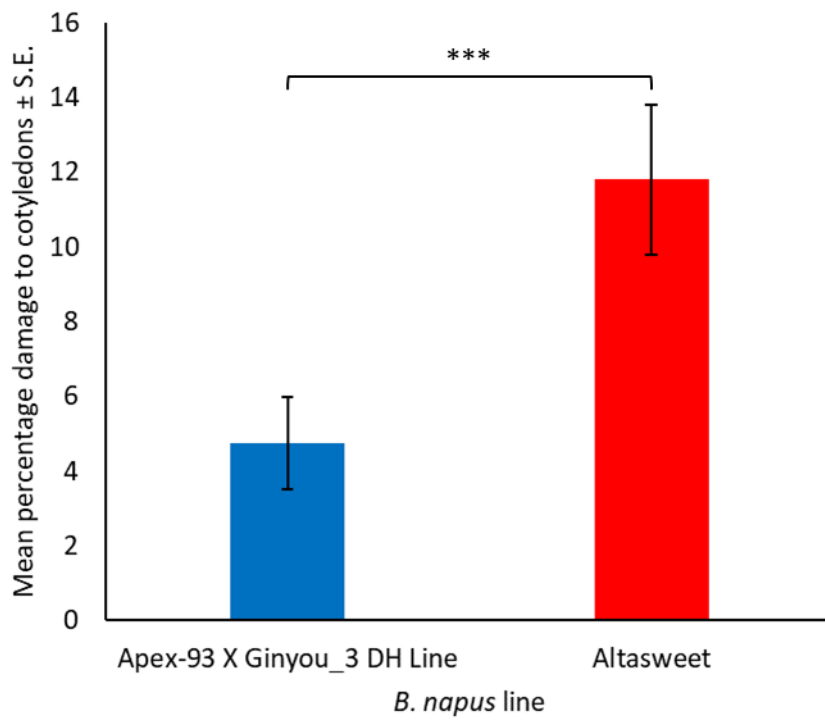
Figure 2.19. Variation in mean total percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in two-way choice assays (***$p < 0.001$) (n = 5).

2.3.2.2 Shot holing feeding damage

Another CSFB feeding trait we recorded was shot holing damage, where beetles eat holes completely through the cotyledons. Altasweet was recorded to have statistically significantly more shot holing damage with a mean score of 11.80% compared with Apex-93_5 X Ginyou_3 DH Line's mean score of 4.20% (Table 2.25) (Figure 2.20). Similarly to total percentage damage, blocks also differed significantly for shot holing damage but an interaction with *B. napus* line was not observed so could be removed from the analysis (Table 2.26). A Tukeys multiple comparisons test was run to see where the differences lied between blocks. This revealed that again block two differed significantly compared with blocks three and four, but additionally differed compared to block five (Table 2.27). Taken with the results from total percentage damage, this demonstrates clearly that block two was behaving differently compared to some of the others.

Despite observing some between block variation in CSFB feeding levels, it was again concluded that Altasweet was more palatable than Apex-93_5 X Ginyou_3 DH Line when scored for shot holing damage in two-way choice assays. Additionally, this is unsurprising given the previously observed strong correlation between total and shot holing damage feeding traits (see section 2.3.1.5).

Table 2.25. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for shot holing percentage feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	13.40	13.398	19.690	0.00005
Block	4	11.56	2.891	4.249	0.00489
Line:Block	4	2.08	0.520	0.764	0.55351
Residuals	50	34.02	0.680		

Table 2.26 Summary output of a two-way ANOVA, with the interaction term removed, highlighting significant differences in shot holing feeding damage between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	13.40	13.398	20.040	0.00003
Block	4	11.56	2.891	4.324	0.00417
Residuals	54	36.10	0.669		

Table 2.27. Summary of pairwise differences between blocks from a Tukeys multiple comparisons test.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Two – One	0.295	-0.647	1.237	0.90205
Three – One	-0.700	-1.642	0.242	0.23623
Four – One	-0.689	-1.631	0.253	0.25060
Five – One	-0.788	-1.730	0.154	0.14237
Three – Two	-0.995	-1.937	-0.053	0.03360
Four – Two	-0.984	-1.926	-0.042	0.03659
Five – Two	-1.082	-2.024	-0.140	0.01668
Four – Three	0.011	-0.931	0.953	0.99999
Five – Three	-0.088	-1.030	0.854	0.99892
Five - Four	-0.099	-1.041	0.943	0.99828
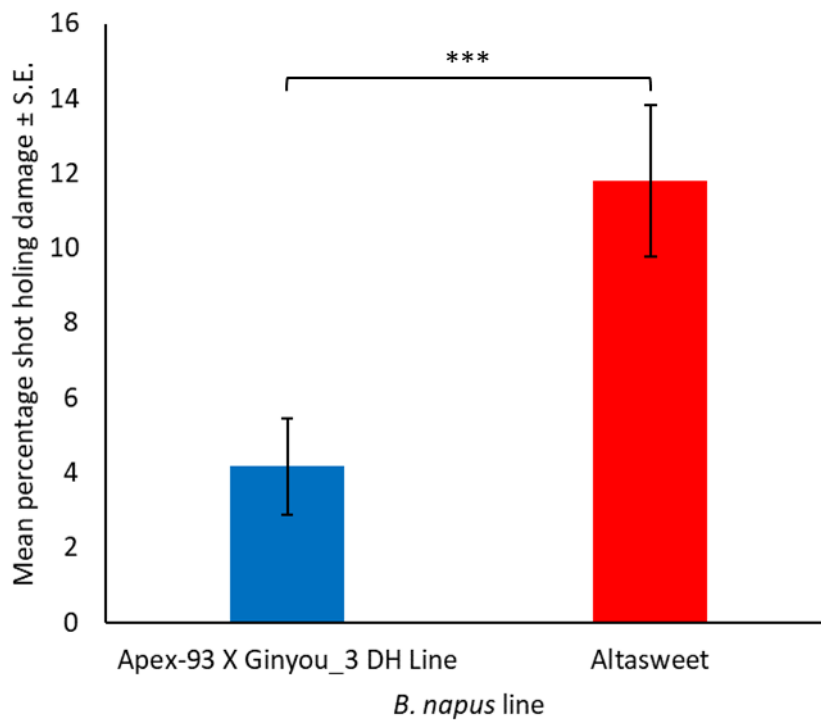
Figure 2.20. Variation in mean shot holing percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in two-way choice assays (***$p < 0.001$) (n = 5).

2.3.2.3 Grazing feeding damage

Herbivory data was also collected for grazing damage, where beetles eat just the surface of the cotyledon as opposed to creating shot holing damage the entire way through the leaf. The key observation was a slight but statistically significant difference between the mean grazing score of Altasweet and Apex-93 X Ginyou_3 DH Line (Table 2.28), with scores of 0.77% and 1.07%, respectively (Figure 2.21). When the non-significant term of block was removed there remained a significant but even weaker difference in grazing between Altasweet and Apex-93_5 X Ginyou_3 DH Line (Table 2.29). Note the narrow difference in grazing damage scores is expected as this has not been observed to be a main component of CSFB herbivory (see section 2.3.1.3).

Therefore, it was concluded that there was a weak difference between Apex-93_5 X Ginyou_3 DH Line and Altasweet for CSFB grazing herbivory.

Table 2.28. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for grazing percentage feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.274	0.274	4.350	0.0421
Block	4	0.481	0.120	1.914	0.1226
Line:Block	4	0.135	0.034	0.536	0.7099
Residuals	50	3.144	0.063		

Table 2.29. Summary output of a one-way ANOVA, with the block and interaction term removed, highlighting significant differences in grazing feeding damage between *B. napus* lines.

|	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.274	0.274	4.219	0.0445
Residuals	58	3.760	0.065		
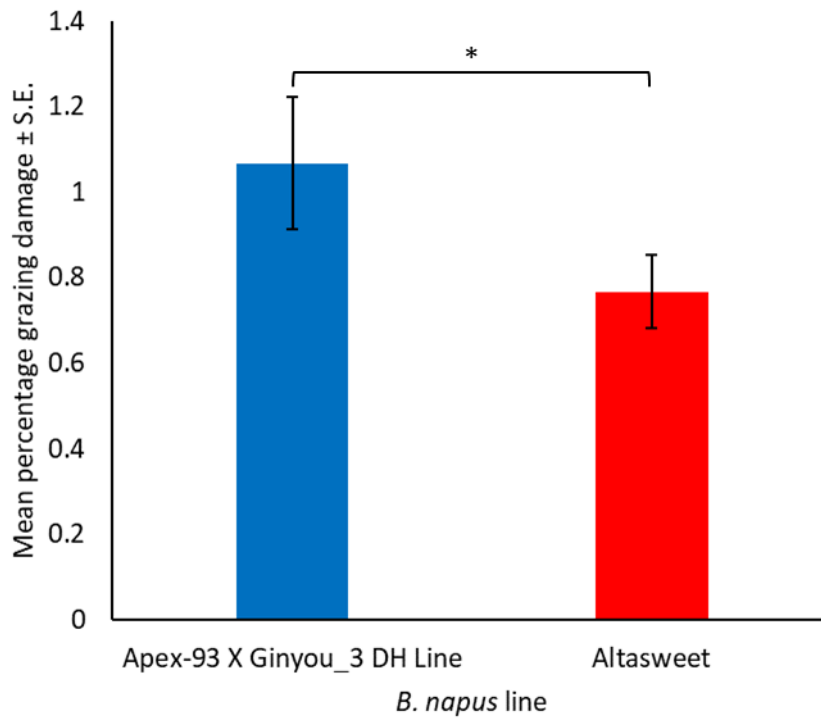
Figure 2.21. Variation in mean grazing percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in two-way choice assays (*$p < 0.05$) (n = 5).

2.3.2.4 Stem feeding damage

The final adult CSFB feeding trait investigated in two-way choice assays was stem damage, where beetles feed on the stem of the seedling rather than cotyledons. To record this, seedlings were given a score of 0, indicating no damage to the stem or 1, indicating damage to the stem. Mean stem damage scores for Altasweet and Apex-93_5 X Ginyou_3 DH Line were similar, with 0.23 and 0.33, respectively (Figure 2.22). Analysis with a two-way ANOVA demonstrated no significant effects of *B. napus* variety, block or an interaction for stem damage in two-way choice assays of Altasweet and Apex-93_5 X Ginyou_3 DH Line (Table 2.30). A one-way ANOVA was ran removing the non-significant factors for block and the line and block interaction (Table 2.31), however differences between *B. napus* varieties remained statistically insignificant. Therefore, the conclusion was that there was no difference in stem damage between these two *B. napus* lines.

Table 2.30. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for stem feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.150	0.1500	0.692	0.409
Block	4	0.433	0.1083	0.500	0.736
Line:Block	4	0.767	0.1917	0.885	0.480
Residuals	50	10.833	0.2167		

Table 2.31. Summary output of a one-way ANOVA, with the block and interaction term removed, demonstrating a non-significant difference in stem feeding damage between *B. napus* lines.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.15	0.1500	0.723	0.399
Residuals	58	12.03	0.2075		
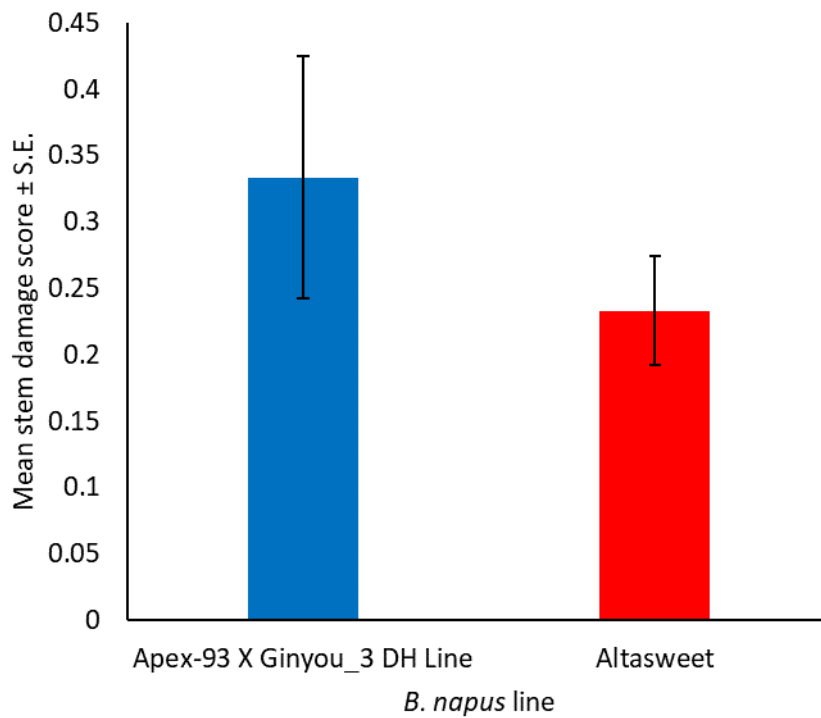
Figure 2.22. Variation in mean stem damage scores (± standard error) between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in two-way choice assays (n = 5).

Overall, from two-way choice assays of Altasweet and Apex-93_5 X Ginyou_3 DH Line, *B. napus* varieties at the extreme ends of the distribution for more or less for total damage in six-way choice assays, the following was concluded; 1) Altasweet received statistically significantly more total and shot hole feeding damage than Apex-93_5 X Ginyou_3 DH Line, supporting our hypotheses, 2) There was no statistically significant difference in amount of stem damage these two lines received as hypothesised and 3) conclusions cannot be drawn about whether grazing damage was the same for both line as we observed a weak but statistically significant difference, with Apex-93_5 X Ginyou_3 DH Line receiving higher levels of damage.

2.3.3 Altasweet recorded to receive even higher levels of feeding damage than Apex-93_5 X Ginyou_3 DH Line in non-choice assays

From observing such clear differences in two-way choice chambers, herbivory differences to Altasweet and Apex-93_5 X Ginyou_3 DH Line were investigated when beetles were presented with only one food option. Hypotheses were the same as reported in the previous section; 1) Altasweet would receive more total and shot holing damage that Apex-93_5 X Ginyou_3 DH Line and 2) There would be no difference in grazing and stem damage between the two lines. An additional hypothesis was that total and shot holing feeding differences would become more extreme in a non-choice setting compared with two-way and six-way choice assays.

To test these hypotheses, non-choice chambers were run with only either Altasweet or Apex-93_5 X Ginyou_3 DH Line seedlings present. The experiment was scored for total, shot holing, grazing and stem damage traits, and analysed in the same way as described for two-way choice assays in the previous section.

2.3.3.1 Total feeding damage

For total percentage damage in non-choice assays, a two-way ANOVA revealed that Altasweet received significantly higher levels of damage (Table 2.32), scoring 19.77%, compared with Apex-93_5 X Ginyou_3 DH Line at 2.37% (Figure 2.22). Block was also a significant factor so retained in the model. However, there was no interaction between *B. napus* line and block, thus that term was removed from the model (Table 2.33). Both *B. napus* line and block remained significant for total feeding damage. Table 2.34 summarises the results of a Tukeys HSD multiple comparisons test highlighting significant differences between three pairs of blocks; four and two, four and five, five and three.

Therefore, although there is an effect of block on total feeding percentage damage, it was concluded that Altasweet was damaged more than Apex-93 X Ginyou_3 DH Line and that the difference between these two *B. napus* is stronger in non-choice assays than previously ran six-way and two-way choice experiments.

Table 2.32. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for total feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	130.08	130.08	392.733	< 0.00001
Block	4	6.46	1.62	4.877	0.00117
Line:Block	4	0.93	0.23	0.703	0.59138
Residuals	110	36.43	0.33		

Table 2.33. Summary output of a two-way ANOVA, with the interaction term removed, highlighting significant differences in total percentage feeding damage between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	130.08	130.08	396.866	< 0.00001
Block	4	6.46	1.62	4.928	0.00106
Residuals	114	37.36	0.33		

Table 2.34. Summary of pairwise differences between blocks from a Tukeys multiple comparisons test.

|	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Two – One	0.184	-0.274	0.642	0.79881
Three – One	-0.204	-0.662	0.254	0.73042
Four – One	-0.331	-0789	0.127	0.27092
Five – One	0.291	-0.167	0.749	0.40155
Three – Two	-0.388	-0.846	0.067	0.13697
Four – Two	-0.515	-0.973	-0.057	0.01919
Five – Two	0.107	-0.351	0.565	0.96695
Four – Three	-0.127	-0.585	0.331	0.93933
Five – Three	0.495	0.037	0.953	0.02718
Five - Four	0.622	0.164	1.080	0.00242
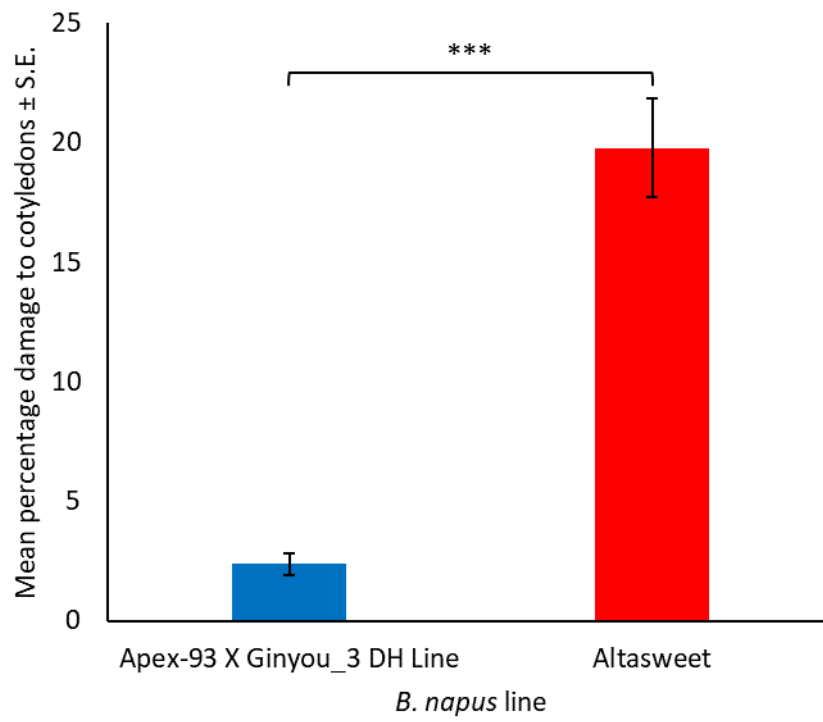
Figure 2.22. Variation in mean total percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in non-choice assays (***$p < 0.001$) (n = 5).

2.3.3.2 Shot holing feeding damage

For shot holing, Altasweet demonstrated higher levels of damage with a mean score of 19.60%, compared with 2.18% that Apex-93_5 X Ginyou_3 DH Line received in non-choice assays (Figure 2.23). As previously, running a two-way ANOVA revealed these differences in shot holing to be statistically significant. Similarly to total damage, the differences observed in non-choice assays for shot holing herbivory were more extreme than those in two-way choice assays, where Altasweet received 11.80% and Apex-93_5 X Ginyou_3 DH Line 4.20% mean shot holing damage. No interaction was observed between line and block so that term was removed from the analysis and re-run demonstrating the significance of *B. napus* line and block (Table 2.36). Running a Tukey's HSD multiple comparisons test demonstrated three statistically significant differences for shot holing damage between blocks, summarised in Table 2.37.

Therefore, whilst again understanding there were differences between blocks, the conclusion was that Altasweet received more shot holing herbivory compared with Apex-93_5 X Ginyou_3 DH Line, and that these difference in damage levels were greater in non-choice compared with six-way and two-way choice chambers.

Table 2.35. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for shot holing feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	139.25	139.25	387.943	< 0.00001
Block	4	7.40	1.85	5.152	0.00077
Line:Block	4	1.77	0.44	1.233	0.30117
Residuals	110	39.48	0.36		

Table 2.36. Summary output of a two-way ANOVA, with the interaction term removed, highlighting significant differences in shot holing percentage feeding damage between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	139.25	139.25	384.80	< 0.00001
Block	4	7.40	1.85	5.11	0.00080
Residuals	114	41.25	0.36		

Table 2.37. Summary of pairwise differences between blocks from a Tukeys multiple comparisons test.

|	Mean difference	95% C.I.		p value
		Lower	Upper	
Two – One	0.115	-0.366	0.596	0.96403
Three – One	-0.367	-0.848	0.114	0.22169
Four – One	-0.331	-0.812	0.150	0.31965
Five – One	0.266	-0.215	0.747	0.54383
Three – Two	-0.482	-0.963	-0.001	0.04959
Four – Two	-0.446	-0.927	0.035	0.08312
Five – Two	0.151	-0.330	0.632	0.90719
Four – Three	0.036	-0.446	0.517	0.99959
Five – Three	0.633	0.152	1.114	0.00364
Five - Four	0.597	0.116	1.079	0.00716
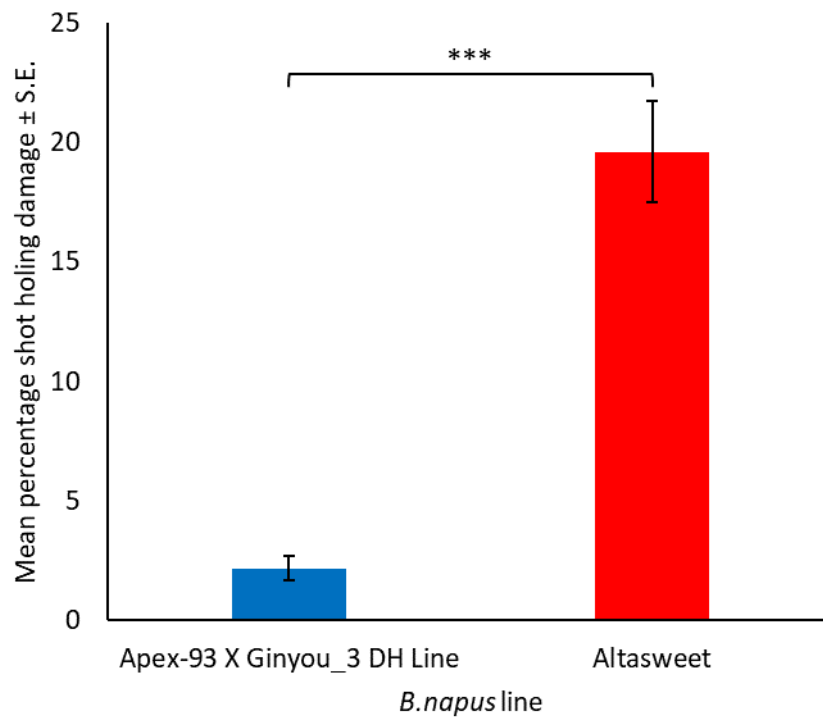
Figure 2.23. Variation in mean shot holing percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in non-choice assays (***$p < 0.001$) (n = 5).

2.3.3.3 Grazing feeding damage

Grazing herbivory was additionally scored for Altasweet and Apex-93_5 X Ginyou_3 DH Line non-choice assays, with mean percentage grazing damage of 1.12% and 0.92%, respectively (Figure 2.24). Note, compared to the two-way choice chambers where Apex-93_5 X Ginyou_3 DH Line received higher grazing damage, Altasweet was observed to have higher grazing levels in non-choice assays, i.e. the relationship has reversed. A two-way ANOVA indicated a weak statistically significant difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line's grazing scores in non-choice assays, as well as a slight statistically significant effect from block (Table 2.38). However, there was no significance of the *B. napus* line and block interaction, thus this term was removed from the model, with outputs summarised in Table 2.39. Tukey's HSD multiple comparisons test revealed the mean percentage grazing for block three to differ statistically significantly compared with block four (Table 2.40). From this data it could not be concluded whether there was difference in CSFB grazing herbivory between Altasweet and Apex-93_5 X Ginyou_3 DH Line in non-choice assays.

Table 2.38. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for grazing feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.285	0.285	5.295	0.0233
Block	4	0.577	0.144	2.685	0.0351
Line:Block	4	0.119	0.030	0.555	0.6960
Residuals	110	5.913	0.054		

Table 2.39. Summary output of a two-way ANOVA, with the interaction term removed, highlighting significant differences in grazing percentage feeding damage between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	0.285	0.285	5.379	0.0222
Block	4	0.577	0.144	2.727	0.0327
Residuals	114	6.032	0.053		

Table 2.40. Summary of pairwise differences between blocks from a Tukeys multiple comparisons test.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Two – One	-0.030	-0.215	0.154	0.99078
Three – One	0.089	-0.096	0.273	0.67113
Four – One	-0.125	-0.309	0.060	0.33654
Five – One	-0.050	-0.234	0.134	0.94402
Three – Two	0.119	-0.065	0.303	0.38339
Four – Two	-0.094	-0.278	0.090	0.61822
Five – Two	-0.019	-0.203	0.165	0.99840
Four – Three	-0.213	-0.397	-0.029	0.01466
Five – Three	-0.138	-0.322	0.046	0.23442
Five - Four	0.075	-0.109	0.259	0.79293
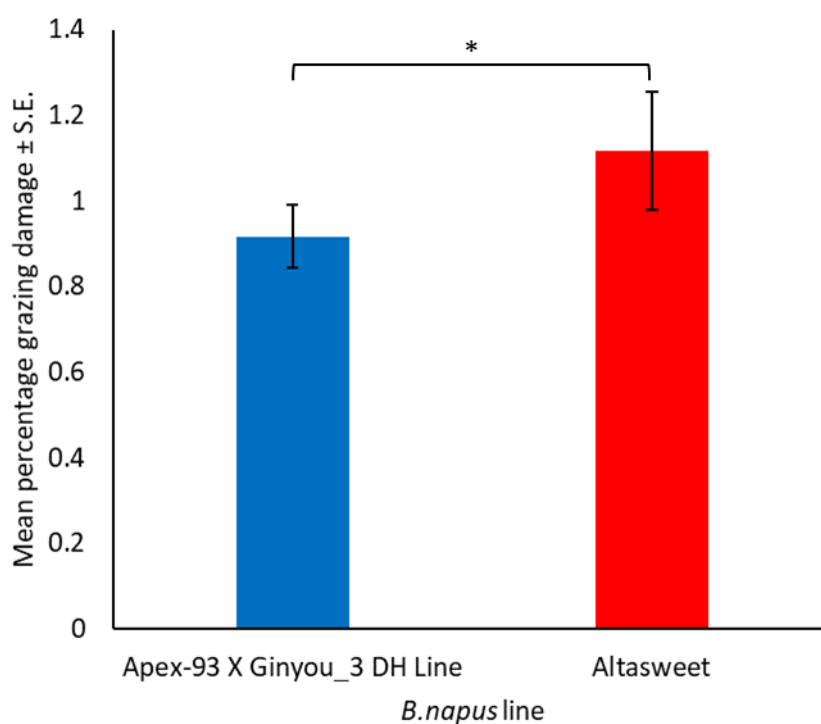
Figure 2.24. Variation in mean grazing percentage damage (± standard error) to cotyledons between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in non-choice assays (*$p < 0.05$) (n = 5).

2.3.3.4 Stem feeding damage

The final CSFB herbivory trait we scored was stem damage, where seedlings were scored a value of 1 indicating stem herbivory or 0 indicating an intact stem. Here, Altasweet seedlings were recorded to have received significantly more stem damage compared to Apex-93_5 X Ginyou_3 DH Line (Table 2.41), with mean stem damage scores of 0.45 and 0.27, respectively (Figure 2.25). Additionally observed was no significant effect of block or and interaction between block and *B. napus* line, so these terms were removed from the model and a one-way ANOVA run, as summarised in Table 2.42. It was concluded that in non-choice assays Altasweet received more damage compared to Apex-93_5 X Ginyou_3 DH Line.

Table 2.41. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for stem feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	1.008	1.008	4.393	0.0384
Block	4	0.467	0.1167	0.508	0.7298
Line:Block	4	0.867	0.2167	0.944	0.4415
Residuals	110	25.250	0.2295		

Table 2.42. Summary output of a one-way ANOVA, with the block and interaction term removed, highlighting significant differences in stem feeding damage between *B. napus* lines.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	1	1.008	1.008	4.476	0.0365
Residuals	118	26.583	0.2253		
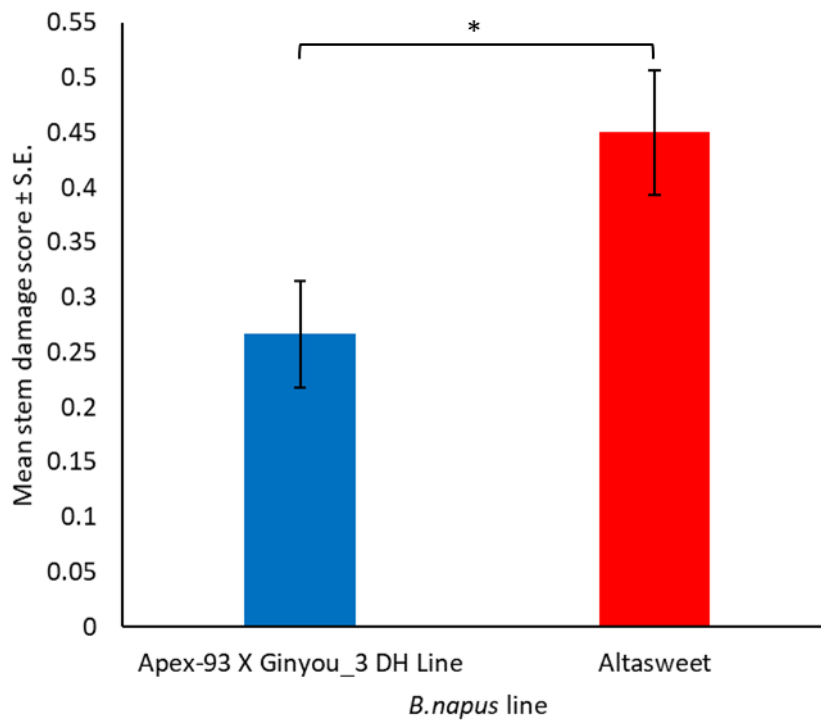
Figure 2.25: Variation in mean stem damage (± standard error) between two *B. napus* lines Apex-93 X Ginyou_3 DH Line and Altasweet, in non-choice assays (*$p < 0.05$) (n = 5).

In conclusion, support was found for the hypotheses that Altasweet would receive more total and shot holing damage and where Apex-93_5 X Ginyou_3 DH Line in both two-way and non-choice chambers. Additionally, the hypothesis that non-choice assays demonstrate herbivory differences more clearly than choice assays was supported. However, the hypotheses that grazing and stem damage would be the same for both *B. napus* lines could not be supported or disproved. Moving forward, scoring of grazing and stem damage was not pursued due to lack of differences between the liens of interest. Additionally, shot holing was not taken further due to this feeding trait being largely captured in total damage scoring.

2.3.4.1 Strong differences recorded from choice assays in amount of feeding damage for Altasweet compared to an F1 cross of Altasweet and Apex-93_5 X Ginyou_3 DH Line

Based on previous results from six-way, two-way and non-choice assays, it was hypothesised that Altasweet and Apex-93_5 X Ginyou_3 DH Line will receive significantly different levels of feeding from adult CSFB. Additionally, it was hypothesised that an F1 cross of these accessions will differ in levels of feeding damage compared with one or both parental lines. A final hypothesis was that visually obtained percentage damage data positively correlates with data obtained from computerised image analysis (using ImageJ software, see Methods 2.2.3.3). To test differences in palatability between these *B. napus* lines, three-way choice assays were conducted using the same petri dish set up as described in Methods 2.2, with four seedlings of each accession in each assay. As with previous experiments in this chapter, total percentage damage to cotyledons was scored by visually estimating damage to the nearest 5%. Additionally, images of cotyledons were put through image analysis, using ImageJ software, with the aim to obtain a more accurate, computerised score of damage.

Percentage data was transformed for analysis using the LOGIT+ function (Equation 2.1). Data was analysed using a two-way ANOVA, including a blocking factor of date the assay and interaction term between *B. napus* line and block.

For data derived from visual scoring, a weak but statistically significant effect of *B. napus* line on mean percentage damage to cotyledons in three-way choice assays was observed (Table 2.43). Block and the *B. napus* line and block interaction term were found to be non-significant thus removed from the model and re-run as a one-way ANOVA (Table 2.44) where *B. napus* line remained significant (Figure 2.26a). Running Tukey's HSD Test for multiple comparisons revealed that the mean percentage damage score of Altasweet of 6.88% differed statistically significantly from the mean percentage damage score of the F1 crossed line of 2.25% (Table 2.45). However, there was no statistically significant difference between the mean damage score of Altasweet and Apex-93_5 X Ginyou_3 DH Line (4.44%) or Apex-93_5 X Ginyou_3 DH Line and the F1 crossed line.

Table 2.43. Summary output of a two-way ANOVA assessing visual score differences between *B. napus* line, block and their interaction for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	4.401	2.201	3.412	0.044
Block	3	2.161	0.720	1.117	0.355
Line:Block	6	4.075	0.679	1.053	0.408
Residuals	36	23.218	0.645		

Table 2.44. Summary output of a one-way ANOVA assessing visual score differences between *B. napus* lines for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	4.401	2.201	3.362	0.0436
Residuals	45	29.454	0.655		

Table 2.45. Summary output of a Tukeys multiple comparisons test demonstrating a significant difference in visually scored feeding damage between the F1 crossed line and Altasweet.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Altasweet - Apex-93_5 X Ginyou_3 DH Line	0.497	-0.196	1.190	0.20228
F1 - Apex-93_5 X Ginyou_3 DH Line	-0.228	-0.921	0.465	0.70670
F1 - Altasweet	-0.725	-1.419	-0.032	0.03851

Running a two-way ANOVA on data derived from image analysis using ImageJ for the same three-way assays again revealed a statistically significant effect of *B. napus* line on mean percentage damage to cotyledons (Table 2.46) (Figure 2.26b). However, the analysis also demonstrated a statistically significant effect of block on mean percentage damage, so this term was retained within the model (Table 2.47). Tukey's HSD multiple comparisons test highlighted that again that the mean percentage damage of Altasweet (3.53%) is significantly different compared with the F1 crossed line (1.07%) (Table 2.48). Similarly, the mean percentage damage score of Apex-93_5 X Ginyou_3 DH Line (2.02%) does not differ statistically significantly from scores of Altasweet or the F1 crossed line. This multiple comparisons test also revealed that the mean percentage damage score for block one differed statistically significantly compared to block four (Table 2.49). There were no statically significant differences between the mean percentage scores for any of the other blocks.

Table 2.46. Summary output of a two-way ANOVA assessing image analysis differences between *B. napus* line, block and their interaction for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	3.205	1.603	6.155	0.00502
Block	3	2.696	0.899	3.452	0.02645
Line:Block	6	2.686	0.448	1.719	0.14465
Residuals	36	9.373	0.260		

Table 2.47. Summary output of a two-way ANOVA assessing image analysis differences between *B. napus* line and block for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	3.205	1.603	5.581	0.00709
Block	3	2.696	0.899	3.130	0.03553
Residuals	42	12.059	0.260		

Table 2.48. Summary output of a Tukeys HSD multiple comparisons test, demonstrating a significant difference between the F1 cross and Altasweet for damage scores derived from image analysis.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Altasweet - Apex-93_5 X Ginyou_3 DH Line	0.445	-0.016	0.905	0.06022
F1 - Apex-93_5 X Ginyou_3 DH Line	-0.168	-0.628	0.292	0.65206
F1 - Altasweet	-0.612	-1.073	-0.152	0.00661

Table 2.49. Summary output of a Tukeys HSD multiple comparisons test, demonstrating a significant difference between blocks four and one.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Two – one	0.338	-0.247	0.924	0.41928
Three – one	0.149	-0.436	0.734	0.90408
Four – one	0.634	0.049	1.220	0.02900
Three – two	-0.190	-0.775	0.395	0.82168
Four – two	0.296	-0.289	0.881	0.53506
Four – three	0.486	-0.099	1.071	0.13433
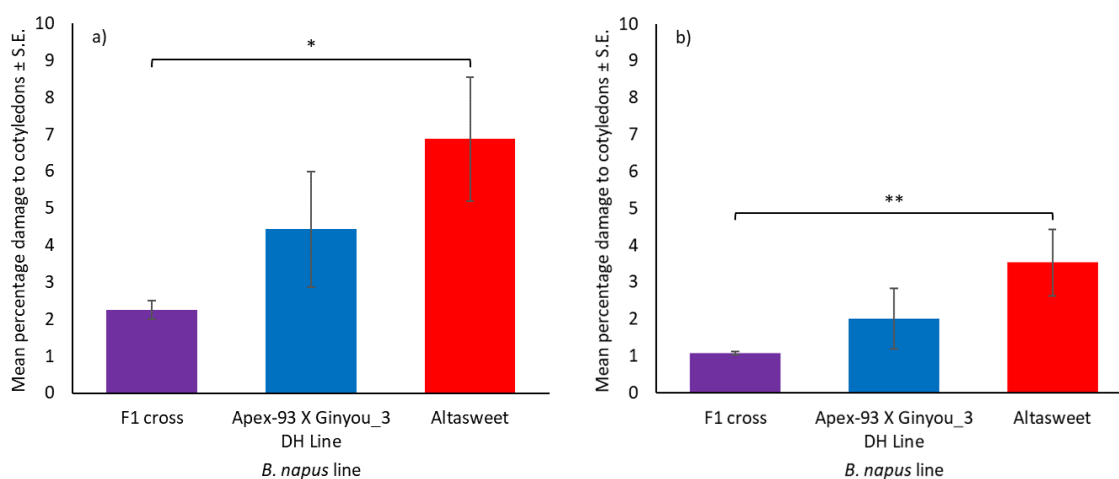
Figure 2.26. Variation in the mean percentage damage to cotyledons (± standard error) from three-way choice chambers for three *B. napus* lines; Apex-93_5 X Ginyou_3 DH Line, Altasweet and F1 cross with these as parental lines. Data displayed is derived from a) by-eye estimated scores to the nearest 5% (* $p < 0.05$) and b) Image analysis derived scores utilising the software ImageJ (** $p < 0.01$) (n = 4).

To better understand how visual estimates of percentage damage compared to scores obtained from image analysis using ImageJ, a Pearson's correlation coefficient analysis was run for damage scores for each of the three focal *B. napus* lines; Altasweet, Apex-93_5 X Ginyou_3 DH Line and the F1 cross of these parental lines. For all three lines, a statistically significant, positive correlation between the visual estimated scores and computer-generated data was observed (Figures 2.27a, b and c). Apex-93_5 X Ginyou_3 DH Line had the strongest correlation (r (14) = 0.965, *p* < 0.001) followed closely by Altasweet (r (14) = 0.923, *p* < 0.001). The F1 cross had a slightly weaker positive correlation between visual estimated and ImageJ derived scores but is nonetheless still strong and statistically significant (r (14) = 0.800, *p* < 0.001). For all three lines, the visual estimated scores were always larger than the ImageJ data, demonstrating an overestimation of damage when scoring by eye.
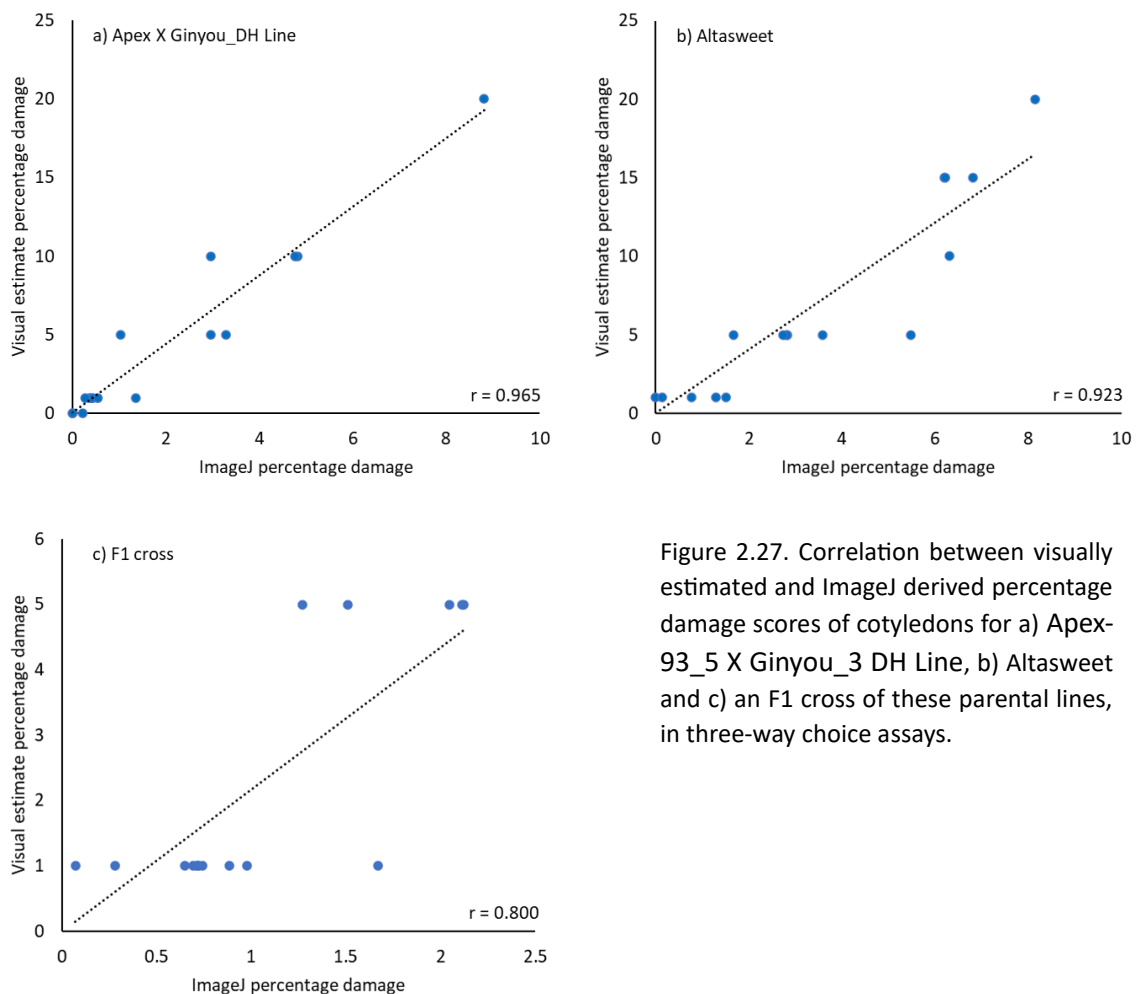
Figure 2.27. Correlation between visually estimated and ImageJ derived percentage damage scores of cotyledons for a) Apex-93_5 X Ginyou_3 DH Line, b) Altasweet and c) an F1 cross of these parental lines, in three-way choice assays.

Therefore, the conclusions from these three-way choice assays of Apex-93_5 X Ginyou_3 DH Line, Altasweet and their F1 cross were that; (1) the F1 crossed line significantly differed in damage levels compared with Altasweet, but not Apex-93_5 X Ginyou_3 DH Line and; (2) visually estimated and ImageJ derived data correlated strongly and positively, despite the overestimation of by-eye scoring.

2.3.4.2 Again non-choice assays demonstrate more extreme differences in CSFB herbivory between Altasweet compared with Apex-93_5 X Ginyou_3 DH Line and their F1 cross

Alongside the three-way choice assays, reported in the previous section, a separate experiment was run conducting non-choice assays (i.e. a single *B. napus* accession in each chamber, see Methods 2.2.3.3) of Apex-93_5 X Ginyou_3 DH Line, Altasweet and their F1 cross, with the aim to further elucidate differences in adult CSFB feeding damage between the F1 cross and parental lines. The hypothesise were that 1) there will be differences in cotyledon damage between these three *B. napus* lines and that they are more extreme in a non-choice compared to a choice setting and 2) by-eye estimated scores will positively correlate with scores generated from image analysis using ImageJ software.

Focusing on visually estimated data first, a statistically significant effect of *B. napus* line on mean percentage damage to cotyledons in non-choice assays was observed (Table 2.50) (Figure 2.28a). Block and the interaction term between *B. napus* and block were observed to be non-significant so removed from the model. A one-way ANOVA demonstrated a strong statistically significant effect of *B. napus* line (Table 2.51). Running Tukey's HSD as a multiple comparisons test revealed that the mean percentage damage score of Altasweet, 9.98%, differed statistically significantly from that of both Apex-93_5 X Ginyou_3 DH Line, 1.98% and their F1 cross, 1.67% (Table 2.52). These differences were more extreme in non-choice than in choice assays. As previously with three-way choice assays, the mean percentage damage scores of the F1 cross and Apex-93_5 X Ginyou_3 DH Line did not differ statistically significantly (Table 2.52).

Table 2.50. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	54.19	27.096	66.830	< 0.00001
Block	3	3.01	1.004	2.476	0.0642
Line:Block	6	2.74	0.456	1.124	0.3517
Residuals	132	53.52	0.405		

Table 2.51. Summary output of a one-way ANOVA assessing differences between *B. napus* lines.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	54.19	27.096	64.47	< 0.00001
Residuals	141	59.26	0.420		

Table 2.52. Summary output of a Tukeys HSD multiple comparisons test, demonstrating a significant difference between Altasweet with Apex-93_5 X Ginyou_3 DH Line and the F1 cross.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Altasweet - Apex-93_5 X Ginyou_3 DH Line	1.295	0.982	1.609	< 0.00001
F1 - Apex-93_5 X Ginyou_3 DH Line	-0.012	-0.326	0.301	0.99518
F1 - Altasweet	-1.307	-1.621	-0.994	< 0.00001

Analysis of data obtained from ImageJ also revealed a statistically significant effect of *B. napus* line on cotyledon percentage damage (Table 2.53) (Figure 2.28b). As with visually obtained data, block and the interaction term between *B. napus* line and block was found to be non-significant and thus removed from the model and re-run as a one-way ANOVA (Table 2.54). Tukey's multiple comparisons test highlighted similar differences to that shown in the visually estimated data, with the mean percentage damage score of Altasweet at 5.52% being statistically significantly different from that of Apex-93_5 X Ginyou_3 DH Line at 0.95% and the F1 cross at 0.79% (Table 2.55). Similarly, these differences observed in non-choice assays were stronger than in choice assays. Consistent with previous results, there was no statistically significant difference between the mean percentage damage scores of Apex-93_5 X Ginyou_3 DH Line and the F1 cross (Table 2.55).

Table 2.53. Summary output of a two-way ANOVA assessing differences between *B. napus* line, block and their interaction for feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	45.12	22.560	115.307	< 0.00001
Block	3	0.99	0.329	1.684	0.174
Line:Block	6	1.43	0.238	1.216	0.302
Residuals	132	25.83	0.196		

Table 2.54. Summary output of a one-way ANOVA assessing differences between *B. napus* lines.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	2	45.12	22.56	112.6	< 0.00001
Residuals	141	28.24	0.20		

Table 2.55. Summary output of a Tukeys HSD multiple comparisons test, demonstrating a significant difference between Altasweet with Apex-93_5 X Ginyou_3 DH Line and the F1 cross.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Altasweet - Apex-93_5 X Ginyou_3 DH Line	1.163	0.947	1.380	< 0.00001
F1 - Apex-93_5 X Ginyou_3 DH Line	-0.047	-0.263	0.170	0.86696
F1 - Altasweet	-1.210	-1.426	-0.994	< 0.00001
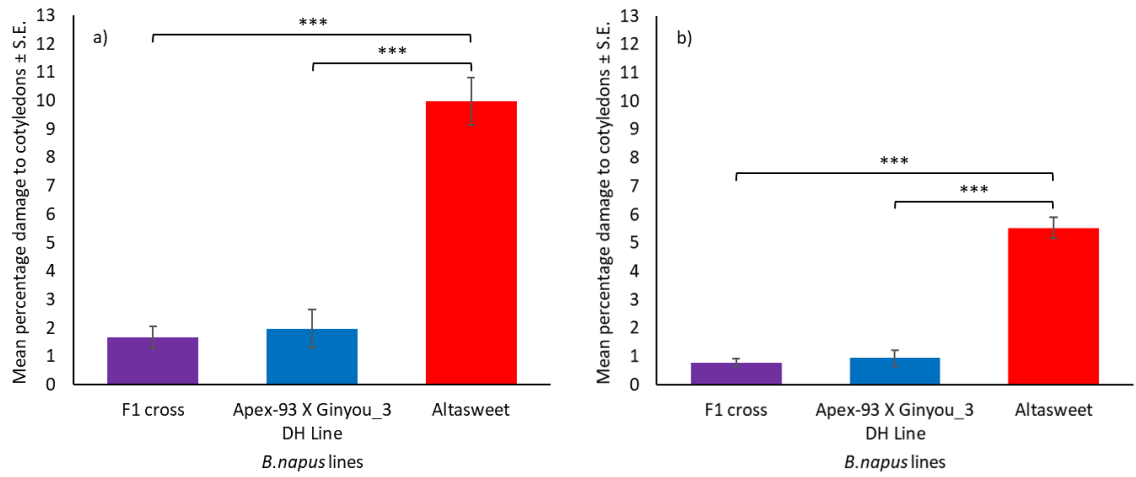
Figure 2.28. Variation in the mean percentage damage to cotyledons (± standard error) from non-choice chambers for three *B. napus* lines; Apex-93_5 X Ginyou_3 DH Line, Altasweet and F1 cross with these as parental lines. Data displayed is derived from a) by-eye estimated scores to the nearest 5% and b) Image analysis derived scores utilising the software ImageJ (*** $p < 0.001$) (n = 4).

A Pearson's correlation coefficient was calculated between visually estimated and ImageJ derived percentage damage data for each *B. napus* line in non-choice assays. All three *B. napus* lines demonstrated a statistically significant positive correlation between the two types of data (Figures 2.29a, b and c), with Altasweet being the strongest (r (46) = 0.904, *p* < 0.001). Visually estimated and ImageJ derived data for Apex-93_5 X Ginyou_3 DH Line and the F1 cross displayed similar levels of correlation ((r (46) = 0.874, *p* < 0.001) and (r (46) = 0.762, *p* < 0.001), respectively). Again, there was a consistent overestimation in the visually estimated scores compared with ImageJ derived scores.
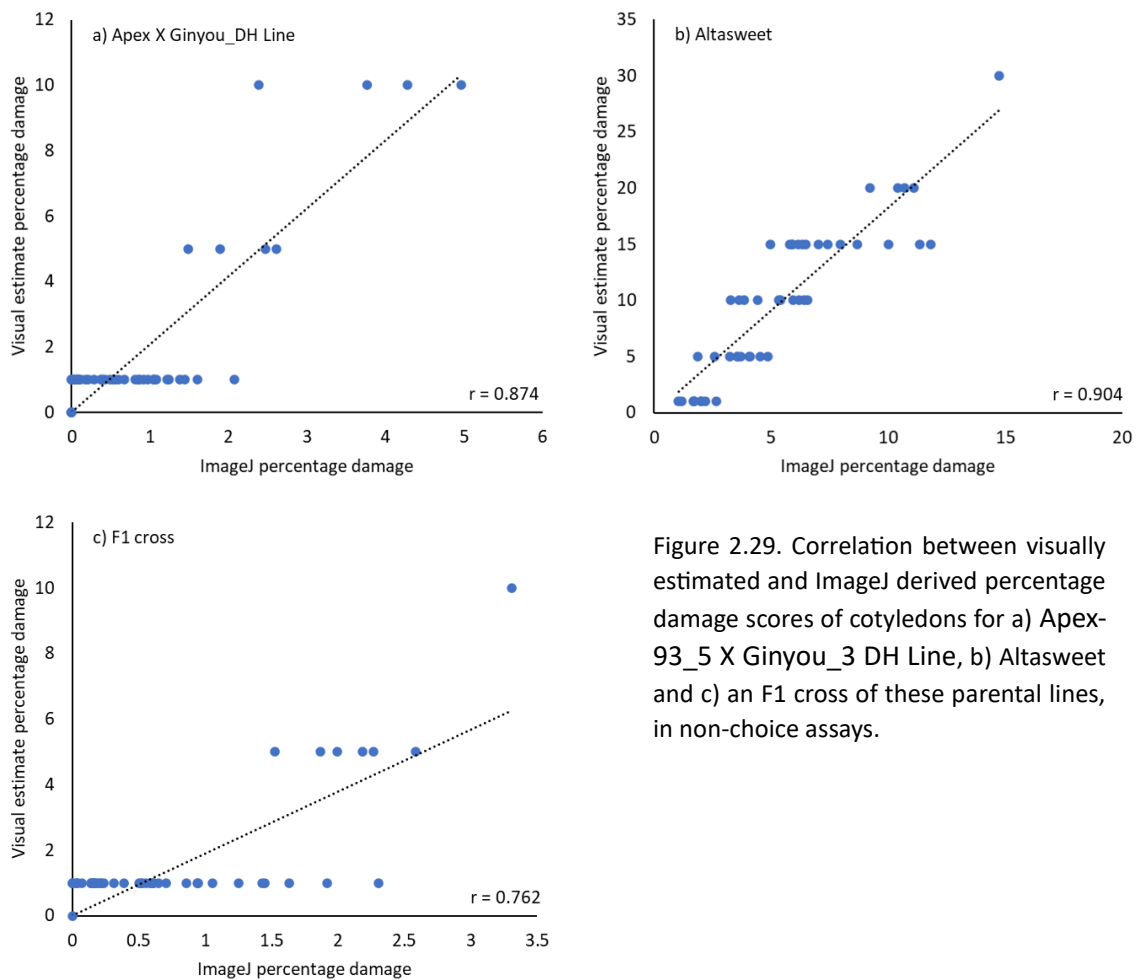
Figure 2.29. Correlation between visually estimated and ImageJ derived percentage damage scores of cotyledons for a) Apex-93_5 X Ginyou_3 DH Line, b) Altasweet and c) an F1 cross of these parental lines, in non-choice assays.

Therefore, from these non-choice assays the following conclusions were made; 1) Altasweet differs statistically significantly differently in levels of feeding damage compared with Apex-93_5 X Ginyou_3 DH Line and the F1 cross of these parental lines, 2) the F1 cross demonstrated similar levels of damage as the Apex-93_5 X Ginyou_3 DH Line parent and 3) despite overestimation of the by-eye scores, again the visually estimated and ImageJ generated data correlated strongly and positively.

In conclusion, it was observed that cotyledon percentage damage from adult CSFB to Altasweet did not significantly differ from Apex-93_5 X Ginyou_3 DH Line in choice assays but did in a non-choice setting (as also demonstrated in Chapter 2.3.3). Furthermore, the F1 cross of these parents received similar feeding levels of that of Apex-93_5 X Ginyou_3 DH Line but significantly lower levels compared to Altasweet in both choice and non-choice assays. Therefore, support is evident for the hypothesis that Altasweet is more palatable than Apex-93_5 X Ginyou_3 DH Line but that this difference is clearer in non-choice assays. Additionally, support was demonstrated for the hypothesis that the F1 cross receiving different levels of damage compared to one parental line. Furthermore, it was observed that significant differences between lines in choice assays were even more distinct in non-choice assays. Finally, it was concluded that by-eye estimation of percentage damage led to overestimation. However, the by-eye estimated data correlated well with ImageJ derived data for all *B. napus* lines, thus both are useful in distinguishing differences in palatability between varieties. Therefore, we provided support for our final hypothesis that visual estimates and computerised scores would positively correlate.

2.3.5 High levels of adult CSFB feeding damage end the field trial early, but variation in establishment and damage still successfully recorded

After observing some significant differences in feeding damage from adult CSFB in a laboratory setting, the next aim was to conduct a field trial to discern whether these differences are maintained in a field environment. Six *B. napus* lines were selected from the 96 laboratory tested lines that showed varying amounts of feeding damage in six-way choice assays (Altasweet, York and Shannon X Winner DH Line demonstrating high, Cabriolet demonstrating medium and Apex and Apex-93_5 X Ginyou_3 DH Line demonstrating low levels of damage (see Results 2.3.1.1)) and two commercial varieties, Skye and Kielder. Varieties were organised in a complete randomised balanced block design with each *B. napus* line appearing once per block (Figure 2.13 in Methods 2.2). The hypothesis was that there will be variation in the amount of feeding damage between *B. napus* varieties, specifically with Altasweet, York and Shannon X Winner DH Line receiving highest levels of damage compared to Apex and Apex-93_5 X Ginyou_3 DH with lowest levels of damage.

 To test for damage differences in the field, 20 seedlings were sampled from border plots 20 days after drilling and scored visually to the nearest 5% for percentage damage to cotyledons. Percentage damage data has been LOGIT+ transformed (Equation 2.1) for analysis with back transformed (Equation 2.2), adjusted data presented below. Additionally, plant counts per plot were obtained from a drone image taken 35 days after drilling to better understand establishment differences between varieties (see Methods 2.2.4). Seedling count data was Logit transformed due to the data being non-normally distributed (see Appendix 5). Due to poor seed quality and establishment scores, not characteristic of commercial lines, Skye and Kielder were removed from analyses. Both damage and establishment data were analysed running a two-way ANOVA with *B. napus* line as the main effect and block as a blocking factor. The assumption of a blocked design in field trials states there should not be an interaction between line and block, thus this interaction term was not included in the model (Buchse et al., 2000).

2.3.5.1 Strong differences recorded in establishment between six *B. napus* lines in the field

Seedling counts per plot were obtained from drone images with the aim of identifying how well different *B. napus* lines established in the field. Mean seedling count scores ranged from 11.6 for Altasweet to 50.2 for Apex-93_5 X Ginyou_3 DH Line (Figure 2.30). Running a two-way ANOVA of Line and Block revealed a strong significant effect of Line (Table 2.56). A Tukey's HSD test for multiple comparisons highlighted a number of statistically significant differences between *B. napus* lines, summarised in Table 2.57 and highlighted on Figure 2.42. Analyses also demonstrated a significant effect of block, and the post hoc test additionally revealed a significant difference in seedling counts between blocks one and five (Table 2.58) with all other blocks not differing significantly (all $p > 0.1$). Overall, seedling counts for the field trial was poor but significant differences were observed between *B. napus* lines, indicating some varieties were better able to establish than others.

Table 2.56. Output summary from a two-way ANOVA assessing variation in seedling counts between *B. napus* lines and blocks.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	5	24.433	4.887	15.852	< 0.00001
Block	4	4.142	1.035	3.359	0.0294
Residuals	20	6.165	0.308		

Table 2.57. Summary of Tukey's HSD multiple comparisons test for *B. napus* line on LOGIT transformed seedling count data.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Apex - Altasweet	1.923	0.819	3.030	0.00029
Apex-93_5 X Ginyou_3 DH Line - Altasweet	2.065	0.961	3.168	0.00012
Cabriolet – Altasweet	0.735	-0.368	1.839	0.32935
Shannon X Winner DH Line – Altasweet	-0.195	-1.299	0.908	0.99280
York – Altasweet	0.173	-0.930	1.277	0.99585
Apex-93_5 X Ginyou_3 DH Line - Apex	0.141	-0.962	1.245	0.99842
Cabriolet – Apex	-1.188	-2.292	-0.084	0.03036
Shannon X Winner DH Line - Apex	-2.118	-3.222	-1.015	0.00009
York – Apex	-1.750	-2.854	-0.646	0.00088
Cabriolet – Apex-93_5 X Ginyou_3 DH Line	-1.329	-2.433	-0.226	0.01274
Shannon X Winner DH Line – Apex-93_5 X Ginyou_3 DH Line	-2.260	-3.363	-1.156	0.00004
York – Apex-93_5 X Ginyou_3 DH Line	-1.891	-2.994	-0.787	0.00036
Shannon X Winner DH Line – Cabriolet	-0.931	-2.034	0.173	0.13070
York – Cabriolet	-0.562	-1.666	0.542	0.60782
York – Shannon X Winner DH Line	0.369	-0.735	1.472	0.89503

Table 2.58. Summary of Tukey's HSD multiple comparisons test for block.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Two – One	-0.476	-1.435	0.483	0.58322
Three – One	-0.369	-1.328	0.590	0.77753
Four – One	-0.430	-1.389	0.530	0.67053
Five – One	-1.148	-2.106	-0.188	0.01436
Three – Two	0.107	-0.852	1.066	0.99711
Four – Two	0.046	-0.913	1.006	0.99989
Five – Two	-0.671	-1.630	0.288	0.26106
Four – Three	-0.060	-1.020	0.899	0.99969
Five – Three	-0.778	-1.737	0.181	0.14881
Five - Four	-0.718	-1.677	0.242	0.20627
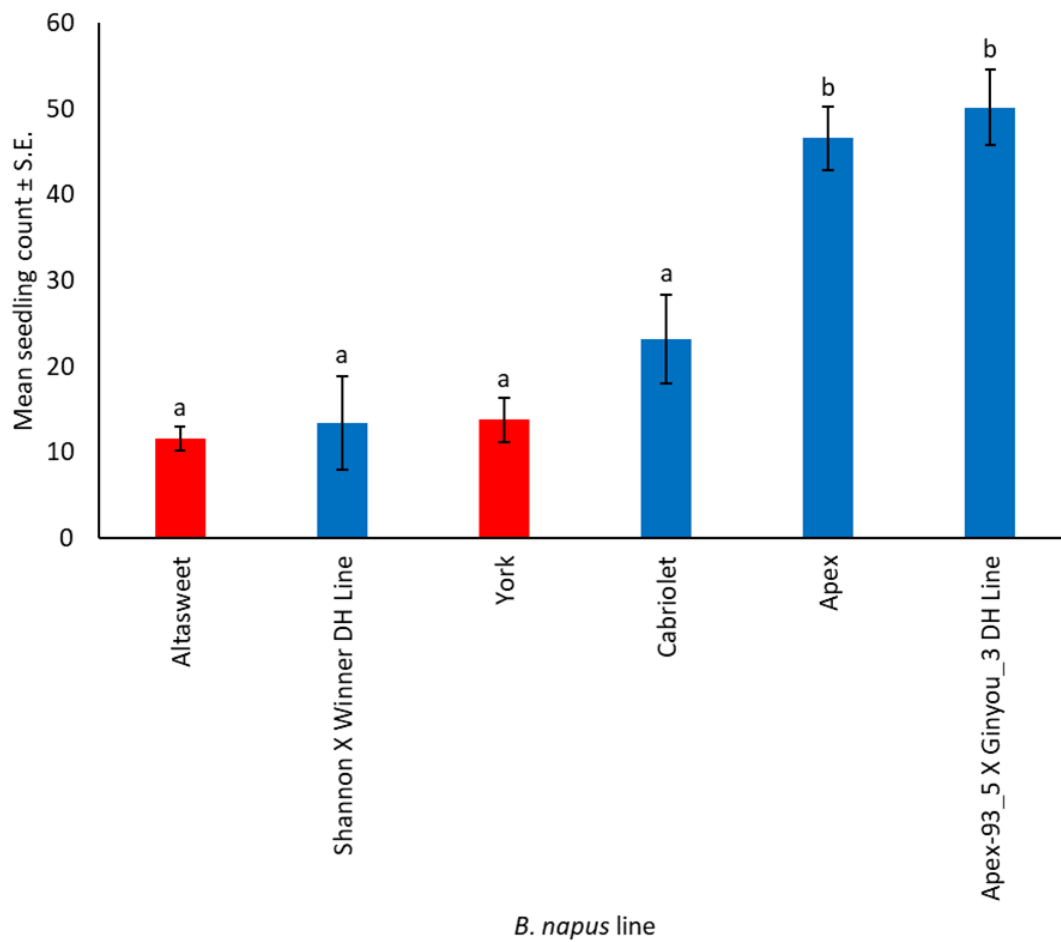
Figure 2.30. Variation in the mean seedling count for six *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 5. Letters denote statistically significant differences between lines, summarised in Table 2.55

2.3.5.2 Variation in adult CSFB feeding damage for six *B. napus* lines in the field

To quantify adult CSFB damage in the field, 20 seedlings per plot were sampled and visually scored percentage damage to cotyledons in the laboratory. From this, it was determined that adjusted mean feeding damage ranging from 17.21% to 26.22%, for Apex and Shannon X Winner DH Line, respectively (Figure 2.31). A two-way ANOVA demonstrated a strong significant effect of both *B. napus* line and block (Table 2.59). Significant differences between *B. napus* lines were revealed by a Tukey's HSD test and are summarised in Table 2.60. This test also demonstrated significant variation between some blocks (Table 2.61).

Table 2.59. Results of a two-way ANOVA of *B. napus* lines and Block showing differences in feeding damage.

	df	Sum of Squares	Mean Square	F value	*p* value
Line	5	21.0	4.196	6.622	< 0.00001
Block	4	22.7	5.678	8.961	< 0.00001
Residuals	584	370.0	0.634		

Table 2.60. Output from a Tukeys HSD multiple comparisons test, highlighting significant differences between *B. napus* lines for CSFB feeding damage.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Apex - Altasweet	-0.332	-0.656	-0.009	0.04024
Apex-93_5 X Ginyou_3 DH Line - Altasweet	-0.288	-0.612	0.0363	0.11449
Cabriolet – Altasweet	0.008	-0.314	0.331	1.00000
Shannon X Winner DH Line – Altasweet	0.172	-0.152	0.496	0.65321
York – Altasweet	0.097	-0.226	0.419	0.95667
Apex-93_5 X Ginyou_3 DH Line - Apex	0.044	-0.280	0.368	0.99884
Cabriolet – Apex	0.341	0.018	0.663	0.03170
Shannon X Winner DH Line - Apex	0.504	0.180	0.829	0.00015
York – Apex	0.429	0.106	0.751	0.00221
Cabriolet – Apex-93_5 X Ginyou_3 DH Line	0.296	-0.027	0.620	0.09409
Shannon X Winner DH Line – Apex-93_5 X Ginyou_3 DH Line	0.460	0.135	0.785	0.00083
York – Apex-93_5 X Ginyou_3 DH Line	0.385	0.061	0.708	0.00938
Shannon X Winner DH Line – Cabriolet	0.164	-0.160	0.487	0.69793
York – Cabriolet	0.088	-0.234	0.410	0.97030
York – Shannon X Winner DH Line	-0.076	-0.399	0.248	0.98535

Table 2.61. Output summary from a Tukeys HSD multiple comparisons test demonstrating the differences in CSDB feeding damage between Blocks.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Two – One	0.044	-0.237	0.327	0.99254
Three – One	0.230	-0.052	0.512	0.16984
Four – One	0.458	0.176	0.740	0.00010
Five – One	0.459	0.177	0.741	0.00010
Three – Two	0.185	-0.098	0.468	0.38105
Four – Two	0.413	0.130	0.696	0.00070
Five – Two	0.414	0.130	0.698	0.00069
Four – Three	0.228	-0.054	0.510	0.17740
Five – Three	0.229	-0.054	0.512	0.17508
Five - Four	0.001	-0.282	0.284	1.00000
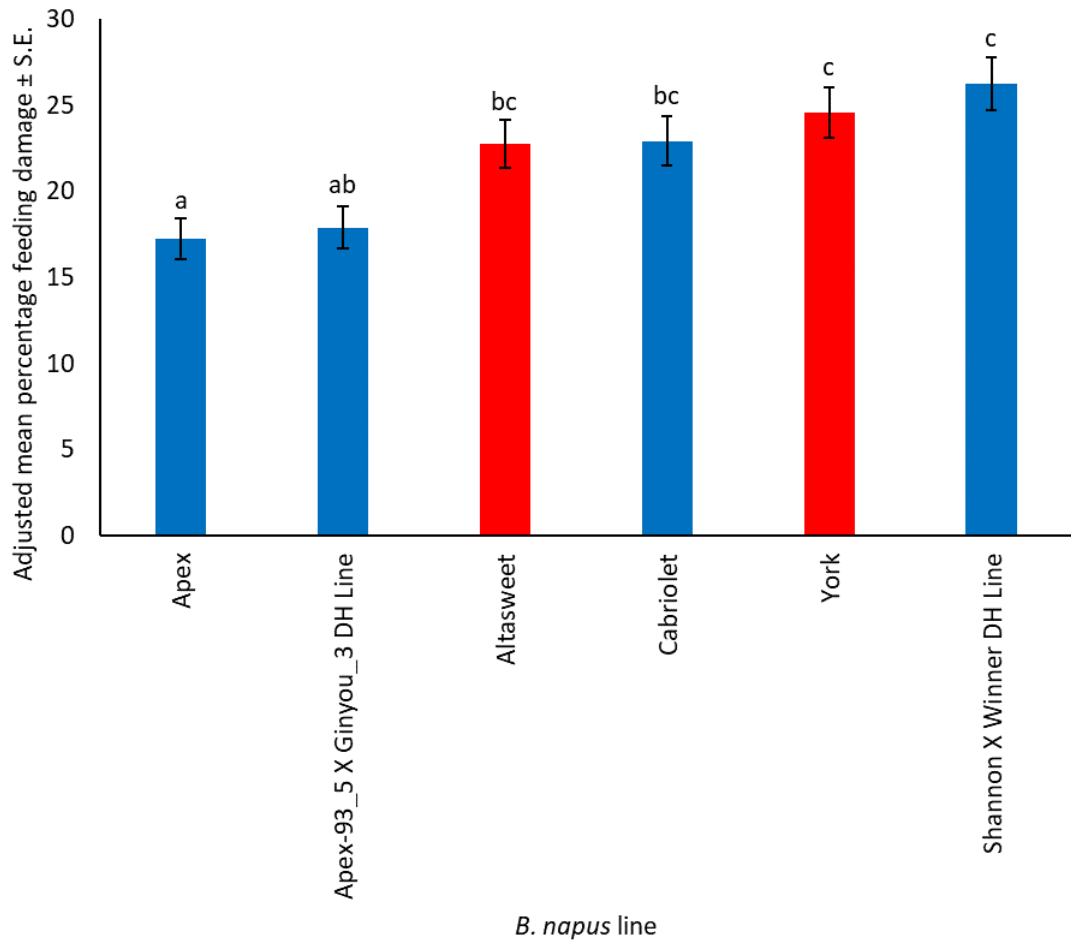
Figure 2.31. Adjusted mean percentage feeding damage for six *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 5. Letters denote statistically significant differences between lines, summarised in Table 2.58.

To better understand if there was a relationship between establishment and CSFB herbivory, a Pearson's correlation coefficient was run between seedling counts and percentage damage scores. A statistically significant, negative relationship between damage and establishment ($r_{(28)}$ = -0.586, $p < 0.001$) was observed, indicating plots with poorer establishment also tended to receive higher levels of feeding damage (Figure 2.32).
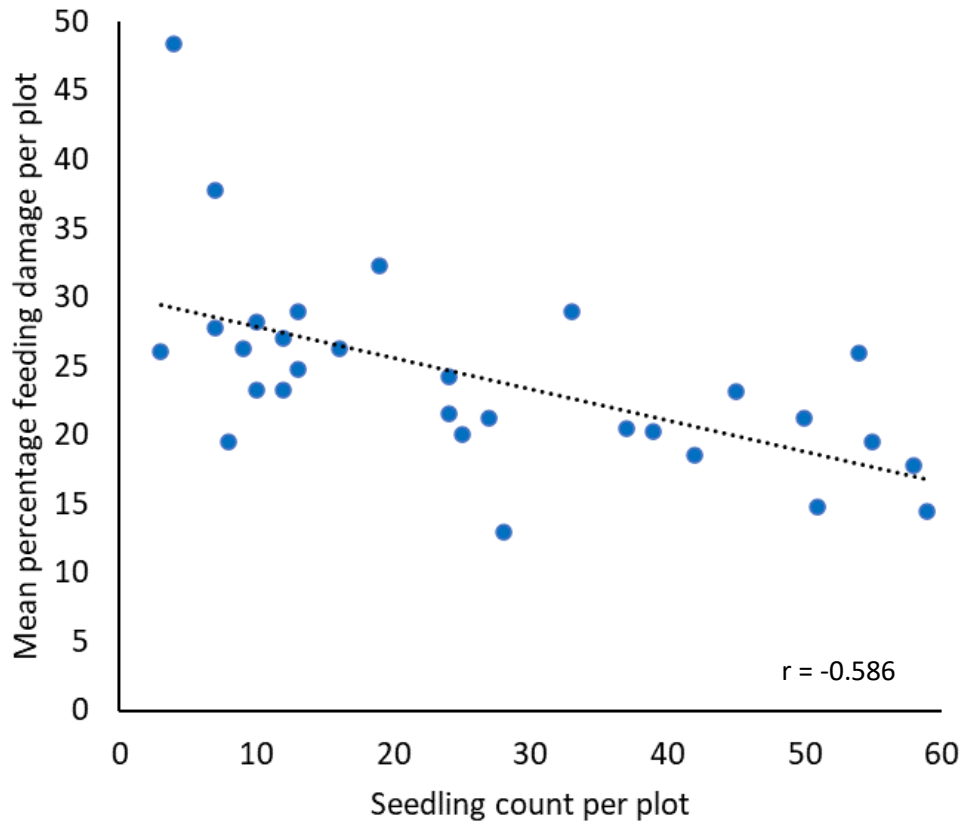
Figure 2.32. Correlation between percentage damage and seedling count scores for all plots.

Additionally, a Pearsons correlation coefficient was run between field damage and laboratory derived scores (from six-way choice chamber assays). This revealed a statistically significant positive correlation (r (4) = 0.894, $p < 0.02$), indicating that *B. napus* lines which received higher levels of damage in the laboratory also received higher damage in the field (Figure 2.33). It also highlights the higher levels of damage that occurred in the field trial compared with laboratory assays.
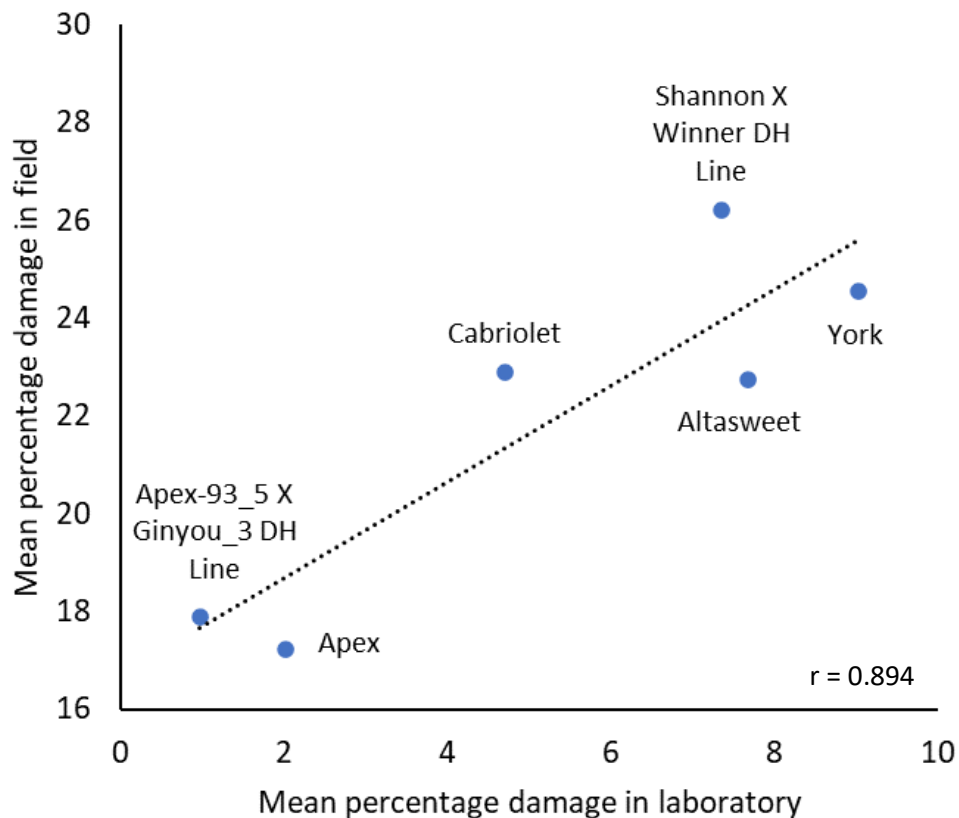
Figure 2.33. Correlation between percentage damage from six-way choice laboratory assays and field scores. Note that mean percentage damage in field axis starts at 16% not 0%.

In conclusion, *B. napus* lines had varying levels of establishment recorded, and lower levels of establishment correlated with higher levels of feeding damage. Despite the effect of block, there was significant variation in feeding damage in the field and this correlated with damage scores obtained from laboratory assays, thus following the expected trend. Therefore, support was demonstrated for the hypothesis that Altasweet, York and Shannon X Winner DH Line would receive higher levels of adult CSFB herbivory compared to Apex and Apex-93_5 X Ginyou_3 DH.

2.3.6 Differences between *B. napus* varieties observed for CSFB damage in both pesticide treated and non-treated field trials in 2020 to 2021

After losing the 2019 field trial prematurely (due to persistent high levels of pest damage) another field trial was conducted in the 2020 – 2021 growing season. This included growing a non-pesticide treated half (as done in 2019) and additionally a pyrethroid treated half as an insurance measure to ensure the trial made it through the winter period when larval CSFB damage occurs. The same eight *B. napus* lines were included as the previous year; Altasweet, York, Shannon X Winner DH Line, Cabriolet, Apex, Apex-93_5 X Ginyou_3 DH Line from the laboratory assays and two commercial varieties, Skye and Kielder. Due to limited seed for some *B. napus* lines, an additional commercial line, Elgar, was included to replace any missing plots of the other eight varieties. Lines were organised in a randomised incomplete block design (Methods 2.2.5). The hypothesis was that there will be variation in feeding damage, expecting lower levels on Apex and Apex-93_5 X Ginyou_3 DH Line and higher levels on Altasweet, York and Shannon X Winner DH Line.

The field trial involved collecting seedling counts from drone images take 25 days after drilling to obtain establishment scores for each *B. napus* line. Adult CSFB herbivory was scored by visually estimating, to the nearest 5%, percentage damage of cotyledons for ten plants per plot. Percentage damage data was LOGIT+ transformed (Equation 2.1) for analysis with back-transformed (Equation 2.2) data presented in the following section. Damage and establishment scores were analysed by running a two-way ANOVA of *B. napus* line and block. Pesticide treated and non-treated plots were considered as two separate trials and thus analysed separately.

2.3.6.1 Non-pesticide treated 2020 field trial demonstrates strong significant differences between *B. napus* lines for establishment.

Drone images were used to obtain plant counts to understand how well different *B. napus* lines established in the non-pesticide treated field trial. Mean seedling counts ranged from 39.7 to 99.8, for Skye and Elgar, respectively (Figure 2.34). Analysing data with a two-way ANOVA of line and block revealed a statistically significant effect of *B. napus* line on seedling count (Table 2.62). As block was found to be a non-significant factor it was dropped from the model. A one-way ANOVA demonstrated a strong statistically significant effect of *B. napus* line (Table 2.63) and a Tukeys HSD multiple comparisons test revealed a number of strong significant differences between some *B. napus* lines, summarised in Table 2.64. Therefore, it was concluded that there were differences in plant establishment between *B. napus* varieties.

Table 2.62. Results from a two-way ANOVA assessing the effects of *B. napus* Line and Block on seedling count for the non-pesticide treated trial.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	15525	1940.7	25.612	< 0.00001
Block	4	803	200.6	2.648	0.056
Residuals	26	1970	75.8		

Table 2.63. Output of a one-way ANOVA with Blocking factor removed for seedling count the non-pesticide treated trial.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	15525	1940.7	21	< 0.00001
Residuals	30	2773	92.4		
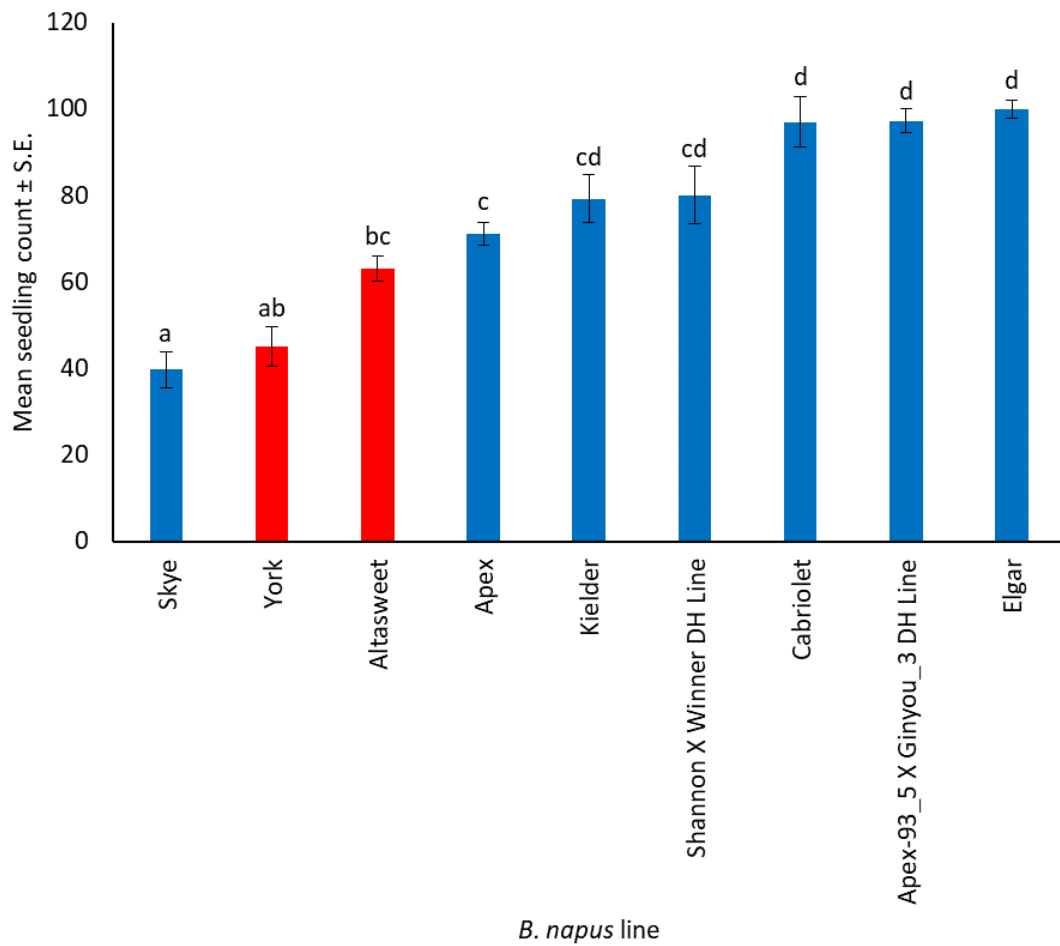
Figure 2.34. Variation in the mean seedling count for nine *B. napus* lines (± standard error) for the non-pesticide treated trial. Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for Shannon X Winner DH Line, Skye and York. n = 4 for Kielder. n = 5 for Altasweet, Apex, Apex-93_5 X Ginyou_3 DH Line and Cabriolet. n = 6 for Elgar. Letters denote statistically significant differences between lines, summarised in Table 2.62.

Table 2.64. Summary of differences between *B. napus* line for seedling count for the non-pesticide treated trial following a Tukeys HSD multiple comparisons test.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Apex - Altasweet	8.000	-12.293	28.293	0.91867
Apex-93_5 X Ginyou_3 DH Line - Altasweet	34.000	13.707	54.293	0.00014
Cabriolet – Altasweet	33.800	13.507	54.093	0.00015
Elgar – Altasweet	36.633	117.205	56.062	0.00002
Kielder – Altasweet	16.050	-5.474	37.574	0.27683
Shannon X Winner DH Line – Altasweet	16.800	-6.632	40.232	0.32367
Skye – Altasweet	-23.533	-46.965	-0.101	0.04837
York – Altasweet	-18.200	-41.632	5.232	0.23153
Apex-93_5 X Ginyou_3 DH Line - Apex	26.000	5.707	46.293	0.00485
Cabriolet – Apex	25.800	5.507	46.093	0.00529
Elgar – Apex	28.633	9.205	48.062	0.00086
Kielder – Apex	8.050	-13.474	29.574	0.93834
Shannon X Winner DH Line - Apex	8.800	-14.632	32.232	0.93696
Skye – Apex	-31.533	-54.965	-8.101	0.00274
York – Apex	-26.200	-49.632	-2.768	0.01951
Cabriolet – Apex-93_5 X Ginyou_3 DH Line	-0.200	-20.493	20.093	1.00000
Elgar – Apex-93_5 X Ginyou_3 DH Line	2.633	-16.795	22.032	0.99994
Kielder – Apex-93_5 X Ginyou_3 DH Line	-17.950	-39.474	3.574	0.16244
Shannon X Winner DH Line – Apex-93_5 X Ginyou_3 DH Line	-17.200	-40.432	6.232	0.29527
Skye – Apex-93_5 X Ginyou_3 DH Line	-57.533	-80.965	-34.101	< 0.00001
York – Apex-93_5 X Ginyou_3 DH Line	-52.200	-75.632	-28.768	< 0.00001
Elgar – Cabriolet	2.833	-16.595	22.262	0.99989
Kielder – Cabriolet	-17.750	-39.274	3.774	0.17241
Shannon X Winner DH Line – Cabriolet	-17.000	-40.432	6.432	0.30927
Skye – Cabriolet	-57.333	-80.765	-33.901	< 0.00001
York – Cabriolet	-52.000	-75.432	-28.568	< 0.00001
Kielder – Elgar	-20.583	-41.294	0.128	0.05241
Shannon X Winner DH Line – Elgar	-19.833	-42.521	2.855	0.12444
Skye – Elgar	-60.167	-82.855	-37.479	< 0.00001
York – Elgar	-54.833	-77.521	-32.145	< 0.00001

Shannon X Winner DH Line – Kielder	0.750	-23.756	25.256	1.00000
Skye – Kielder	-39.583	-64.089	-15.078	0.00024
York – Kielder	-34.250	-58.756	-9.744	0.00172
Skye – Shannon X Winner DH Line	-40.333	-66.531	-14.136	0.00047
York – Shannon X Winner DH Line	-35.000	-61.198	-8.802	0.00299
York - Skye	5.333	-20.864	31.531	0.99872

To compare to the previous establishment scores from the 2019 field trial, a Pearson's correlation was run against 2020 seedling counts (excluding Elgar, Skye and Kielder as these were not represented in the 2019 trial). This revealed a weak positive but non-significant relationship between 2019 and 2020 seedling counts (r (4) = 0.490, p = 0.324) (Figure 2.35). It additionally demonstrates the higher seedling counts observed in 2020 compared with the 2019 field trial.
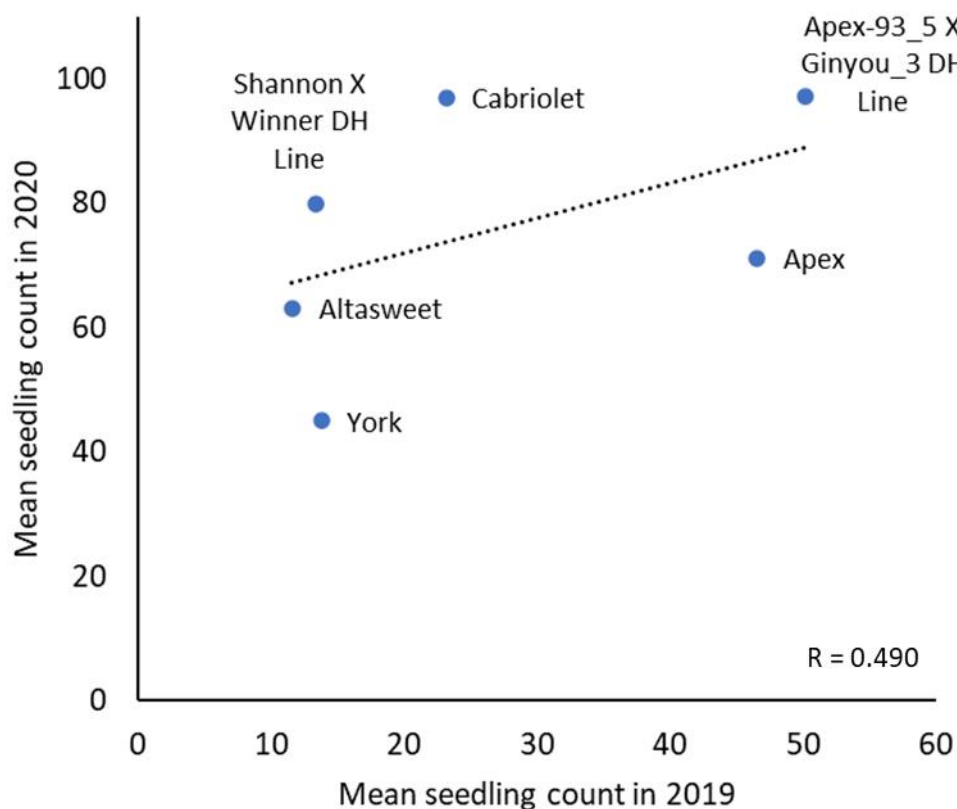
Figure 2.35. Pearson's correlation coefficient between mean seedling counts for six
B. napus lines in 2019 and 2020 for non-pesticide treated trials.

2.3.6.2 Non-pesticide treated 2020 field trial damage scores significantly varied between *B. napus* varieties.

To record variation of adult CSFB in the field, ten seedlings per plot were scored in-field for percentage eaten, to the nearest 5%. Damage scores ranged from 10.93% for Shannon X Winner DH Line to 18.79% for Skye (Figure 2.36). Running a two-way ANOVA of Line and Block demonstrated a strong, significant effect of *B. napus* line on percentage feeding damage (Table 2.65). Block was observed to be a non-significant factor thus was removed from the model. Running a one-way ANOVA also demonstrated a strong significant effect of *B. napus* Line on percentage feeding damage in the field (Table 2.66). A Tukey's HSD multiple comparisons test revealed a number of statistically significant differences between *B. napus* lines for damage, as summarised in Table 2.67.

Table 2.65. Output of a two-way ANOVA assessing the effects of *B. napus* line and block on percentage feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	15.71	1.964	7.871	< 0.00001
Block	4	1.44	0.361	1.446	0.218
Residuals	364	90.84	0.250		

Table 2.66. Results of a one-way ANOVA showing the significant effect of *B. napus* line on percentage feeding damage.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	15.71	1.964	7.833	< 0.00001
Residuals	368	92.28	0.251		
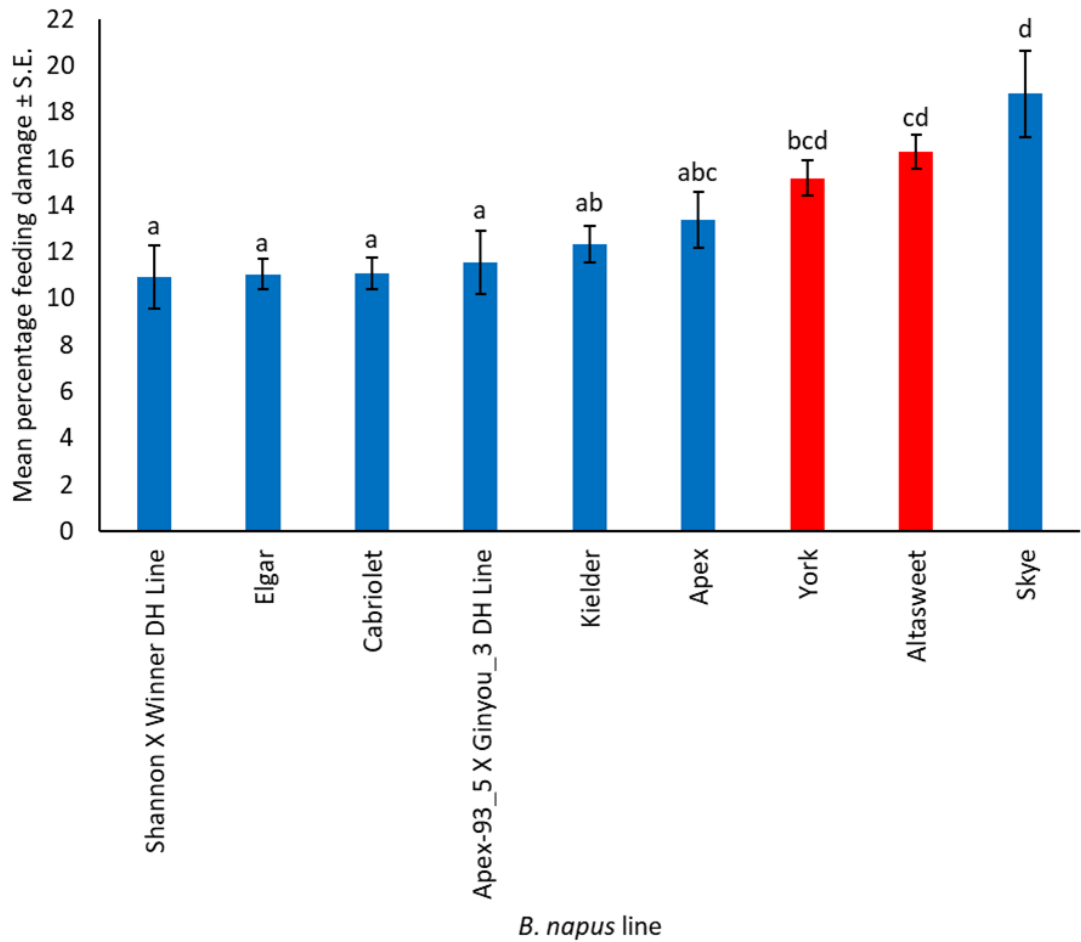
Figure 2.36. Variation in the mean percentage feeding damage for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for Shannon X Winner DH Line, Skye and York. n = 4 for Kielder. n = 5 for Altasweet, Apex, Apex-93_5 X Ginyou_3 DH Line Cabriolet and Kielder. n = 6 for Elgar. Letters denote statistically significant differences between lines, summarised in Table 2.65.

Table 2.67. Results from a Tukeys HSD multiple comparisons tests comparing different *B. napus* lines for percentage damage in the 2020 non-pesticide treated field trial.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Apex - Altasweet	-0.280	-0.599	0.039	0.13817
Apex-93_5 X Ginyou_3 DH Line - Altasweet	-0.469	-0.784	-0.153	0.00017
Cabriolet – Altasweet	-0.440	-0.767	-0.113	0.00111
Elgar – Altasweet	-0.453	-0.756	-0.150	0.00015
Kielder – Altasweet	-0.346	-0.660	-0.032	0.01870
Shannon X Winner DH Line – Altasweet	-0.493	-0.866	-0.120	0.00150
Skye – Altasweet	0.116	-0.249	0.481	0.98635
York – Altasweet	-0.066	-0.430	0.299	0.99976
Apex-93_5 X Ginyou_3 DH Line - Apex	-0.188	-0.512	0.134	0.66672
Cabriolet – Apex	-0.160	-0.494	0.173	0.85614
Elgar – Apex	-0.173	-0.483	0.137	0.72057
Kielder – Apex	-0.066	-00387	0.255	0.99936
Shannon X Winner DH Line - Apex	-0.213	-0.592	0.166	0.71145
Skye – Apex	0.396	0.026	0.767	0.02583
York – Apex	0.215	-0.156	0.585	0.67722
Cabriolet – Apex-93_5 X Ginyou_3 DH Line	0.028	-0.302	0.358	1.00000
Elgar – Apex-93_5 X Ginyou_3 DH Line	0.016	-0.291	0.322	1.00000
Kielder – Apex-93_5 X Ginyou_3 DH Line	0.123	-0.195	0.440	0.95511
Shannon X Winner DH Line – Apex-93_5 X Ginyou_3 DH Line	-0.025	-0.401	0.351	1.00000
Skye – Apex-93_5 X Ginyou_3 DH Line	0.585	0.217	0.952	0.00004
York – Apex-93_5 X Ginyou_3 DH Line	0.403	0.035	0.771	0.01968
Elgar – Cabriolet	-0.013	-0.330	0.305	1.00000
Kielder – Cabriolet	0.094	-0.234	0.423	0.99314
Shannon X Winner DH Line – Cabriolet	-0.053	-0.438	0.332	0.99997
Skye – Cabriolet	0.556	0.179	0.934	0.00020
York – Cabriolet	0.375	-0.003	0.752	0.05315
Kielder – Elgar	0.107	-0.197	0.411	0.97450
Shannon X Winner DH Line – Elgar	-0.040	-0.405	0.325	0.99999
Skye – Elgar	0.569	0.213	0.926	0.00003
York – Elgar	0.387	0.031	0.744	0.02168

Shannon X Winner DH Line – Kielder	-0.147	-0.522	0.227	0.95014
Skye – Kielder	0.462	0.096	0.828	0.00312
York – Kielder	0.280	-0.086	0.647	0.29229
Skye – Shannon X Winner DH Line	0.609	0.191	1.027	0.00025
York – Shannon X Winner DH Line	0.428	0.010	0.846	0.04023
York - Skye	-0.182	-0.592	0.229	0.90437

To better understand the relationship between herbivory and establishment, a Pearson's correlation coefficient was run between mean percentage damage per plot and seedling count per plot. This demonstrated a strong negative correlation (r = (39) -0.722, *p* < 0.001), indicating plots with lower establishment scores received higher adult CSFB feeding damage (Figure 2.37).
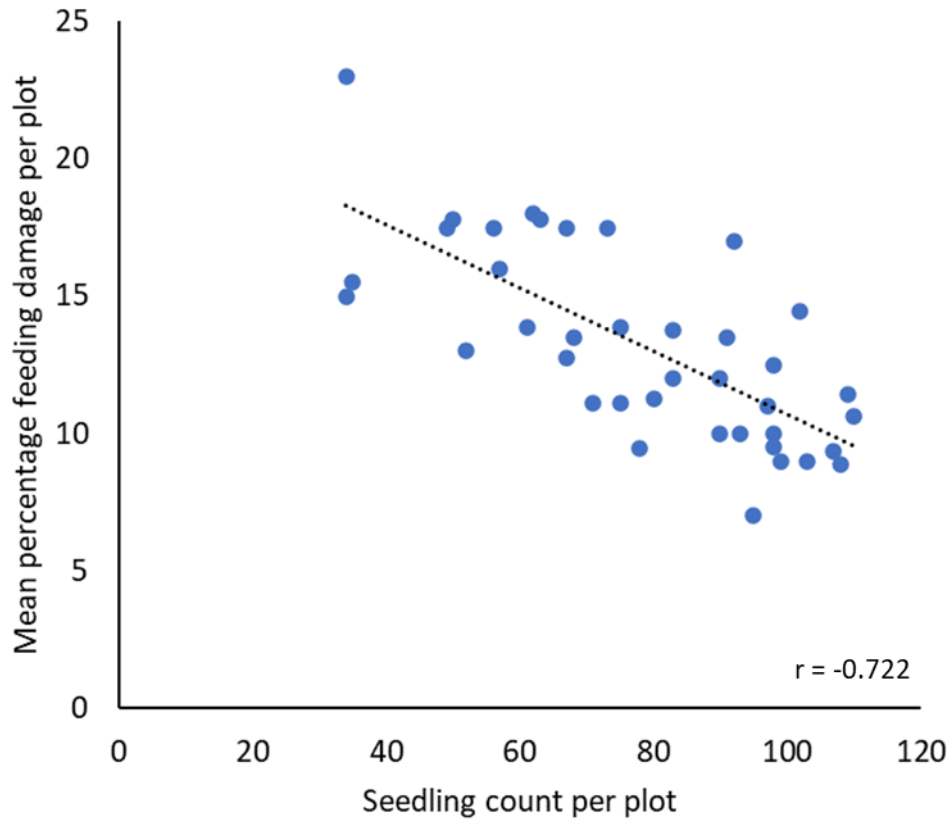
Figure 2.37. Pearson's correlation between mean percentage damage per plot and seedling count per plot.

Additionally, a Pearson's correlation coefficient was run between CSFB damage scores from 2020 to those in the 2019 field trial (excluding Elgar, Skye and Kielder as they were not present in the 2019 trial). This demonstrated a non-significant and weak relationship between the CSFB herbivory between the two trials (r = (4) -0.167, p = 0.752), indicating *B. napus* lines received differing amounts of damage between the two years (Figure 2.38). This additionally demonstrated that plants received higher levels of damage in 2019 compared with 2020.
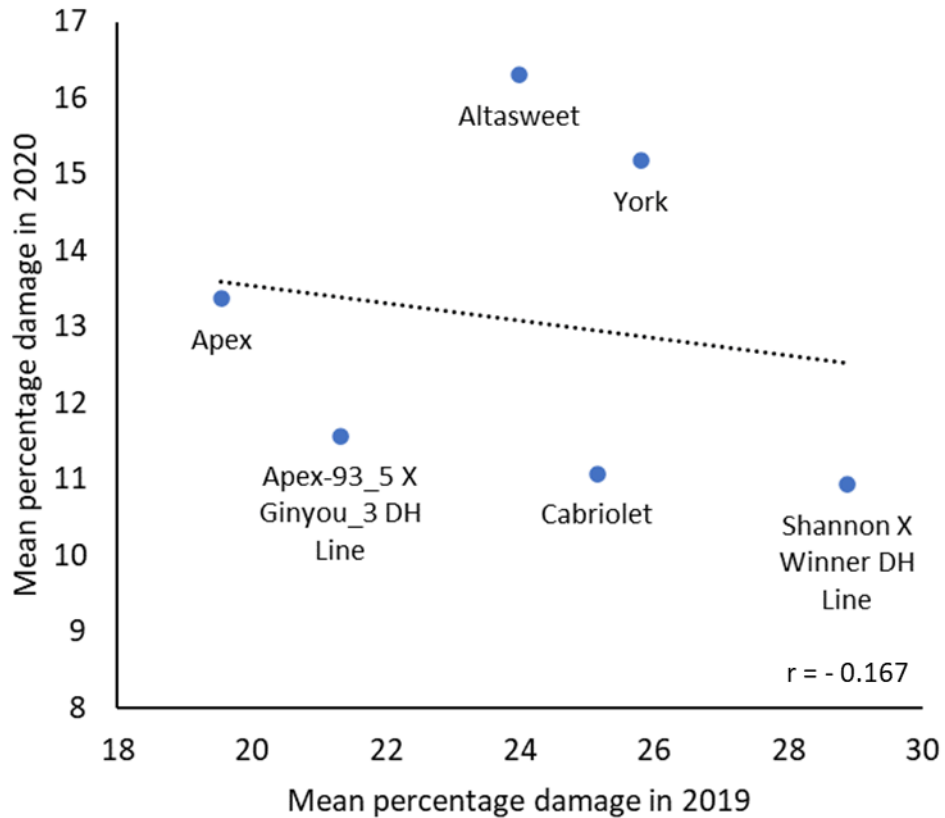
Figure 2.38. Pearson's correlation demonstrating the relationship between mean percentage damage scores in the 2019 and 2020 field trials. Note the x axis starts at 18% and y axis at 8%.

Finally, a Pearson's correlation was run in order to better understand the relationship between 2020 non-pesticide treated field damage and scores derived from six-way choice laboratory assays. This revealed a moderate positive but non-significant relationship between field damage and laboratory damage scores (r = (4) 0.479, *p* = 0.337), indicating that some *B. napus* lines behaved in a similar way in both experiments (Figure 2.39). Additionally, this correlation demonstrated that damage in the field was greater than that observed in the laboratory (Figure 2.39).
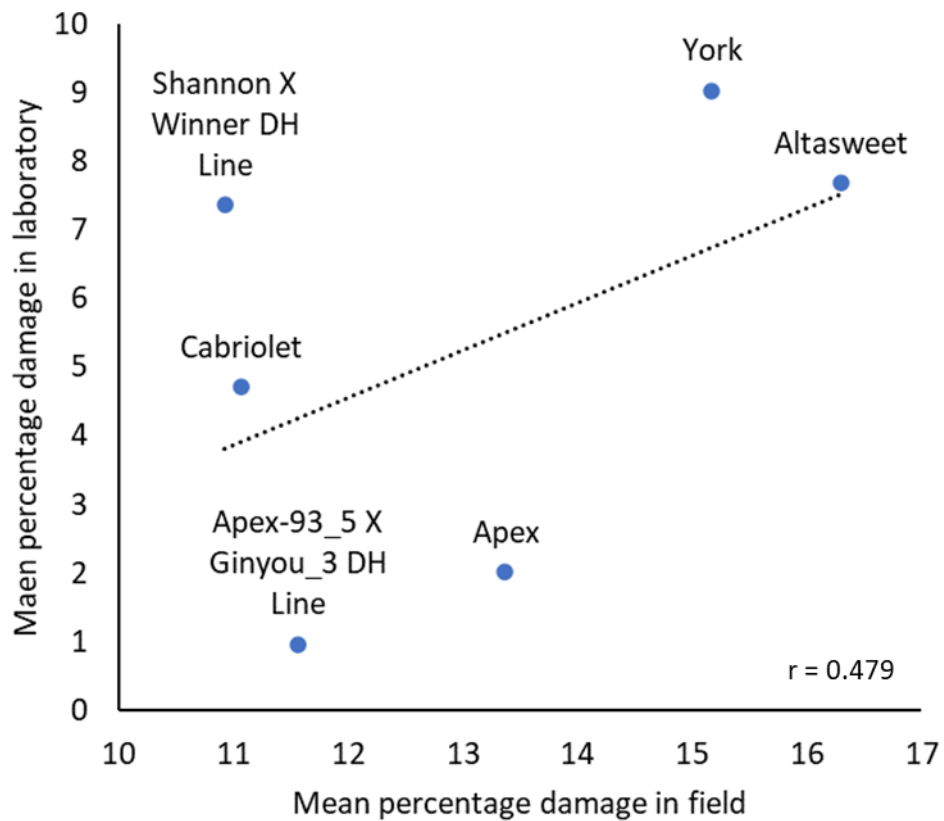
Figure 2.39. Pearson's correlation demonstrating the relationship between mean percentage damage scores in the 2020 field trial and six-way choice assays in the laboratory. Note that the x axis starts at 10%.

In conclusion, differences were observed in establishment between *B. napus* lines and establishment strongly negatively correlated with adult CSFB herbivory, with low seedling counts correlating with higher levels of damage. Like the 2019 field trial, also observed was significant differences between *B. napus* lines for CSFB herbivory, particularly Altasweet receiving higher levels of damage compared to Apex-93_5 X Ginyou_3 DH. Damage in the 2020 field trial was lower than the 2019 trial and it did not correlate as strongly with laboratory scores, particularly with Shannon X Winner DH Line receiving lower levels of damage compared with the previous year. However, the expected trend of Altasweet and York receiving higher levels of herbivory compared with Apex and Apex-93_5 X Ginyou_3 DH was still observed. Therefore, support was demonstrated for the hypothesis that Altasweet and York would receive higher levels of damage in comparison to Apex and Apex-93_5 X Ginyou_3 DH.

2.3.6.3 Pesticide treated *B. napus* establishment varied between *B. napus* varieties

In addition to the non-pesticide treated field trial, a pyrethroid and neonicotinoid treated trial was conducted with the aim to ensure the plants made it through the winter larval CSFB season (see Appendix 3 for full application schedule). This trial consisted of the same *B. napus* lines as the non-treated trial and was organised in a randomised incomplete block design (see Methods 2.2.5). The hypothesis was that plots in the treated trial will receive lower levels of adult CSFB feeding damage compared to the non-treated trial. Establishment and CSFB herbivory scores were obtained and analysed in the same way as the non-pesticide treated field trial (see Methods 2.2.5).

To understand how well different *B. napus* lines established in the pesticide treated field trial, seedling counts were collected from a drone image taken on 15.09.2020, 25 days after sowing. There was a range of establishment, from 39.67 to 99.83 seedlings, for Skye and Elgar, respectively (Figure 2.40). Running a two-way ANOVA demonstrated a statistically significant difference between *B. napus* lines (Table 2.68). Block was also a significant factor in seedling count so retained in the model. A Tukey's multiple comparisons test demonstrated where significant differences occurred between *B. napus* lines (Table 2.69). This test additionally revealed a significant difference between certain blocks, summarised in Table 2.70. It was again concluded that there was variation in establishment between *B. napus* varieties for the pesticide treated field trial.

Table 2.68. Output of a two-way ANOVA assessing the effects of *B. napus* Line and Block on seedling count in the 2020 pesticide treated field trial.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	20917	2614.6	18.727	<0.00001
Block	4	2594	648.5	4.645	0.00554
Residuals	27	3770	139.6		

Table 2.69. Summary of Tukeys HSD multiple comparisons test demonstrating the differences between *B. napus* lines for seedling count in the 2020 pesticide treated field trial.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Apex – Altasweet	-0.400	-25.545	24.745	1.00000
Apex-93_5 X Ginyou_3 DH Line – Altasweet	31.800	6.655	56.945	0.00594
Cabriolet – Altasweet	37.800	12.655	62.945	0.00076
Elgar – Altasweet	26.800	1.655	51.945	0.03017
Kielder – Altasweet	4.200	-20.945	29.345	0.99967
Shannon X Winner DH Line – Altasweet	4.733	-24.302	33.768	0.99972
Skye – Altasweet	-39.600	-68.635	-10.565	0.00255
York – Altasweet	-24.850	-51.520	1.820	0.08240
Apex-93_5 X Ginyou_3 DH Line – Apex	32.200	7.055	57.345	0.00519
Cabriolet – Apex	38.200	13.055	63.345	0.00066
Elgar – Apex	27.200	2.055	52.345	0.02663
Kielder – Apex	4.600	-20.545	29.745	0.99935
Shannon X Winner DH Line – Apex	5.133	-23.902	34.168	0.99949
Skye – Apex	-39.200	-68.235	-10.165	0.00287
York – Apex	-24.450	-51.120	2.220	0.91604
Cabriolet – Apex-93_5 X Ginyou_3 DH Line	6.000	-19.145	31.145	0099582
Elgar – Apex-93_5 X Ginyou_3 DH Line	-5.000	-30.256	20.145	0.99882
Kielder – Apex-93_5 X Ginyou_3 DH Line	-27.600	-52.745	-2.455	0.02347
Shannon X Winner DH Line – Apex-93_5 X Ginyou_3 DH Line	-27.067	-56.102	1.968	0.08212
Skye – Apex-93_5 X Ginyou_3 DH Line	-71.400	-100.435	-42.365	< 0.00001
York – Apex-93_5 X Ginyou_3 DH Line	-56.650	-83.320	-29.980	< 0.00001
Elgar – Cabriolet	-11.000	-36.145	14.145	0.85816
Kielder – Cabriolet	-33.600	-58.745	-8.455	0.00323
Shannon X Winner DH Line – Cabriolet	-33.067	-62.102	-48.365	0.01686
Skye – Cabriolet	-77.400	-106.435	-48.365	< 0.00001
York – Cabriolet	-62.650	-89.320	-35.980	< 0.00001
Kielder – Elgar	-22.600	-47.745	2.545	0.10379
Shannon X Winner DH Line – Elgar	-22.067	-51.102	6.968	0.25034
Skye – Elgar	-66.400	-95.435	-37.365	< 0.00001
York – Elgar	-51.650	-78.320	-24.980	0.00002

	Mean difference	-28.502	29.568	1.00000
Shannon X Winner DH Line – Kielder	0.533	-28.502	29.568	1.00000
Skye – Kielder	-43.800	-72.835	-14.765	0.00072
York – Kielder	-29.050	-55.720	-2.380	0.02509
Skye – Shannon X Winner DH Line	-44.333	-76.795	-11.871	0.00251
York – Shannon X Winner DH Line	-29.583	-59.949	0.782	0.06056
York - Skye	14.750	-15.616	45.116	0.77820

Table 2.70. Summary of Tukeys HSD multiple comparisons test demonstrating the differences between blocks for seedling count in the 2020 pesticide treated field trial.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Two – One	-2.427	-19.683	14.829	0.99366
Three – One	-20.469	-37.724	-3.213	0.01415
Four – One	-14.927	-32.183	2.329	0.11427
Five – One	-14.594	-31.849	2.662	0.12764
Three – Two	-18.042	-35.297	-0.786	0.03714
Four – Two	-12.500	-29.756	4.756	0.24272
Five – Two	-12.167	-29.422	5.089	0.26642
Four – Three	5.542	-11.714	22.797	0.87951
Five – Three	5.875	-11.381	23.131	0.85553
Five – Four	0.333	-16.922	17.589	1.00000
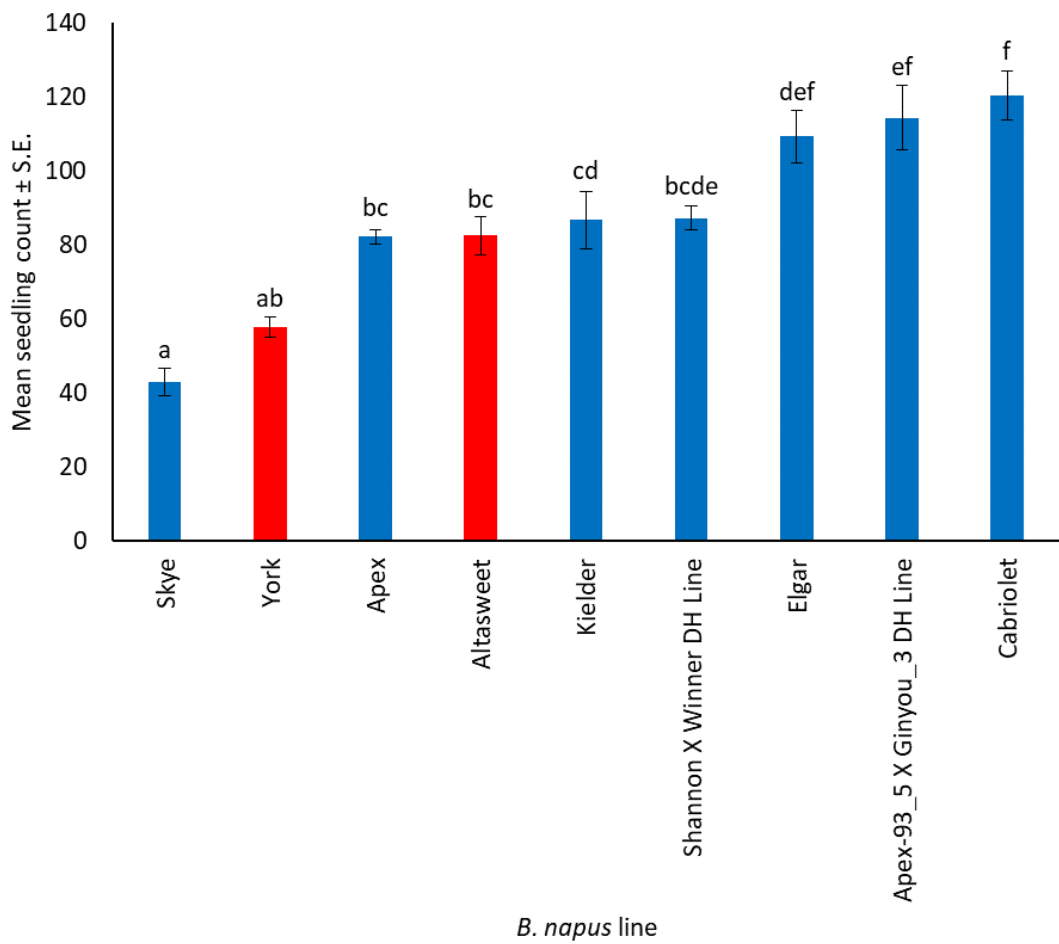
Figure 2.40. Variation in the mean seedling count for nine *B. napus* lines for the pesticide treated trial (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for Shannon X Winner DH Line and Skye. n = 4 for York. n = 5 for Altasweet, Apex, Apex-93_5 X Ginyou_3 DH Line, Cabriolet, Elgar and Kielder. Letters denote statistically significant differences between lines, summarised in Table 2.67.

It was also interested whether there was a relationship between pesticide treated and non-treated seedling counts. Running a Pearsons correlation revealed a statistically significant strong positive correlation between the two trials (r = (7) 0.973, *p* = 0.00001), indicating that establishment was similar for *B. napus* lines between treated and non-treated plots (Figure 2.41). Figure 2.41 also demonstrates a slightly higher seedling count for *B. napus* lines in the pesticide treated field trial compared to the non-treated trial.
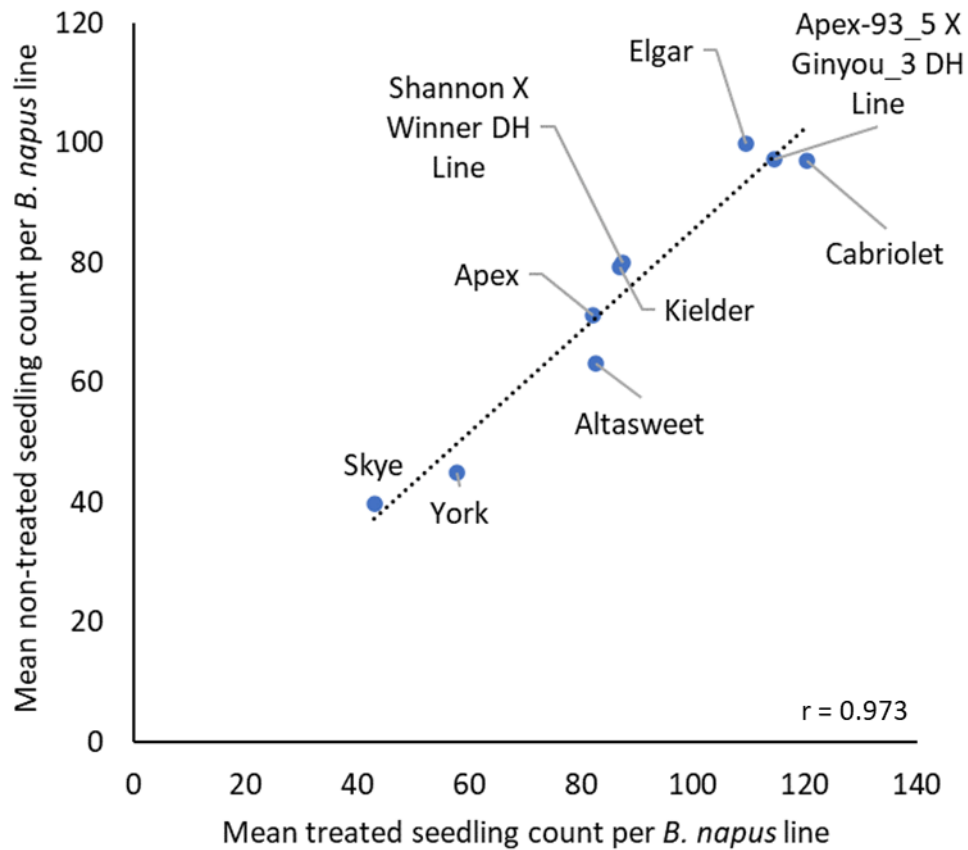
Figure 2.41. Pearsons correlation between pesticide treated and non-pesticide treated *B. napus* lines for mean seedling count.

2.3.6.4 Pesticide treated *B. napus* CSFB damage varied between varieties

To assess differences in adult CSFB damage in the 2020 – 2021 pesticide treated field trial, percentage damage scores to cotyledons for 10 plants per plot were collected, by visually estimation to the nearest 5%. Variation of feeding damage was observed, with scores ranging from 9.59% for Apex-93_5 X Ginyou_3 DH Line and 14.26% for Altasweet (Figure 2.42). Analysing with a two-way ANOVA revealed a strongly statistically significant effect of *B. napus* line on percentage damage (Table 2.71). Additionally, block was also demonstrated to be statistically significant, and thus is retained in the model. To assess differences in damage between individual *B. napus* lines, a Tukeys HSD multiple comparisons test was run. This demonstrated only one statistically significant difference between two *B. napus* lines, Apex-93_5 X Ginyou_3 DH Line and Altasweet ($p < 0.008$, 95% C.I. = -0.797, -0.061), as demonstrated in Figure 2.42. A statistically significant difference between blocks four and five was also observed ($p < 0.001$, C.I. = -0.617, -0.113). Therefore, although there was less variation in damage for the treated field trial, it was concluded that there were significant differences between Altasweet and Apex-93_5 X Ginyou_3 DH Line.

Table 2.71. Summary of the output from a two-way ANOVA, assessing the effects of *B. napus* line and block on percentage damage from adult CSFB in the 2020 – 2021 pesticide treated field trial.

	df	Sum of Squares	Mean Square	*F* value	*p* value
Line	8	7.02	0.8781	2.769	0.00557
Block	4	6.01	1.5017	4.736	0.00098
Residuals	353	111.94	0.3171		
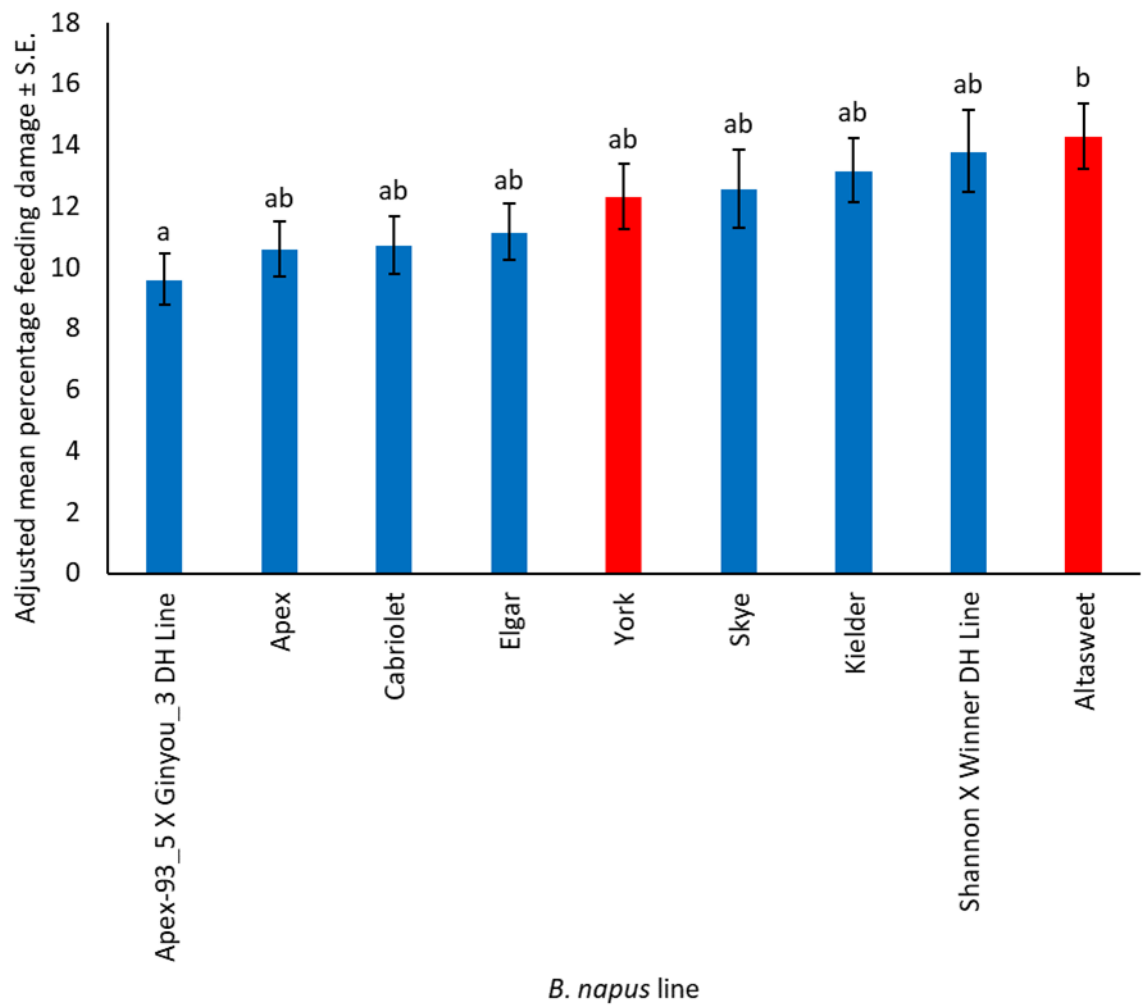
Figure 2.42. Variation in the adjusted mean percentage damage to cotyledons for nine *B. napus* lines (± standard error). Colours equate to crop type; Red = Swede, Blue = WOSR. n = 3 for Shannon X Winner DH Line and Skye. n = 4 for York. n = 5 for Altasweet, Apex, Apex-93_5 X Ginyou_3 DH Line, Cabriolet, Elgar and Kielder. Letters denote statistically significant differences between lines.

To better understand how herbivory related to establishment scores for the pesticide treated trial, a Pearsons correlation was run between percentage damage scores and seedling counts, which demonstrated no relationship between the two (r = (38) -0.082, p = 0.614) (Figure 2.43). This indicates that herbivory damage and seedling establishment were not significant in this trial.
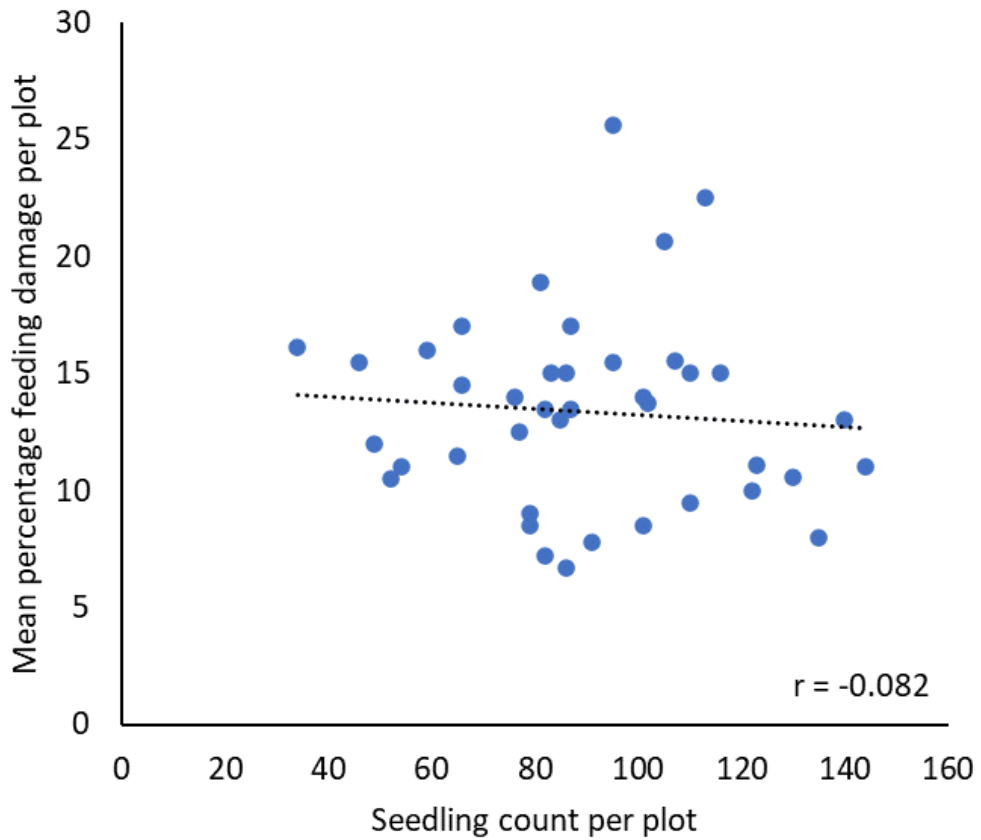
Figure 2.43. Pearsons correlation between mean percentage damage and seedling counts per plot for the pesticide treated 2020 – 2021 field trial.

Finally, a Pearsons correlation was run to better understand how pesticide treated plants compared to non-treated plants for CSFB damage. This demonstrated a moderate but non-significant interaction between pesticide treated and non-treated damage scores (r (7) = 0.410, p = 0.273) (Figure 2.44). This indicated that for some *B. napus* lines there were similar levels of damage between the two trials.
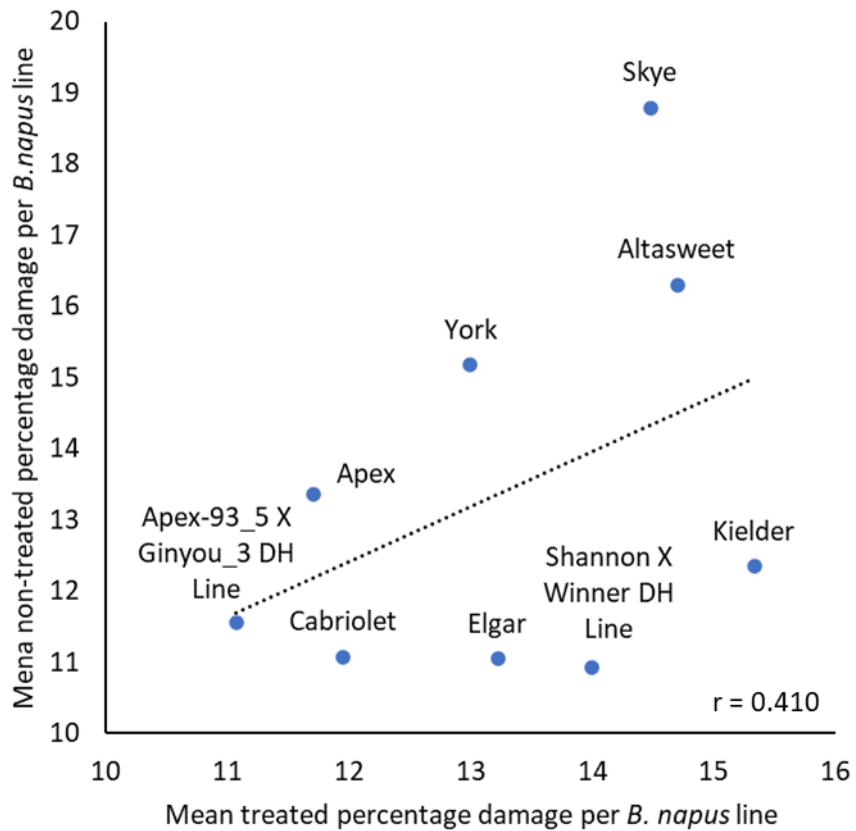
Figure 2.44. Pearsons correlation between mean non-pesticide treated and pesticide treated CSFB percentage damage.

In summary of the 2020 – 2021 pesticide treated field trial, a significant difference was observed between *B. napus* lines for seedling count and this correlated strongly with establishment scores from the non-treated field trial. For adult CSFB feeding damage, less extreme differences were recorded for the treated trial compared to the non-treated trial. However, a significant difference was still observed in damage levels between Altasweet and Apex-93_5 X Ginyou_3 DH Line. Additionally, although there was only a weak correlation between damage scores for the non-treated and treated field trials, it was observed that Apex-93_5 X Ginyou_3 DH Line and Apex were at the lower end and Altasweet at the higher end of the distribution. Therefore, the pesticide treated field trial ran in 2020 – 2021 provides additional support for the hypothesis that Altasweet would receive higher levels of damage compared to Apex-93_5 X Ginyou_3 DH Line. Overall, for both 2019 and 2020 field trials, there was support for Altasweet being more highly damaged by adult CSFB than Apex-93_5 X Ginyou_3 DH Line.

2.4 Discussion

Novel six-way choice assays examining adult CSFB of intact *B. napus* seedlings allowed identification of some variation, albeit non-significant (other than for the grazing feeding trait), between varieties under laboratory conditions. This could potentially be because there were no actual differences in adult feeding preferences between *B. napus* varieties. However, it could have also been that this experimental design is not suitable for establishing CSFB herbivory differences, with low replication levels (n = 3) and multiple choice of food source in a relatively limited spatial range, with poor air flow. Differentiation between food sources by CSFB in such an environment may be problematic if olfaction is involved in host selection (Henderson et al., 2004). Indeed Bartlet et al. (1999) identified sensilla likely involved in olfactory sensing on CSFB.

The one feeding trait where differences were observed in damage levels was grazing, characterised by surface level herbivory on cotyledons, unlike shot holing where beetles create feeding holes the entire way through the leaf material. Altasweet, a swede type, demonstrated the lowest levels of grazing damage but one of the highest levels of total damage. Additionally, when comparing grazing damage between crop types, we observed swede types differing significantly compared to WOSR and semi-winter OSR *B. napus* varieties. As Henderson et al. (2004) demonstrate with *P. cruciferae*, flea beetles go through a number of sensory detection behaviours when selecting food plants. One of the observed behaviours was test biting, where contact with mouthparts, that contain many chemosensilla, is made with the surface of cotyledons. It may be that the grazing feeding trait observed in CSFB in these experiments is them test biting the plant to determine whether it is a food source they want to consume. This could explain why Altasweet received low levels of grazing damage but high levels of total damage, indicating CSFB test bit their food source before committing to proper feeding.

To test this idea further, a potential experiment could be presenting beetles with a choice between two *B. napus* seedlings in a well-ventilated Y-tube setup and record feeding behaviours as they are happening. This may determine whether CSFB test bite or graze before committing to a food source. Additionally, it may reveal more about how they detect food plants, for example do beetles approach the food source they then stay and feed at, or do they sample both before making a decision?

From examining other published experimental work, it appears that no other research has quantified the CSFB herbivory differences between a large number of *B. napus* lines from a genetically diverse panel under laboratory conditions. Most studies have focused on smaller numbers of *Brassica* varieties, samples of leaf tissue rather than complete plants or other pests of cruciferous crops. However, Barlet and Mithen (1996) do assess differences in CSFB on whole plants between eight *B. napus* varieties. They found a significant difference between lines for number of

CSFB shot holes in leaves under laboratory conditions. However, they only discuss these differences further in the context of glucosinolate content and do not find a relationship between CSFB herbivory and glucosinolate content.

Other research has looked at more diverse plant material but not with CSFB. Gavoski et al. (2000) tested a variety of Brassicaceae species, including *B. napus* varieties for resistance against *Phyllotreta cruciferae*, a closely related flea beetle to the CSFB. Using multiple choice assays and a zero to 10 scoring system for damage to cotyledons, they did not find any repeatable resistance in *B. napus* varieties to *P. cruciferae* herbivory. This is consistent with other studies where they have also failed to find significant differences in damage to *B. napus* against *P. cruciferae* (Palaniswamy and Lamb, 1992). This is perhaps because there are no differences between these *B. napus* varieties, but also may be that a multiple-choice experimental system is not suitable for revealing differences in flea beetle herbivory. This again may indicate that beetles find it difficult to differentiate between food sources in a relatively air-tight environment, particularly as detection of volatiles is involved in their choice whether to feed or not (Henderson et al., 2004).

Results from conducting preliminary six-way choice assays of *96 B. napus* vartities in this project allowed selection of two *B. napus* varieties to further characterize for CSFB herbivory. Altasweet was selected as a variety with high and Apex-93_5 X Ginyou_3 DH Line with low levels of CSFB herbivory, indicating a more susceptible and more resistant/tolerance variety, respectively.

As it was suspected a six-way choice was not an appropriate assay system for detecting differences in CSFB herbivory between *B. napus* lines, the assay was refined to include either a two-way choice between *B. napus* varieties or no choice, i.e. a single variety. In these experiments a strong difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line was recorded, with Altasweet receiving much higher levels of total feeding damage, thus confirming the preliminary finding from six-way choice assays. Interestingly, when beetles were given no choice of food source the difference in feeding levels between the two *B. napus* lines became even more exacerbated, i.e. Altasweet received even higher and Apex-93_5 X Ginyou_3 DH Line even lower damage in comparison to two-way choices. This further indicates that decisions to feed may be initiated by volatile detection, which can get confused in assays containing multiple *B. napus* varieties.

Whilst there is no previous research into CSFB herbivory on *B. napus* varieties in two-way or non-choice assays of whole plants, some has investigated *P. cruciferae* and Brassicaceae species. Soroka et al. (2011) investigated herbivory between two *B. napus* varieties, Westar and a transgenic line with enhanced trichome density. Hairier plants received significantly less damage from flea beetles, indicating that trichomes offer some resistance against herbivory. Investigating the CSFB herbivory response to different plant architectures would also be beneficial, although *P. cruciferae* are smaller than CSFB and likely more impacted by trichomes.

In earlier research, Bodnaryk and Lamb (1991) compared *S. alba* variety Ochre with *B. napus* variety Westar in two-way and non-choice assays for *P. Cruciferae* herbivory, finding that *B. napus* was fed upon about twice as much as *S. alba*. They also demonstrated a similar result in non-choice assays, but the differences between *B. napus* and *S. alba* we more extreme. This is similar to what was observed when running non-choice assays compared to two-way choice assays in this project. Taken together, although running assays with multiple varieties provides a higher throughput method for screening plant material, this indicates CSFB research into palatability of different *B. napus* varieties may benefit from more non-choice experiments as opposed to multiple choice assays.

When investigating other CSFB feeding traits grazing and stem damage, unpredictable results for Altasweet and Apex-93_5 X Ginyou_3 DH Line were obtained. Although these feeding behaviours are interesting, they do not make up the main part of adult CSFB feeding damage to *B. napus* seedlings. Shot holing constitutes the majority of herbivory inflicted by adult CSFB and is largely captured by total percentage damage scores to cotyledons. Therefore, whilst these damage traits were not pursued further in this project, more refined studies into CSFB herbivory types would be beneficial to improve understanding of *B. napus* palatability and what governs potential feeding decisions in adult CSFB.

In the next set of laboratory experiments, the aim was to further clarify differences in herbivory levels between Altasweet and Apex-93_5 X Ginyou_3 DH Line as well as an F1 cross of these parental lines. In three-way choice assays a significant difference in CSFB feeding damage between Altasweet and the F1 crossed line, but not with Apex-93_5 X Ginyou_3 DH Line was observed. Interestingly, when non-choice assays were conducted a significant difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line, as well as with the F1 crossed line, was recorded. This again reinforces the idea that non-choice assays are more suitable for distinguishing CSFB herbivory differences than multiple choice.

In both multiple choice and non-choice experiments, CSFB damage to the F1 cross did not differ significantly from parental line Apex-93_5 X Ginyou_3 DH Line. Additionally, the F1 seedlings were similar in appearance to their Apex-93_5 X Ginyou_3 DH Line parent (personal observations). Given the visual and damage level similarities, it is possible that the resistance/tolerance trait of Apex-93_5 X Ginyou_3 DH Line is the dominant phenotype and susceptibility of Altasweet is the recessive. If time had permitted, the next experiment would have been to further explore the dominant and recessive phenotypes of resistance and susceptibility by screening a panel of seedlings from the F2 generation for CSFB herbivory. From examining published experimental work, no other research has crossed susceptible and resistant/tolerant *B. napus* varieties and tested the resulting F1 cross for CSFB palatability.

In this same experiment, an additional aim was to test a new cotyledon damage scoring method using the software ImageJ and compare this to human estimated damage scores. Percentage damage scores derived from ImageJ were consistently about half the value of visually estimated scores. However, the scores from ImageJ followed the same pattern as those from visual estimates, with Altasweet differing significantly from the F1 cross in three-way choice assays and differing from both the F1 cross and Apex-93_5 X Ginyou_3 DH Line in non-choice assays. A strong, positive correlation was observed between the scoring methodologies, demonstrating both as valid techniques for capturing differences in adult CSFB cotyledon herbivory. ImageJ provides more precise damage values, but visual estimates are still an appropriate way to distinguish differences in *B. napus* palatability.

Currently, there is no published work on the use of ImageJ software for quantifying flea beetle damage to crop species. However, this software has been used to assess herbivory damage of other important crop pest species successfully, such as snails (Stawarczyk and Stawarczyk, 2015) and thrips (Visschers et al., 2018). Therefore, it may be beneficial for future studies to utilise such computation methods for obtaining damage data for CSFB on *B. napus*.

Field trials in 2019 and 2020 – 2021 assessing CSFB damage levels for a subset of *B. napus* varieties from laboratory assays and commercial lines provided support for previous laboratory results on adult CSFB herbivory damage. The 2019 trial suffered high levels of pest damage immediately after drilling. Coupled with dry, hot conditions, establishment was generally poor, and the trial prematurely ended, despite having two field sites.

However, from the Bawburgh (JIC field station) site, we managed to obtain CSFB damage data for six *B. napus* varieties before the trial was lost in November 2019. Whilst there was a significant effect of *B. napus* line on CSFB damage amount, a significant difference between Altasweet and Apex-93_5 X Ginyou_3 DH Line was not observed. This is potentially attributable to the scoring methodology used, where 20 seedlings were sampled from border runs and cotyledons scored in the laboratory for percentage damage (visual estimates to the nearest 5%). This may have introduced bias, as to select 20 seedlings meant they had to be present, and thus does not give a full representation of how empty or damaged a plot may have been. Seedlings where just small bits of plant material remained (i.e. mostly completely eaten) were likely not selected for scoring back in the laboratory.

When inspecting drone images, there was a clear difference in plant coverage between plots and *B. napus* varieties. Swede type *B. napus* varieties Altasweet and York (the highest damaged line in six-way choice laboratory assays) had a significantly lower seedling count compared to Apex-93_5 X Ginyou_3 DH Line and Apex (a close relative of Apex-93_5 X Ginyou_3 DH Line that also received low levels of damage in six-way choice laboratory assays). It is possible that these observed

differences are solely attributable to different establishment levels. However, was additionally recorded that establishment and herbivory scores negatively and significantly correlated, indicating that *B. napus* varieties with lower plant counts also received higher levels of damage. Furthermore, damage in the field correlated strongly with damage scores obtained in the laboratory and followed the expected trend with Altasweet and York at the higher end and Apex-93_5 X Ginyou_3 DH Line and Apex at the lower end of the damage spectrum. Therefore, there was reasonable support for Altasweet being more susceptible and Apex-93_5 X Ginyou_3 DH Line more resistant/tolerant to CSFB herbivory.

Establishment was much more successful in 2020 – 2021 field trials, with less pest pressure and better weather conditions. Two field trials were drilled at the Bawburgh field site for nine *B. napus* varieties, one treated with pyrethroid pesticides to try and ensure the crop would make it through the overwinter larval CSFB season, and a non-treated trial.

In the non-treated trial, it was again observed that there were a significant differences in herbivory between *B. napus* varieties and that damage received by Altasweet and York was significantly higher than that of Apex-93_5 X Ginyou_3 DH Line. For the 2020 – 2021 field trials, percentage damage to cotyledons was scored in the field for a continuous run of 10 seedlings, rather than sampling and scoring in the laboratory. Although it was less accurate to score in the field, this methodology may have been more appropriate as seedlings with high levels of damage would not have been missed, unlike in the 2019 trial.

Likewise with the 2019 trial, a strong negative correlation between seedling count and percentage damage was observed, again indicating low establishment was linked to higher levels of pest damage. However, unlike the 2019 trial, a significant correlation between 2020 non-treated field damage scores and laboratory data was not recorded. Despite this, the pattern is as expected for some *B. napus* lines, specifically Altasweet and York having higher and Apex-93_5 X Ginyou_3 DH Line having lower CSFB damage in both the field and laboratory environments.

Continuing with the non-treated field trial, there was not a correlation for establishment or damage data between the 2020 and 2019 field trials. This is perhaps unsurprising given that damage levels were so high in 2019, and demonstrates how variable establishment and CSFB damage can be year on year.

In pyrethroid treated plots, a significant effect of *B. napus* variety on amount of CSFB damage was recorded, despite these differences being more subtle. It may be that pesticide treatment offered some protection to more susceptible *B. napus* varieties, and thus lessened differences between them and the more tolerant varieties. Nonetheless, Altasweet was observed to be the mostly highly

damaged *B. napus* variety compared with Apex-93_5 X Ginyou_3 DH Line which received the lowest levels of cotyledon herbivory.

Damage scores from pesticide treated plots did not correlate statistically significantly with the non-treated trial. However, Altasweet appears as one of the *B. napus* varieties highest and Apex-93_5 X Ginyou_3 DH Line the lowest in the distribution, thus indicating for at least some *B. napus* varieties they behaved in a similar way between treated and non-treated trials. Additionally, from this correlation it was observed that damage received by treated plots is lower than that of non-treated plots, albeit this difference is minor.

Establishment scores additionally differed significantly between *B. napus* varieties in treated plots, but this time did not correlate with damage scores. However, they did correlate strongly with non-treated establishment scores. This correlation also demonstrated marginally higher establishment scores for the treated trial compared to the non-treated trial. Taking higher establishment scores with lower damage scores for treated plots, there was an indication that pesticide treatment was providing some protection for these seedlings, albeit minor. Later in the growing season the differences between pesticide treated and non-treated plots became more apparent with differing plant sizes.

There has been limited previous research into differences in palatability of *B. napus* varieties to adult CSFB in the field. However, Giamoustaris and Mithen (1995) demonstrate that *B. napus* varieties with enhanced glucosinolate content were found to be fed upon more by CSFB in field trials than regular *B. napus* varieties. This is consistent with research conducted in laboratory conditions demonstrating the link between CSFB herbivory and glucosinolate content (Bartlet and Williams, 1991; Bartlet et al., 1994) and there is currently ongoing research to better understand the role of glucosinolates in the CSFB diet (Beran *et al*., 2018). However, other research failed to find any significant differences between *B. napus* varieties for flea beetle herbivory in the field (Lambdon et al., 1998; Soroka et al., 2013).

From personal scoring of a field trial on behalf of OREGIN (Oilseed RapE Genetic Improvement Network) some variation was observed, albeit limited, in CSFB damage to a panel of 28 *B. napus* varieties. Incidentally, Altasweet was included in this panel and received the third highest amount of damage out of the 28 *B. napus* varieties (unpublished data). However, the results presented in this project appear to represent the first time a more susceptible *B. napus* variety (Altasweet) and a more resistant/tolerance variety (Apex-93_5 X Ginyou_3 DH Line), identified in laboratory assays, also maintained these CSFB damage differences in a field environment.

Overall, the results demonstrate that multiple choice assays are not suitable for picking out significant differences between *B. napus* varieties for CSFB feeding traits. However, they did successfully provide data for using in Associative Transcriptomics (AT, see Chapter 3) and allow selection of more and less palatable varieties for further investigation. Laboratory assays with limited or no choice demonstrated clear differences between Altasweet and Apex-93_5 X Ginyou_3 DH Line for CSFB herbivory, and that both visual estimates and scores derived from ImageJ analysis are useful techniques for determining these differences. Finally, it is demonstrated that Altasweet remains more susceptible, and Apex-93_5 X Ginyou_3 DH Line more resistant/tolerant, to adult CSFB herbivory damage in the field environment.

Chapter 3 – Genetics underlying CSFB feeding

3.1 Background

Previous research has largely focused on identifying phenotypic differences in insect herbivory, with the aim of breeding in potential resistance/tolerance traits. Whilst this has been beneficial to improvement of crop varieties, it can now be taken a step further with modern techniques to investigate the underlying genetics potentially conferring these observable differences. A fairly modern approach is screening for phenotypic differences in a trait such as herbivory or disease incidence, then using associative transcriptomics (AT) pipelines to identify genomic regions and gene expression differences linked to these phenotypes.

One recent study has investigated the effects of leaf trichomes on herbivory from *Plutella xylostella* larvae (Xuan et al., 2020), a prolific pest of oilseed rape. They demonstrated that hairy leaves were less attractive to larvae than smooth (glabrous) leaves. They then applied a Genome Wide Association Study (GWAS) to analyse phenotypic data on leaf hairiness in 290 *Brassica napus* varieties, linking genotypic variation to the trait and identifying candidate genes associated with hairiness. Two such gene identified to be associated with leaf trichome density were BnaC07g24950D and BnaC07g24960D, which are genes involved in auxin homeostasis. Another candidate gene identified was BnaA06g31780D (BnaA.GL1.a), which is a potential ortholog of AtGL1. Further experiments by Xuan et al. (2020) demonstrated that Arabidopsis plants with the gl1-1 mutation, carrying the 35S:BnaA.GL1.a construct, developed trichomes on both leaves and stems. This indicated that *B. napus* may share a similar regulatory pathway with Arabidopsis in controlling trichome development. Overall, Xuan et al (2020) demonstrate genes involved in auxin pathways to be associated with more or less leaf hairiness and that this trichome density influences *P. xylostella* herbivory. Thus, this research demonstrates utilisation of a GWAS approach used to identify genes that could be used to assist in breeding programmes for *B. napus* trichome density, and therefore the potential to enhance resistance to *P. xylostella* larvae.

Another recent study investigated variation in *Pyrenopeziza brassicae*, light leaf spot (LLS), occurrence in 195 *B. napus* varieties (Fell et al., 2023). Using an AT approach, they identified gene loci significantly associated with disease occurrence, including one demonstrating enhanced resistance to LLS. Furthermore, they identified eight gene expression markers, seven of which demonstrated a positive correlation between resistance and gene expression levels. Again, these types of candidate gene discovery can be used to aid future crop improvement.

In the next experiments, the aim was to utilise a similar approach to Fell et al. (2023) and use phenotypic data collected for Cabbage Stem Flea Beetle (CSFB) herbivory traits to screen a diverse panel of *B. napus* varieties for genomic variation and gene expression variation, and ultimately identify potential candidate genes conferring these phenotypic differences for further exploration.

3.2 Methods

Phenotypic scores for four CSFB herbivory traits (total, shot holing, grazing and stem damage) were collected from six-way choice assays using 96 *B. napus* varieties (see section 2.2 Methods for how this data was generated). The resulting phenotypic data from this experiment was used to investigate underlying genetic variation potentially linked to CSFB herbivory traits and thus resistance/tolerance or susceptibility, using Associative Transcriptomics (AT).

3.2.1 Association transcriptomics

Phenotypic datasets for total, shot holing, grazing and stem damage scores were analysed using an associative transcriptomics pipeline developed by Harper et al. (2012) which was demonstrated for use in mapping traits in *B. napus*. Genotype and expression level data for 95 *B. napus* lines (one line was dropped due to insufficient quality of sequence data) were used from published datasets (Trick et al., 2009). Additionally, the population structure used for analysis was also obtained from Harper et al. (2012), determined in the software STRUCTURE.

Gene expression marker (GEM) associations were determined for four CSFB herbivory traits using linear regression with Reads Per Kilobase per Million mapped reads (RPKM) to predict the outcome of trait values. Markers with expression less than 0.5RPKM were removed before analysis.

GWAS was performed using TASSEL v4 after removing Single Nucleotide Polymorphism (SNP) markers with an allele frequency of less than 0.05. Generalised linear models (GLMs) and mixed linear models (MLMs) were run for all four traits to determine the most suitable fit for the data. False discovery rate (FDR) was calculated using the Shiny implementation of the q-value R package (Storey et al., 2020). This R package was adapted and used to visualise data in R version 2.15.1. Allelic effect and linkage disequilibrium (LD) were investigated for the most significantly associated SNPs. LD was examined for three significantly associated markers by calculating the mean pair-wise r^2 for all markers on the chromosome of the focal SNP. LD was considered if r^2 was greater than 0.15.

3.2.2 Arabidopsis feeding assays

3.2.2.1 Plant material

IQ67-domain 2 (IQD2) and ABB8 (IQD1) loss-of-function mutants (in Columbia (Col-0) and Wassilewskija (Ws-0) wild-type backgrounds, respectively) were obtained from Katharina Bürstenbinder (Leibniz-Institut für Pflanzenbiochmie) and are detailed in Zang et al. (2021) and Levy et al. (2005), respectively. Seeds for IQD2 and ABB8 mutants, and wild-type controls of Col-0 and Ws-0 were germinated and grown in Levingtons F2 soil in a controlled environment room CER (22°C:22°C and 16h daylength) for 14 days before being pricked out into custom ''pots'' modified from 50ml Corning centrifuge tube lids (approximately 2.1cm diameter, 1.0cm height) (Figure 3.1). Seedlings were then grown for a further week before use in feeding assays.

Figure 3.1. A: photograph demonstrating modifications made to 50ml Corning centrifuge tube lids, with holes drilled in the bottom and covered in mesh to allow contact with water and B: photographic example of Arabidopsis seedlings in modified corning tube ''pots'', on damp blue roll to allow watering from below.

3.2.2.2 Insect material

CSFB were used from a laboratory stock population maintained in the John Innes Centre (JIC) insectary and screened for feeding activity six days prior to inclusion in assays (as previously described in Methods 2.2). Four beetles per assay were used, in a sex ratio of 2:2 males and females, after being starved for 24 hours.

3.2.2.3 Design and process of running Arabidopsis feeding assays

A petri dish assay was designed alongside Anna Jordan in the JIC insectary as displayed in Figure 3.2. Arabidopsis plants were slotted into holes in an agar bass to maintain moisture levels. Prior to inclusion in assays plants were photographed to have an undamaged document of each seedling. Non-choice (i.e. just a single Arabidopsis line) assays were run for 48 hours before beetle removal and damage scoring. Entire plants were again photographed upon removal, maintaining the same orientation that they went into the assay, to give a document of overall plant damage. The intention was to use these before and after photographs to obtain changes in greenness and thus damage scores utilising ImageJ software. However, during the 48 hours seedlings grew substantially, meaning that greenness scores often increased despite being subjected to CSFB herbivory.

Instead, approximate percentage damage scores were obtained for six leaves per plant after removal and laying out on a white PVC board (Figure 3.3). Leaves were removing starting at a 12:00 o'clock position moving clockwise so that individual leaves were identifiable in photographs. Additionally, this ensured that the same portion of leaves were scored for all plants removing any selection bias whilst scoring. Images were also taken of cut leaves for potential future image analysis.
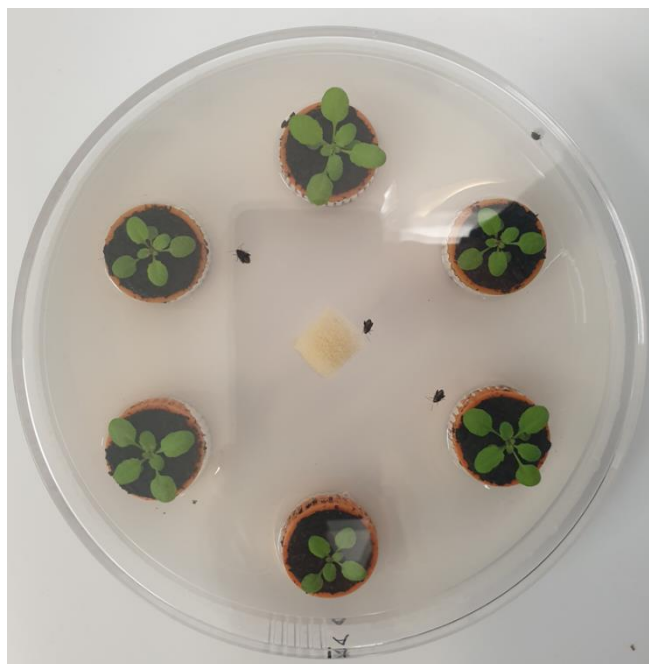
Figure 3.2. Non-choice Arabidopsis feeding assays, of whole plants in soil pots inserted into water agar.
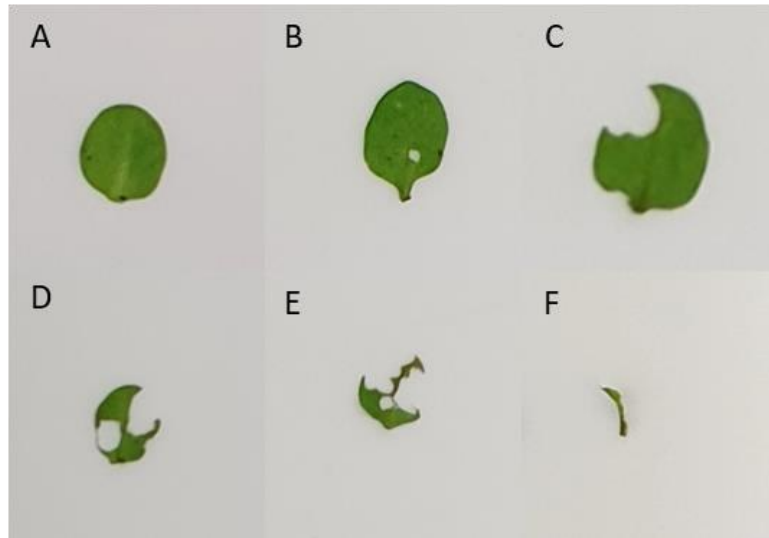
Figure 3.3. Examples of levels of feeding on Arabidopsis leaves. A: 0% (no feeding damage occurred). B: 1% (marginal feeding levels occurred but not 0%). C: 25%. D: 50%. E: 75%. F: 100% (or near to no leaf material left).

3.2.2.4 Statistical analysis

Mean feeding damage levels were analysed for the four Arabidopsis lines with a two-way ANOVA of Arabidopsis line and date of assay and an interaction term between the two. As with previous percentage damage presented in this thesis data was LOGIT+ transformed for analysis (Equation 2.1). Analysis was conducted using Genstat software.

3.3 Results

Phenotypic CSFB feeding data obtained from six-way choice assays for 95 *B. napus* lines (see Results 2.3.1) were analysed using an associative transcriptomics pipeline (as per Harper et al. 2012 protocol) to identify genomic regions or gene expression linked to herbivory. TASSEL identified a mixed linear model (MLM) as the optimal fit for CSFB total, shot holing and grazing phenotypic data and a generalised linear model (GLM) for stem damage phenotypic data (see Appendix 6). Here, first presented is the resulting output for gene expression markers (GEMs), followed by single nucleotide polymorphism (SNP) data.

3.3.1 Gene expression markers found to be associated with CSFB herbivory

Gene expression levels were assessed for 95 transcriptomes from the 96 *B. napus* lines tested for four CSFB feeding traits; total, shot holing, grazing and stem damage. For total CSFB feeding damage, the MLM identified 17 significantly associated GEMs (FDR < 0.05, *p* < 0.00001 (Figure 3.4, Table 3.1)). Table 3.2 demonstrates the RPKM (reads per kilobase per million mapped) values of significant GEMs for total CSFB feeding damage for Altasweet and Apex-93_5 X Ginyou_3 DH Line. 16 out of 17 of these were also shared with the top GEMs for shot holing CSFB herbivory. Examining the Manhattan plot in Figure 3.4 demonstrated a weak but potential peak on chromosome A03. The top GEM on this peak was A_JCVI_17072, a gene encoding Lorelei Like Protein (LLP), which is involved in the regulation of growth and has been linked to plant immunity function. The second GEM in the A03 peak was A_JCVI_36078, a gene encoding Beta-1,2-xylosyltransferase, involved in glycosylation. Another GEM of interest was A_EX137858, located on chromosome A05. This encodes a gene Cinnamate-4-hydroxylase (C4H), which is involved in phenylpropanoid metabolism, growth and development. Finally, GEM C_JCVI_2920, located on chromosome C08, that had a significant top GEM hit homologue on A08, was a gene encoding an alpha-crystallin domain protein.

Linear regressions were plotted for the 17 GEMs with an FDR < 0.05 FDR. All 17 were positively correlated with CSFB total feeding damage, indicating higher gene expression was linked to increased CSFB herbivory. Other than one GEM, all were also found to be significant for shot holing feeding damage with an FDR < 0.05. This is unsurprising as shot holing is the main component of total feeding damage scores.
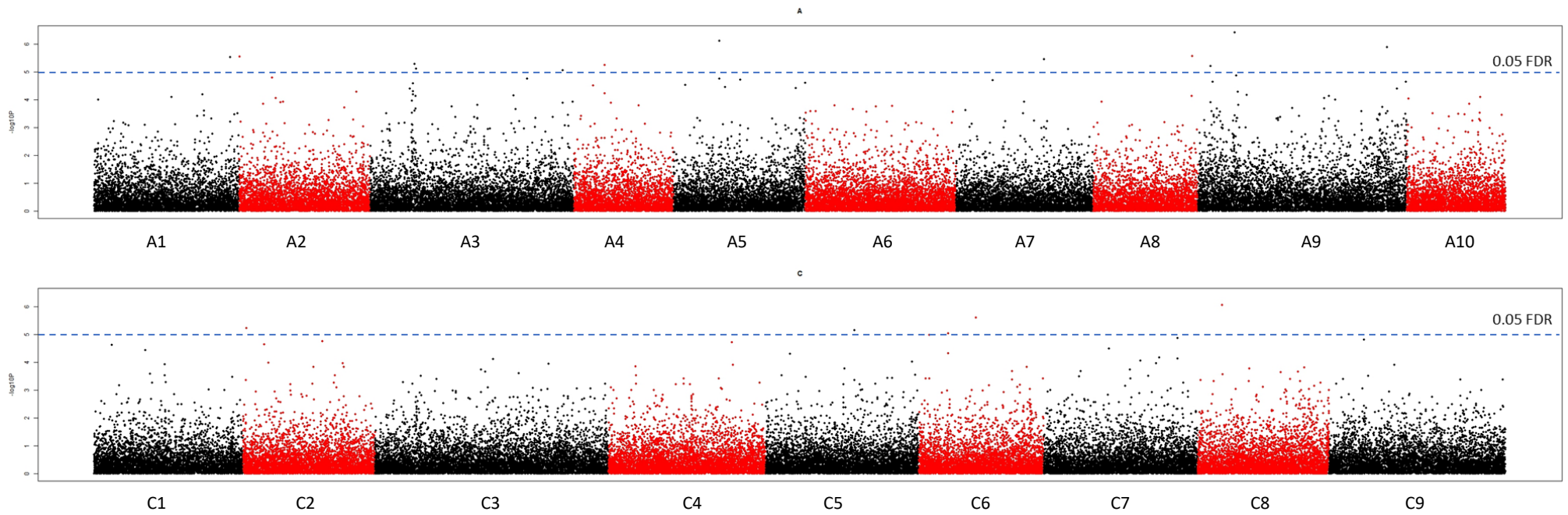
Figure 3.4. Manhattan plot demonstrating GEMs and their association with total CSFB feeding damage. The blue dashed line indicates the 0.05 FDR.

Table 3.1. GEMs correlated with CSFB total and shot holing feeding damage phenotypic scores at an FDR < 0.05. Where there are two Log10p and p values, the first relates to total damage phenotype and the second relates to shot holing damage phenotype.

Brassica unigene	Chromosome	Total or Shot holing	Log10p value	p value	Positive/negative correlation	Arabidopsis orthologue	Gene annotation
C_JCVI_2920	C08	Total & shot holing	6.053/7.070[a]	8.84E-07/8.52E-08	Positive	AT1G06460.1	Alpha-crystallin domain containing protein with homology to small heat shock proteins.
A_JCVI_17072	A03	Total & shot holing	5.288/7.048[a]	5.15E-06/8.95E-08	Positive	AT5G56170.1	LORELEI-LIKE-GPI-ANCHORED PROTEIN 1 involved in regulation of growth.
A_EX137858	A05	Total & shot holing	6.122/6.814[a]	7.56E-07/1.54E-07	Positive	AT2G30490.1	Cinnamate-4-hydroxylase. Mutations in this gene impact phenylpropanoid metabolism, growth and development.
A_JCVI_2920	A08	Total & shot holing	5.561/6.571[a]	2.75E-06/2.69E-07	Positive	AT1G06460.1	Alpha-crystallin domain containing protein with homology to small heat shock proteins.
C_DY009791	C02	Total & shot holing	5.226/6.309[a]	5.94E-06/4.91E-07	Positive	AT3G25110.1	FATA, encodes a FatA acyl-ACP thioesterase.
C_ES266017	Unmapped	Total & shot holing	5.048/6.276[a]	8.95E-06/5.29E-07	Positive	AT1G53290.1	Galactosyltransferase family protein involved in protein amino acid glycosylation.
A_JCVI_23190	A02	Total & shot holing	5.552/5.843	2.81E-06/1.43E-06	Positive	AT5G01530.1	Light harvesting complex photosystem II involved in response to blue light, response to red light, response to far red light.
A_DY009335	A07	Total & shot holing	5.460/5.806	3.47E-06/1.56E-06	Positive	AT1G02660.1	Plastid Lipase2, a glycerolipid A1 lipase with substrate preference for monogalactosyldiacylglycerol.

A_DY009791	A02	Shot holing	5.725	1.88E-06	Positive	AT3G25110.1	FATA, encodes a FatA acyl-ACP thioesterase.
A_JCVI_16258	A09	Total & shot holing	6.414[a]/5.693	3.86e-07/2.03E-06	Positive	AT2G16485.1	NEEDED FOR RDR2-INDEPENDENT DNA METHYLATION
C_JCVI_728	C09	Shot holing	5.680	2.09E-06	Positive	AT2G14890.1	Arabinogalactan protein 9, putative proline-rich protein.
A_JCVI_18907	A05	Shot holing	5.680	2.09E-06	Positive	AT2G42670.1	Protein of unknown function (DUF1637).
A_JCVI_26505	A09	Total & shot holing	5.219/5.607	6.05E-06/2.47E-06	Positive	AT5G48385.1	FRIGIDA-like protein1.
C_DY009335	C05	Total & shot holing	5.162/5.594	6.89E-06/2.55E-06	Positive	AT1G02660.1	Plastid Lipase2, a glycerolipid A1 lipase with substrate preference for monogalactosyldiacylglycerol.
A_JCVI_36078	A03	Total & shot holing	5.117/5.568	7.65E-06/2.70E-06	Positive	AT5G55500.1	Beta-1,2-xylosyltransferase that is glycosylated at two positions.
A_JCVI_22372	A04	Shot holing	5.530	2.95E-06	Positive	AT4G02970.1	7SL RNA1, signal recognition particle.
A_JCVI_17622	A07	Shot holing	5.496	3.19E-06	Positive	AT1G22450.1	Subunit bB of cytochrome C oxidase.
A_AT002090	A03	Shot holing	5.444	3.60E-06	Positive	AT5G59320.1	Lipid transfer protein 3, predicted to encode pathogenesis-related protein.
A_JCVI_19271	A03	Shot holing	5.299	5.03E-06	Positive	AT4G37870.1	Phosphoenolpyruvate carboxykinase 1.
A_JCVI_2624	A01	Shot holing	5.290	5.13E-06	Positive	AT4G37870.1	Phosphoenolpyruvate carboxykinase 1.
C_EX056051	C02	Shot holing	5.262	5.47E-06	Positive	AT5G15780.1	Pollen Ole e 1 allergen and extensin family protein.
A_JCVI_27501	A09	Total & shot holing	5.896/5.246	1.27E-06/5.68E-06	Positive	AT1G13120.1	Embryo defective 1745 involved in embryo development ending in seed dormancy.
A_JCVI_121	A05	Shot holing	5.204	6.26E-06	Positive	AT2G30490.1	Cinnamate-4-hydroxylase. Mutations in this gene impact phenylpropanoid metabolism, growth and development.

A_EV196428	A04	Total & shot holing	5.248/5.190	5.65E-06/6.46E-06	Positive	AT1G71400.1	RECEPTOR LIKE PROTEIN 12.
C_JCVI_24072	C07	Shot holing	5.180	6.61E-06	Positive	AT4G30020.1	PA-domain containing subtilase family protein.
A_EV215941	Unmapped	Shot holing	5.160	6.92E-06	Positive	AT5G60920.1	Glycosylphosphatidylinositol-anchored protein localized primarily in root cells.
C_AT002090	Unmapped	Shot holing	5.122	7.55E-06	Positive	AT5G59320.1	Lipid transfer protein 3, predicted to encode pathogenesis-related protein.
A_JCVI_37206	Unmapped	Shot holing	5.074	8.43E-06	Positive	AT3G03710.1	PIGMENT DEFECTIVE 326.
A_EV210619	A09	Shot holing	5.069	8.53E-06	Positive	AT5G27150.1	Na+/H+ exchanger 1, involved in salt tolerance, ion homeostasis and leaf development.
A_JCVI_41243	A03	Total	5.053	8.86E-06	Positive	AT4G33180.1	Alpha/beta-Hydrolases superfamily protein.
A_JCVI_27166	A04	Shot holing	5.027	9.39E-06	Positive	AT3G59790.1	MAP kinase 10.
A_JCVI_41281	A03	Shot holing	5.025	9.44E-06	Positive	AT5G57270.1	Core-2/I-branching beta-1,6-N-acetylglucosaminyltransferase family protein.
C_EV040764	C09	Shot holing	4.987	1.03E-05	Positive	AT2G18910.1	Hydroxyproline-rich glycoprotein family protein.
A_EX123292	A03	Shot holing	4.914	1.22E-05	Positive	AT4G15470.1	Bax inhibitor-1 family protein.
A_EV009430	A01	Total & shot holing	5.535/4.868	2.92E-06/1.36E-05	Positive	AT1G71400.1	RECEPTOR LIKE PROTEIN 12.
A_JCVI_14594	A10	Shot holing	4.835	1.46E-05	Positive	AT1G01430.1	TRICHOME BIREFRINGENCE-LIKE 25.
A_JCVI_11653	A05	Shot holing	4.822	1.51E-05	Positive	AT1G55900.1	Haloacid dehalogenase-like hydrolase superfamily protein.
A_EV202339	Unmapped	Shot holing	4.822	1.51E-05	Positive	AT1G63140.2	O-methyltransferase family protein.
C_EV009430	C06	Total & shot holing	5.604/4.805	2.49E-06/1.57E-05	Positive	AT1G71400.1	RECEPTOR LIKE PROTEIN 12.

C_JCVI_10356	C07	Shot holing	4.752	1.77E-05	Positive	AT4G17640.1	Encodes casein kinase II beta (regulatory) subunit.
A_JCVI_6849	A09	Shot holing	4.749	1.78E-05	Negative	AT3G50790.1	Esterase/lipase/thioesterase family protein.
C_JCVI_27690	C03	Shot holing	4.749	1.78E-05	Positive	AT5G19000.1	BTB-POZ and MATH domain 1.
A_JCVI_292	A09	Shot holing	4.731	1.86E-05	Positive	AT2G17480.1	Seven transmembrane MLO family protein, homologs of the barley mildew resistance locus o protein.
C_JCVI_39384	C06	Shot holing	4.713	1.93E-05	Positive	AT1G15890.1	Disease resistance protein (CC-NBS-LRR class) family.
A_EV178195	A09	Shot holing	4.709	1.95E-05	Positive	AT1G22870.1	Protein kinase family protein with ARM repeat domain.
A_JCVI_18163	A03	Shot holing	4.699	2.00E-05	Positive	AT5G57840.1	HXXXD-type acyl-transferase family protein.
C_JCVI_14895	C07	Shot holing	4.698	2.00E-05	Positive	AT4G19230.2	Encodes a Protein with ABA 8'-hydroxylase activity, involved in ABA catabolism, post-germination growth.
C_EE474874	C08	Shot holing	4.679	2.10E-05	Positive	AT1G13790.1	XH/XS domain-containing protein.
C_JCVI_24016	C06	Shot holing	4.662	2.18E-05	Positive	AT1G53300.1	Tetratricopetide-repeat thioredoxin-like 1.
A_JCVI_21432	A01	Shot holing	4.658	2.20E-05	Positive	AT4G14605.1	Mitochondrial transcription termination factor family protein.
A_JCVI_10374	A06	Shot holing	4.612	2.45E-05	Positive	AT3G32940.1	RNA-binding KH domain-containing protein.
A_JCVI_69	A09	Shot holing	4.603	2.49E-05	Positive	AT1G33590.1	Leucine-rich repeat (LRR) family protein.
A_CD837356	C09	Shot holing	4.596	2.53E-05	Positive	AT5G10960.1	CCR4-ASSOCIATED FACTOR 1I.
C_JCVI_14717	C02	Shot holing	4.569	2.70E-05	Positive	AT2G01170.1	Bidirectional amino acid transporter 1, expression localised in vascular tissues.
A_CX267110	A06	Shot holing	4.555	2.78E-05	Positive	AT1G62050.1	Ankyrin repeat family protein.

A_JCVI_7792	A03	Shot holing	4.555	2.78E-05	Positive	AT5G57100.1	Nucleotide/sugar transporter family protein.
A_EX131104	A03	Shot holing	4.545	2.85E-05	Positive	AT5G57100.1	Nucleotide/sugar transporter family protein.
C_JCVI_23060	C08	Shot holing	4.541	2.88E-05	Negative	AT1G06690.1	NAD(P)-linked oxidoreductase superfamily protein.
A_JCVI_26804	A09	Shot holing	4.532	2.93E-05	Positive	AT3G27230.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein.
A_JCVI_37339	A03	Shot holing	4.515	3.06E-05	Positive	AT2G12400.1	A plasma membrane fusion protein.
A_JCVI_12400	A09	Shot holing	4.512	3.08E-05	Positive	AT1G12820.1	Auxin receptor involved in primary and lateral root growth inhibition in response to nitrate.
C_EV210619	Unmapped	Shot holing	4.504	3.13E-05	Positive	AT5G27150.1	Na+/H+ exchanger 1, involved in salt tolerance, ion homeostasis and leaf development.
A_DY009587	A10	Shot holing	4.468	3.40E-05	Positive	AT5G19000.1	BTB-POZ and MATH domain 1.
A_EX089761	A09	Shot holing	4.455	3.51E-05	Positive	AT3G63190.1	Ribosome recycling factor, chloroplast precursor.
A_CN830809	A09	Shot holing	4.449	3.56E-05	Positive	AT1G64380.1	Integrase-type DNA-binding superfamily protein.
A_EV124240	A08	Shot holing	4.445	3.59E-05	Positive	AT1G54090.1	Exocyst subunit exo70 family protein D2.

[a] indicates a GEM which was also found to be significant at the 0.05 bonferroni level for that specific feeding phenotype.

Table 3.2. RPKM (reads per kilobase per million mapped) for GEMs correlated with CSFB total feeding damage scores at an FDR < 0.05, for *B. napus* lines Altasweet and Apex-93_5 X Ginyou_3 DH Line.

Brassica unigene	Altasweet	Apex-93_5 X Ginyou_3 DH Line
Total damage score (%)	7.68279	0.96448
C_JCVI_2920	17.25894	1.38729
A_JCVI_17072	26.17343	6.83999
A_EX137858	24.23918	7.92401
A_JCVI_2920	13.89309	1.02221
C_DY009791	10.65280	0.00000
C_ES266017	3.52026	0.39879
A_JCVI_23190	258.63580	5.97438
A_DY009335	19.02871	2.72296
A_JCVI_16258	5.62695	5.73706
A_JCVI_26505	7.57229	4.17082
C_DY009335	21.58813	1.47493
A_JCVI_36078	0.45633	0.23263
A_JCVI_27501	2.56598	1.07725
A_EV196428	3.05175	0.00000
A_JCVI_41243	8.22216	1.59432
A_EV009430	11.59628	0.00000
C_EV009430	9.73733	0.00000

Focusing on shot holing damage, the MLM identified 65 significantly associated GEMs (FDR < 0.05, p < 0.00001 (Figure 3.5, Table 3.1)). Table 3.3 demonstrates the RPKM values of significant GEMs for shot holing CSFB feeding damage for Altasweet and Apex-93_5 X Ginyou_3 DH Line. The Manhattan plot (Figure 3.5) demonstrated a similar but stronger peak on chromosome A03 compared to total damage, along with similar top hits and included those already highlighted for above. Another top GEM of shot holing not mentioned above was identified as C_DY009791 located on chromosome C02, representing a gene which encodes FATA, a FatA acyl-ACP thioesterase, which also had a top homologous hit on A02.

Linear regressions were plotted for these 65 GEMs. 63 were positively associated with CSFB shot holing, indicating that higher feeding levels were linked to increased gene expression. One of the strongest associations was for observed for GEM hit A_JCVI_17072 (R^2 = 0.2571), a gene encoding LLP located on chromosome A03 (Table 3.1, Figure 3.6). This marker was also positively associated with total damage. A_JCVI_6849 was a top GEM marker located on chromosome A09, representing a gene encoding an esterase/lipase/thioesterase family protein, demonstrated a negative association (R^2 = 0.1476) with shot holing herbivory (Figure 3.7A). C_JCVI_23060, a gene encoding a NAD(P)-linked oxidoreductase superfamily protein, located on chromosome C08, also demonstrated a negative association (R^2 = 0.0786) with shot holing feeding damage (Figure 3.7B). This indicated that higher gene expression for these two genes was associated with lower levels of CSFB shot holing feeding damage.

Table 3.3. RPKM (reads per kilobase per million mapped) for GEMs correlated with
CSFB shot holing feeding damage scores at an FDR < 0.05, for *B. napus* lines Altasweet
and Apex-93_5 X Ginyou_3 DH Line.

Brassica unigene	Altasweet	Apex-93_5 X Ginyou_3 DH Line
Shot holing damage score (%)	8.00653	0.96448
C_JCVI_2920	17.25894	1.38729
A_JCVI_17072	26.17343	6.83999
A_EX137858	24.23918	7.92401
A_JCVI_2920	13.89309	1.02221
C_DY009791	10.65280	0.00000
C_ES266017	3.52026	0.39879
A_JCVI_23190	258.63580	5.97438
A_DY009335	19.02871	2.72296
A_DY009791	10.10043	0.08045
A_JCVI_16258	5.62695	5.73706
C_JCVI_728	1.45428	0.00000
A_JCVI_18907	2.43698	1.76687
A_JCVI_26505	7.57229	4.17082
C_DY009335	21.58813	1.47493
A_JCVI_36078	0.45633	0.23263
A_JCVI_22372	10.94893	0.00000
A_JCVI_17622	34.86822	22.09313
A_AT002090	22.07497	1.41936
A_JCVI_19271	8.66933	0.49449
A_JCVI_2624	66.41735	22.57232
C_EX056051	11.09895	1.14594
A_JCVI_27501	2.56598	1.07725
A_JCVI_121	21.05403	4.68798
A_EV196428	3.05175	0.00000
C_JCVI_24072	7.21158	1.53894
A_EV215941	4.25638	0.00000
C_AT002090	19.29074	2.43318
A_JCVI_37206	3.80180	0.25001
A_EV210619	3.15061	0.00000

A_EV009430	11.59628	0.00000
C_EV009430	9.73733	0.00000
A_JCVI_27166	64.77823	1.01870
A_JCVI_41281	2.91365	1.54035
C_EV040764	0.99400	1.46387
A_EX123292	6.50835	0.99040
A_JCVI_14594	3.74946	1.37622
A_JCVI_11653	1.34507	0.72603
A_EV202339	36.78049	0.00000
C_JCVI_10356	3.51592	0.81937
A_JCVI_6849	1.95427	14.38500
C_JCVI_27690	2.71136	0.98290
A_JCVI_292	2.95591	1.34657
C_JCVI_39384	2.07068	0.60320
A_EV178195	2.00535	0.72550
A_JCVI_18163	13.78735	5.51260
C_JCVI_14895	0.27003	0.20649
C_EE474874	1.18239	0.34444
C_JCVI_24016	2.02988	1.00344
A_JCVI_21432	3.95123	3.27746
A_JCVI_10374	0.90844	0.55573
A_JCVI_69	24.97371	7.79461
A_CD837356	6.95779	2.52022
C_JCVI_14717	2.48414	0.46050
A_CX267110	0.86952	0.17731
A_JCVI_7792	2.65332	0.56621
A_EX131104	2.23463	0.52077
C_JCVI_23060	12.19561	44.39213
A_JCVI_26804	6.04709	1.95029
A_JCVI_37339	1.21955	0.33476
A_JCVI_12400	28.64645	7.91363
C_EV210619	2.48732	0.00000
A_DY009587	2.31932	0.00000
A_EX089761	9.75406	3.40221
A_CN830809	3.36121	0.74097
A_EV124240	0.81759	0.98515
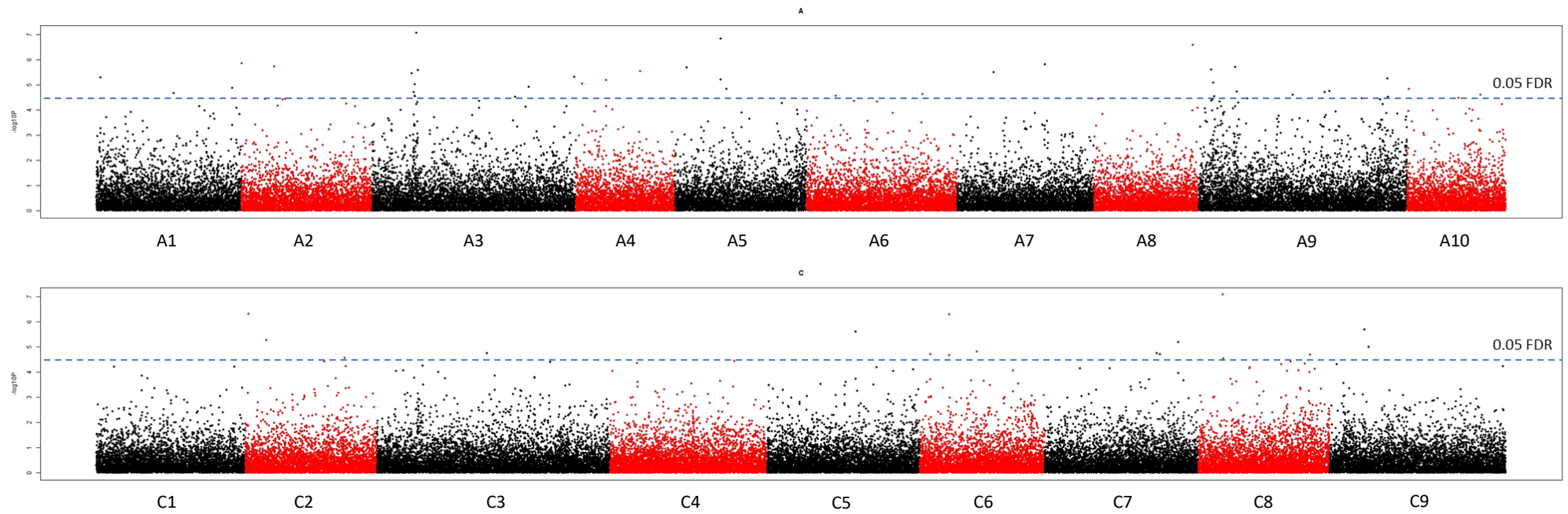
Figure 3.5. Manhattan plot demonstrating GEMs and their association with shot holing CSFB feeding damage. The blue dashed line indicates the 0.05 FDR.
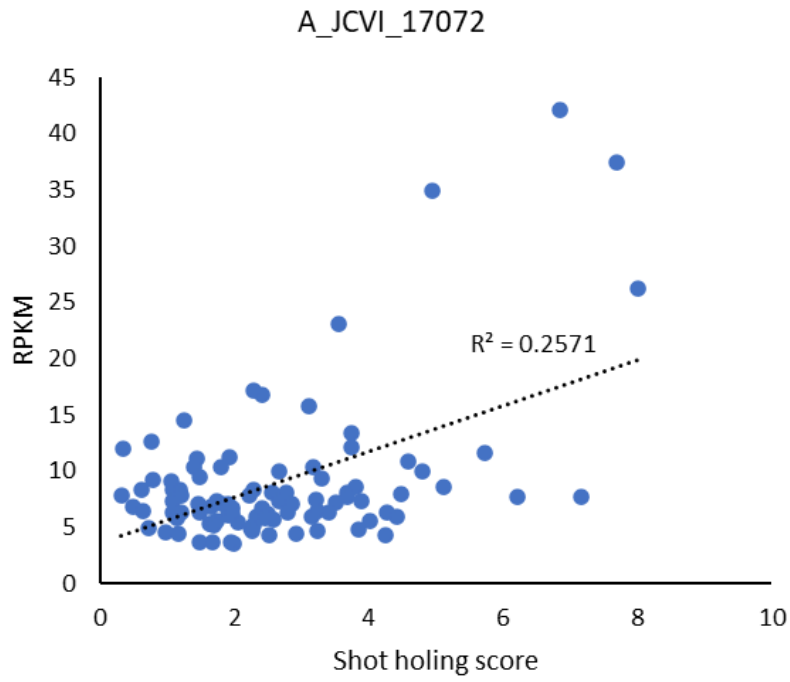
Figure 3.6. Regression of CSFB shot holing damage scores against gene expression (reads per kilobase per million mapped) for A_JCVI_17072, demonstrating a positive association (R^2 = 0.2571).

Figure 3.7. Regression of CSFB shot holing damage scores against gene expression (reads per kilobase per million mapped) for A: A_ACVI_6849 and B: C_JCVI_23060, both demonstrating a negative association (R^2 = 0.1476 and R^2 = 0.0786, respectively).

For grazing damage, no GEMs were identified with an FDR < 0.05. Therefore, a log10p threshold of 4 was set, revealing two GEM hits with *p* values < 0.0001 (Table 3.4). Table 3.5 demonstrates the RPKM values of significant GEMs for grazing CSFB feeding damage for Altasweet and Apex-93_5 X Ginyou_3 DH Line. A Manhattan plot did not highlight any significant peaks of interest (Figure 3.8). Linear regressions were plotted for the two GEMs above the log10p threshold and are displayed in Figure 3.9A and 3.9B. Both A_JCVI_16446, a gene encoding metacaspase 3 located on chromosome A02 and C_JCVI_39264, located on C09, encoding a galactose oxidase/kelch repeat superfamily protein were positively associated (R^2 = 0.1460 and R^2 = 0.1851, respectively) with grazing feeding damage. This indicated higher gene expression to be associated with greater levels of grazing herbivory. These GEMs were unique to the grazing and not shared by total or shot holing feeding phenotypes.

Table 3.4. GEMs correlated with CSFB grazing feeding damage phenotypic scores with a log10p > 4.

Brassica unigene	Chromosome	Log10p value	*p* value	Arabidopsis orthologue	Gene annotation
A_JCVI_16446	A02	4.628	2.35E-05	AT5G64240.2	Metacaspase 3, involved in proteolysis.
C_JCVI_39264	C09	4.438	3.65E-05	AT5G18590.1	Galactose oxidase/kelch repeat superfamily protein.

Table 3.5. RPKM (reads per kilobase per million mapped) for GEMs correlated with CSFB total feeding damage phenotypic scores with a Log10p > 4, for *B. napus* lines Altasweet and Apex-93_5 X Ginyou_3 DH Line.

| Brassica unigene	Altasweet	Apex-93_5 X Ginyou_3 DH Line
Total damage score (%)	0.36759	0.58849
A_JCVI_16446	1.56905	1.15845
C_JCVI_39264	1.70248	1.60227
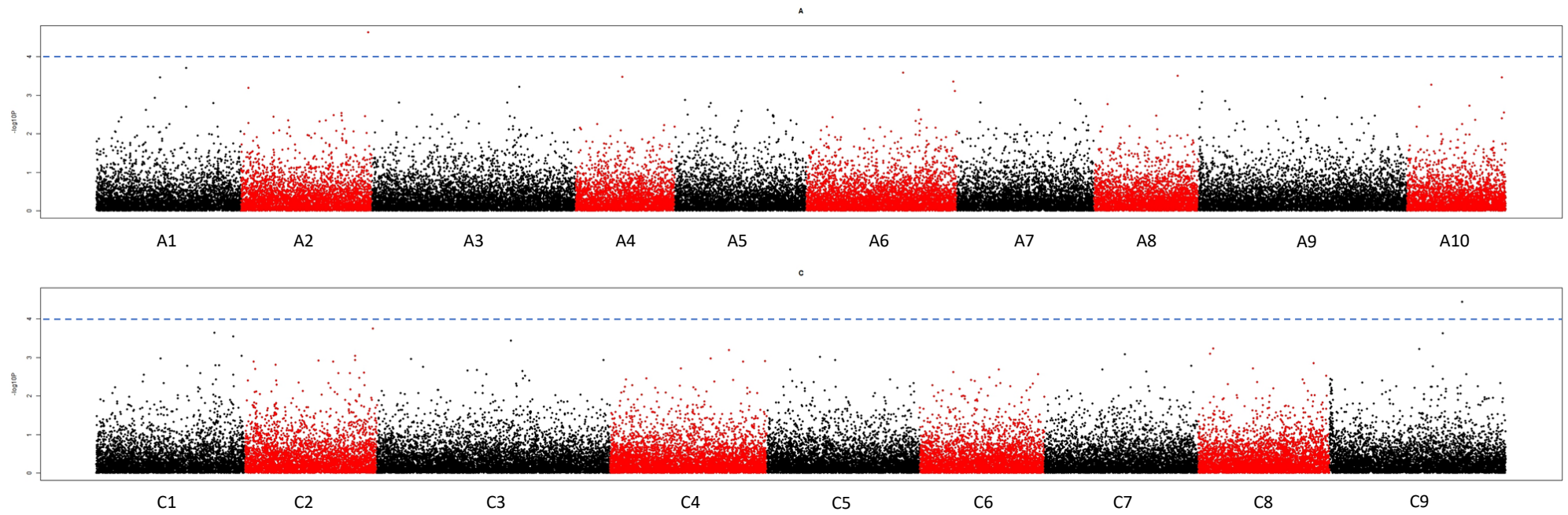
Figure 3.8. Manhattan plot demonstrating GEMs and their association with grazing CSFB feeding damage. The blue dashed line indicates an arbitrary threshold of 4 log10p.
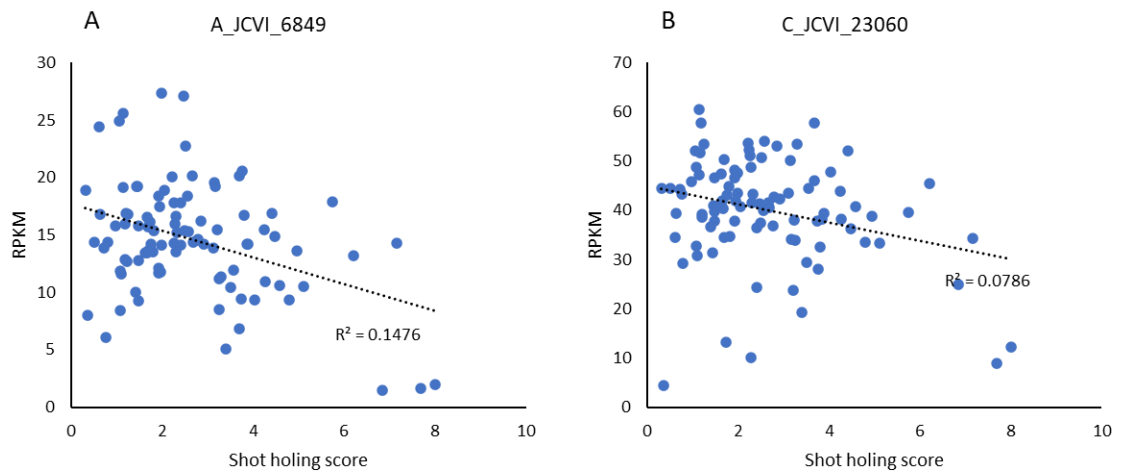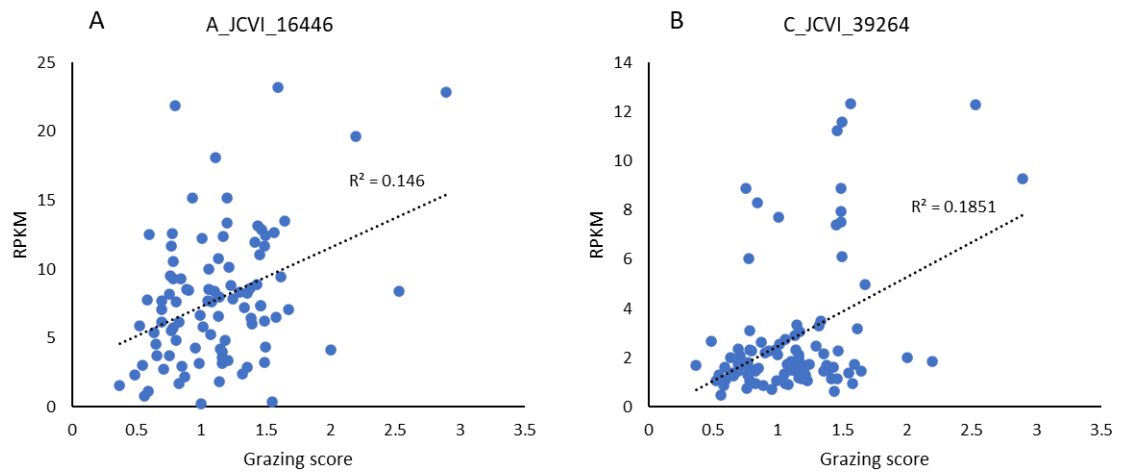
Figure 3.9. Regression of CSFB grazing damage scores against gene expression (reads per kilobase per million mapped) for A: A_ACVI_16446 and B: C_JCVI_39264, both demonstrating positive associations (R^2 = 0.1460 and R^2 = 0.1851, respectively).

Lastly, stem feeding damage did not reveal any GEMs above the 4log10p threshold, as demonstrated in Figure 3.10. However, it was notable that there is a slight GEM peak on chromosome A03, in the same region as observed for total and shot holing CSFB herbivory traits. The top GEM marker on this A03 chromosome peak is A_ES905950, a gene encoding a nuclear-localized NOT (negative on TATA-less) domain-containing protein. This was unique to stem damage and not recorded in the top hits for other CSFB feeding phenotypes.
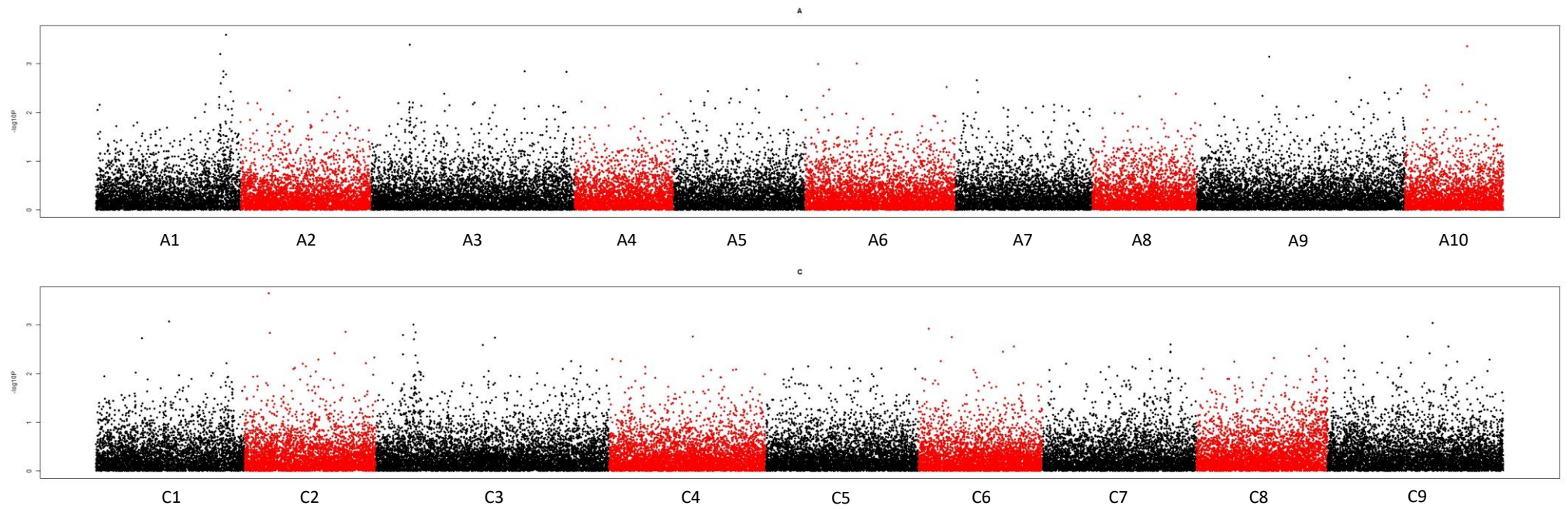
Figure 3.10. Manhattan plot demonstrating GEMs and their association with stem CSFB feeding damage.

3.3.2 SNP markers found to be associated with CSFB herbivory

The same 95 *B. napus* lines assessed for gene expression variation were used to identify single nucleotide polymorphisms (SNPs) associated with total, shot holing, grazing and stem CSFB herbivory. For all feeding traits, no SNPs were identified with an FDR < 0.05. Therefore, a log10p threshold of 4 was set.

For total CSFB herbivory scores there were 16 markers observed to be significantly associated with this trait (log10p > 4, p < 0.00001), eight of which were also found to be associated with shot holing damage (Table 3.6). Association peaks on both A02/C02 and A03/C03 chromosomes (Figure 3.11) were observed. On the A02 the most highly associated marker was JCVI_10519:503 (and two other highly associated SNP markers for the same gene were also observed), a gene encoding Pseudo-Response Regulator 7 (PRR7). For A03, JCVI_1507:55, a gene encoding ubiquitin-conjugating enzyme 30 (UBC30). From examining the Manhattan plot, the homologue for A03 JCVI_1507:55, can be observed located on C06. Another SNP of interest was JCVI_4396:57, a gene encoding a RPM1-interacting protein 4 (RIN4) family protein.

Table 3.6. SNPs associated with CSFB total and shot holing feeding damage phenotypic scores above 4log10p. Where there are two Log10p and *p* values, the first relates to total damage phenotype and the second relates to shot holing damage phenotype.

Brassica SNP marker	Chromosome	Total or Shot holing damage	Log10p value	*p* value	Arabidopsis orthologue	Gene annotation
JCVI_1507:55	A03	Total & shot holing	5.182/5.482	6.57E-06/3.30E-06	AT5G56150.1	Ubiquitin-conjugating enzyme 30.
JCVI_4396:57	A03	Total & shot holing	4.431/5.052	3.71E-05/8.87E-06	AT5G55850.1	RPM1-interacting protein 4 (RIN4) family protein.
JCVI_22654:430	C07	Total & shot holing	4.245/4.869	5.68E-05/1.35E-05	AT5G47970.1	Aldolase-type TIM barrel family protein.
JCVI_26688:438	A03	Shot holing	4.784	1.64E-05	AT4G30440.1	UDP-D-glucuronate 4-epimerase 1.
JCVI_18361:964	A03	Total & shot holing	4.300/4.679	5.01E-05/2.10E-05	AT5G57850.1	D-aminoacid aminotransferase-like PLP-dependent enzymes superfamily protein.
JCVI_27545:652	A10	Total & shot holing	4.063/4.585	8.64E-05/2.60E-05	AT5G14720.1	Protein kinase superfamily protein.
JCVI_26003:544	A03	Shot holing	4.379	4.17E-05	AT4G30690.1	Translation initiation factor 3 protein.
JCVI_28000:793	A09	Total & shot holing	4.070/4.363	8.51E-05/4.33E-05	AT3G51800.1	Metallopeptidase M24 family protein.
JCVI_20195:561	A03	Total & shot holing	4.247/4.348	5.66E-05/4.49E-05	AT4G30020.1	PA-domain containing subtilase family protein.
JCVI_4366:371	A03	Shot holing	4.334	4.64E-05	AT5G56350.1	Pyruvate kinase family protein.
JCVI_5180:230	C03	Shot holing	4.327	4.71E-05	AT5G55710.1	NA (uncharacterised).
JCVI_18444:263	Cnng	Shot holing	4.311	4.88E-05	AT2G05530.1	Glycine-rich protein family.
JCVI_6849:531	A09	Total & shot holing	4.188/4.301	6.49E-05/5.00E-05	AT3G50790.1	Esterase/lipase/thioesterase family protein.
JCVI_22673:421	A01	Total	4.240	5.76E-05	AT4G37000.1	Accelerated cell death 2 (ACD2).
JCVI_26087:1613	C03	Shot holing	4.218	6.05E-05	AT5G55910.1	D6 protein kinase.

EV195955:561	C02	Total	4.207	6.21E-05	AT5G03940.1	Chloroplast signal recognition particle 54 kDa subunit.
JCVI_10519:503	A02	Total	4.159	6.93E-05	AT5G02810.1	Pseudo-response regulator 7.
JCVI_6849:534	A09	Shot holing	4.153	7.03E-05	AT3G50790.1	Esterase/lipase/thioesterase family protein.
EX132015:95	A08	Shot holing	4.133	7.36E-05	AT4G33630.1	Protein of unknown function (DUF3506).
JCVI_26688:504	A03	Shot holing	4.120	7.59E-05	AT4G30440.1	UDP-D-glucuronate 4-epimerase 1.
JCVI_10700:314	A09	Shot holing	4.114	7.68E-05	AT1G15710.1	Prephenate dehydrogenase family protein.
JCVI_5180:317	C03	Shot holing	4.100	7.94E-05	AT5G55710.1	NA (uncharacterised).
JCVI_7299:272	C03	Shot holing	4.091	8.11E-05	AT5G57840.1	HXXXD-type acyl-transferase family protein.
JCVI_8639:406	Anng	Shot holing	4.083	8.26E-05	AT5G01010.1	NA (uncharacterised).
JCVI_4938:250	C08	Shot holing	4.068	8.56E-05	AT3G52850.1	Vacuolar sorting receptor homolog 1.
JCVI_19582:609	A06	Total	4.053	8.85E-05	AT5G65110.1	acyl-CoA oxidase 2.
JCVI_26321:511	A04	Total	4.053	8.86E-05	AT2G21270.3	Ubiquitin fusion degradation 1.
JCVI_10519:375	Anng	Total	4.052	8.87E-05	AT5G02810.1	Pseudo-response regulator 7.
JCVI_35451:678	A08	Total	4.051	8.89E-05	AT1G14830.1	DYNAMIN-like 1C.
JCVI_10519:335	Anng	Total	4.002	9.95E-05	AT5G02810.1	Pseudo-response regulator 7.
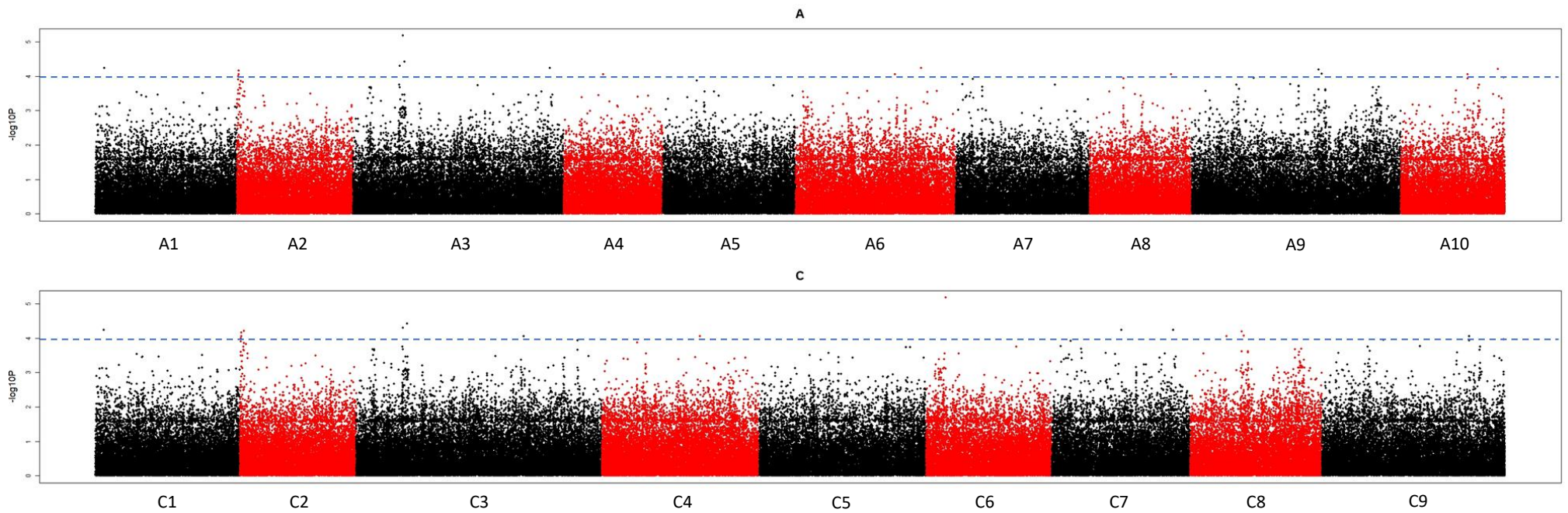
Figure 3.11. Manhattan plot demonstrating SNPs and their association with total CSFB feeding damage. The blue dashed line indicates an arbitrary threshold of 4 log10p.

22 marker associations were identified to be associated with CSFB shot holing herbivory (log10p > 4, $p < 0.0001$) (Table 3.6). Similarly to total CSFB feeding data, two association peaks were observed; one on A02/C02 and the other on A03/C03 (Figure 3.12). This was expected with shot holing being the main component of total damage from CSFB. Notably, the peaks on A03 and C03 appear clearer than that for total damage. The marker at the top of the association peak for A03 is again JCVI_1507:55, UBC30. Another SNP marker of interest on chromosome A03 was JCVI_26688:438 (with also another SNP for the same gene observed to be highly associated with shot holing), a gene encoding UDP-D-glucuronate 4-epimerase 1, and was not shared with total. Additionally, the top marker on association peak A02 was different than that for total phenotypic data. Here it was JCVI_8639:406, which has not been fully characterised but is a retinal binding protein. A final SNP of interest was JCVI_6849:531 on chromosome A09, which also appeared for the total damage phenotype, as a gene encoding an esterase/lipase/thioesterase family protein. Interestingly, this was also one of the significant GEM hits observed for shot holing.
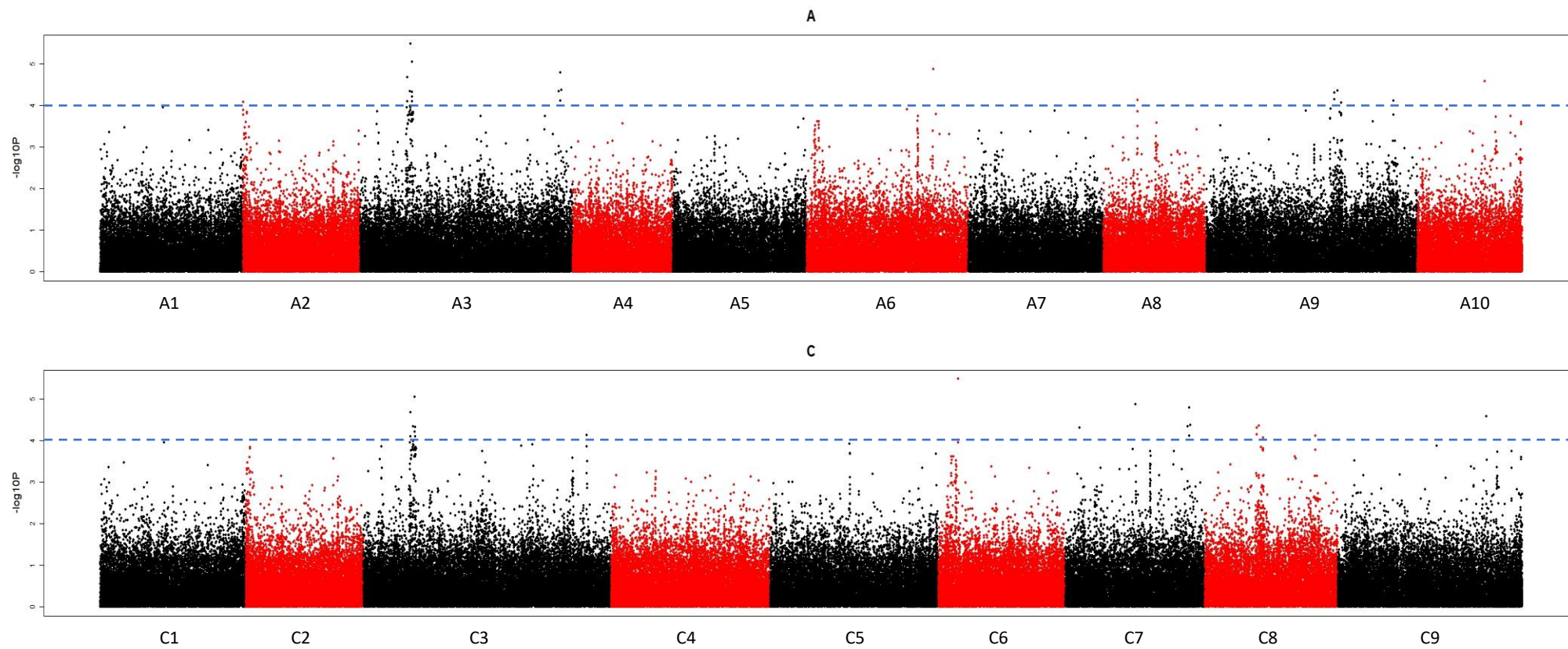
Figure 3.12. Manhattan plot demonstrating SNPs and their association with shot holing CSFB feeding damage. The blue dashed line indicates an arbitrary threshold of 4 log10p.

For grazing and stem damage phenotypes, 13 and four SNPs over the 4 log10p threshold level and *p* values < 0.0001 were observed, respectively (Table 3.7 and Table 3.8). The top SNP marker identified for the CSFB grazing phenotype was JCVI_10830:561, located on A08, a gene predicted to encode a pyridoxal phosphate-dependent enzyme. The top SNP marker for stem damage was JCVI_14759:235 on chromosome C02 but the function of this gene is unknown. However, observation of the Manhattan plots did not reveal any significant association peaks (Figures 3.13 and 3.14), although as with GEM markers a weak peak can be observed on A03 for stem damage in the same region as the peaks for total and shot holing damage. None of the most associated SNPs were shared with total or shot holing herbivory traits.

Table 3.7. SNPs associated with CSFB grazing feeding damage phenotypic scores above 4log10p.

Brassica SNP marker	Chromosome	Log10p value	*p* value	Arabidopsis orthologue	Gene annotation
JCVI_10830:561	A08	5.123	7.53E-06	AT1G11930.1	Predicted pyridoxal phosphate-dependent enzyme.
JCVI_636:366	A03	4.972	1.07E-05	AT3G12390.1	Nascent polypeptide-associated complex (NAC), alpha subunit family protein.
JCVI_35712:134	C02	4.731	1.86E-05	AT5G16120.1	Alpha/beta-Hydrolases superfamily protein.
JCVI_4243:133	A09	4.419	3.81E-05	AT4G09320.1	Nucleoside diphosphate kinase family protein.
JCVI_8421:207	C09	4.392	4.05E-05	AT5G46430.1	Ribosomal protein L32e.
JCVI_24802:338	A10	4.279	5.26E-05	AT5G57800.1	Fatty acid hydroxylase superfamily.
JCVI_22957:392	C01	4.235	5.82E-05	AT4G27520.1	Early nodulin-like protein 2.
JCVI_3305:199	A01	4.225	5.96E-05	AT3G48930.1	Nucleic acid-binding, OB-fold-like protein.
JCVI_2342:529	C03	4.165	6.84E-05	AT3G05590.1	Ribosomal protein L18.
EE430391:172	C02	4.153	7.03E-05	NA	NA
JCVI_12332:674	A04	4.147	7.12E-05	AT3G62980.1	F-box/RNI-like superfamily protein.
JCVI_2605:997	A03	4.099	7.96E-05	AT3G02470.1	S-adenosylmethionine decarboxylase.
JCVI_38337:88	Cnng	4.014	9.69E-05	AT1G26850.1	S-adenosyl-L-methionine-dependent methyltransferases superfamily protein.

Table 3.8. SNPs associated with CSFB stem feeding damage phenotypic scores above 4log10p.

| Brassica SNP marker	Chromosome	Log10p value	p value	Arabidopsis orthologue	Gene annotation
JCVI_14759:235	C02	4.573	2.67E-05	AT1G75810.1	Unknown protein.
JCVI_28646:660	A10	4.487	3.26E-05	AT1G04300.3	TRAF-like superfamily protein.
JCVI_27955:754	A07	4.0972	8.00E-05	AT1G65540.1	LETM1-like protein.
JCVI_18536:222	C06	4.0103	9.77E-05	AT1G47310.1	Unknown protein.
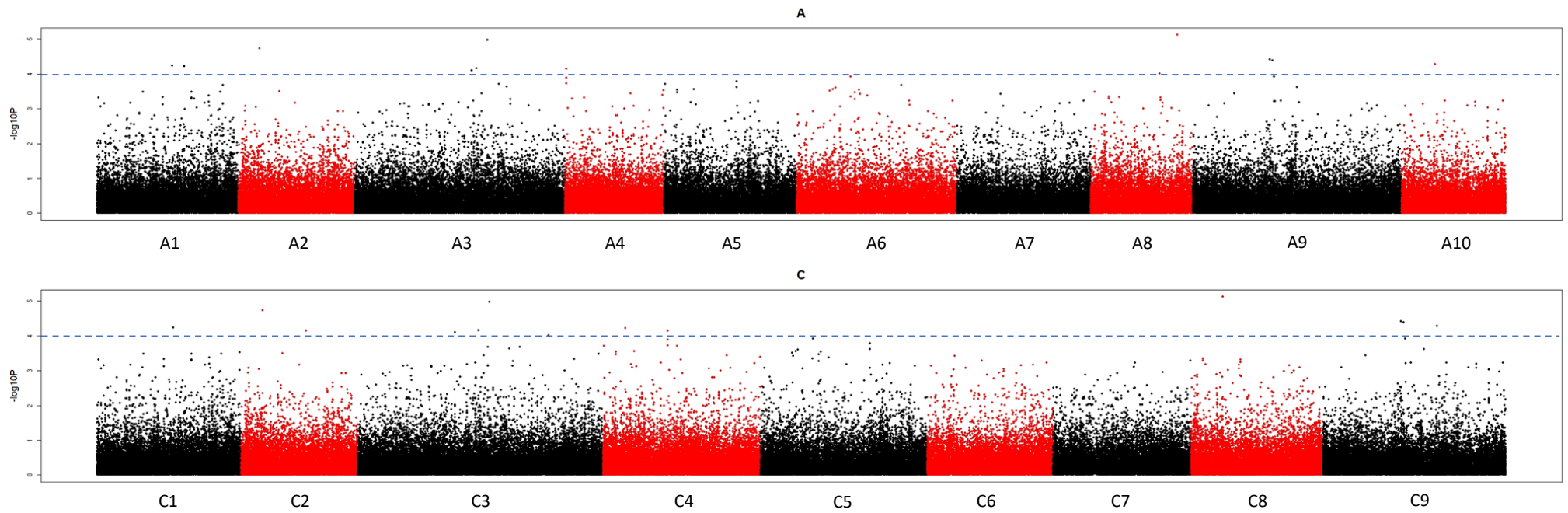
Figure 3.13. Manhattan plot demonstrating SNPs and their association with grazing CSFB feeding damage. The blue dashed line indicates an arbitrary threshold of 4 log10p.
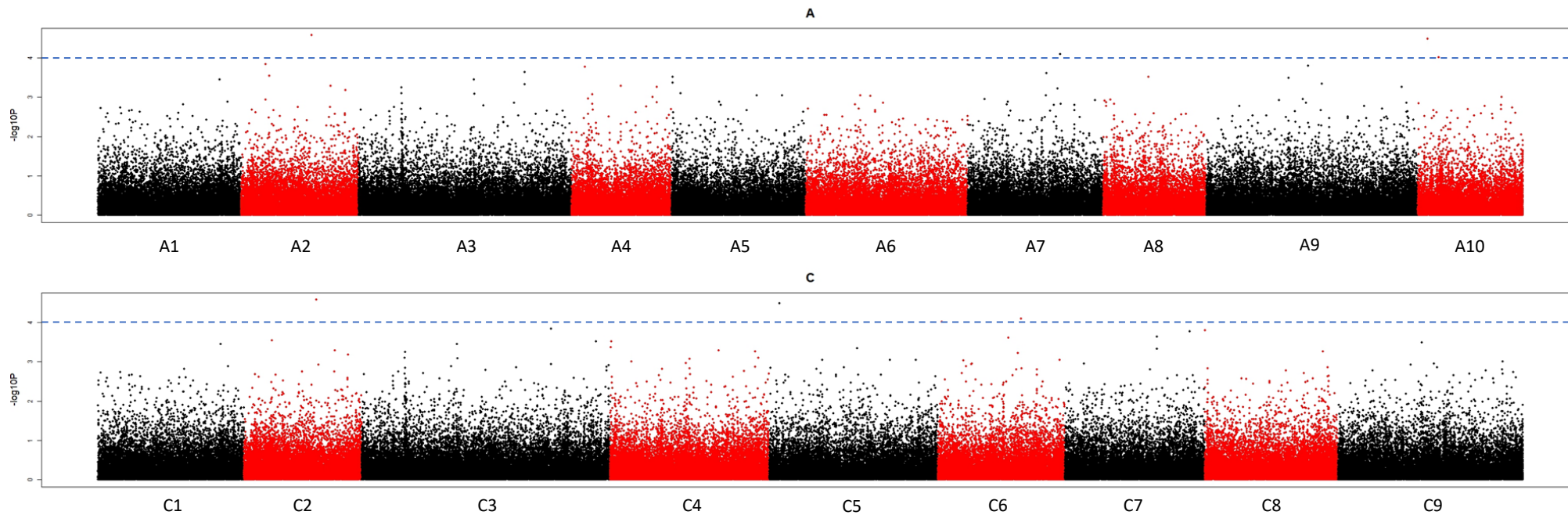
Figure 3.14. Manhattan plot demonstrating SNPs and their association with stem CSFB feeding damage. The blue dashed line indicates an arbitrary threshold of 4 log10p.

The allelic effects of three SNP markers at the top of association peaks were selected for further exploration; JCVI_10519:503, JCVI_8639:406 and JCVI_1507:55. Due to similarities between sequences in the A and C genome, there is some ambiguity in specific allele call for SNPs. Nonetheless, JCVI_10519:503, the most highly associated SNP for total CSFB damage on chromosome A02 demonstrated some identifiable variation in allele calls (Figure 3.15A). The majority of *B. napus* varieties assessed carried the C allele (n=78), which was associated with lower total CSFB herbivory than those with the T allele (n=8).

For the shot holing phenotype, the most highly associated SNP on A02 was JCVI_8639:406 and demonstrated more extreme differences in allele calls (Figure 3.15B). The majority of *B. napus* varieties carried the A allele (n=88) which was associated with lower levels of CSFB shot holing damage compared to varieties with an ambiguity allele call of A or G (n=4).

Focusing on the most highly associated SNP for total and shot holing damage on A03, JCVI_1507:55, unsurprisingly similar allelic calls between the two were observed (Figure 3.15C and D). Interestingly, the opposite pattern for this SNP was observed, with *B. napus* varieties carrying the A allele receiving higher levels of total and shot holing CSFB damage compared to others (n=5 for both total shot holing damage traits). Furthermore, four out of five of these *B. napus* lines were swede types. However, the majority fell under the ambiguity call for either A/G which demonstrated lower levels of total and shot holing herbivory (n=71 for both total and shot holing phenotypes).
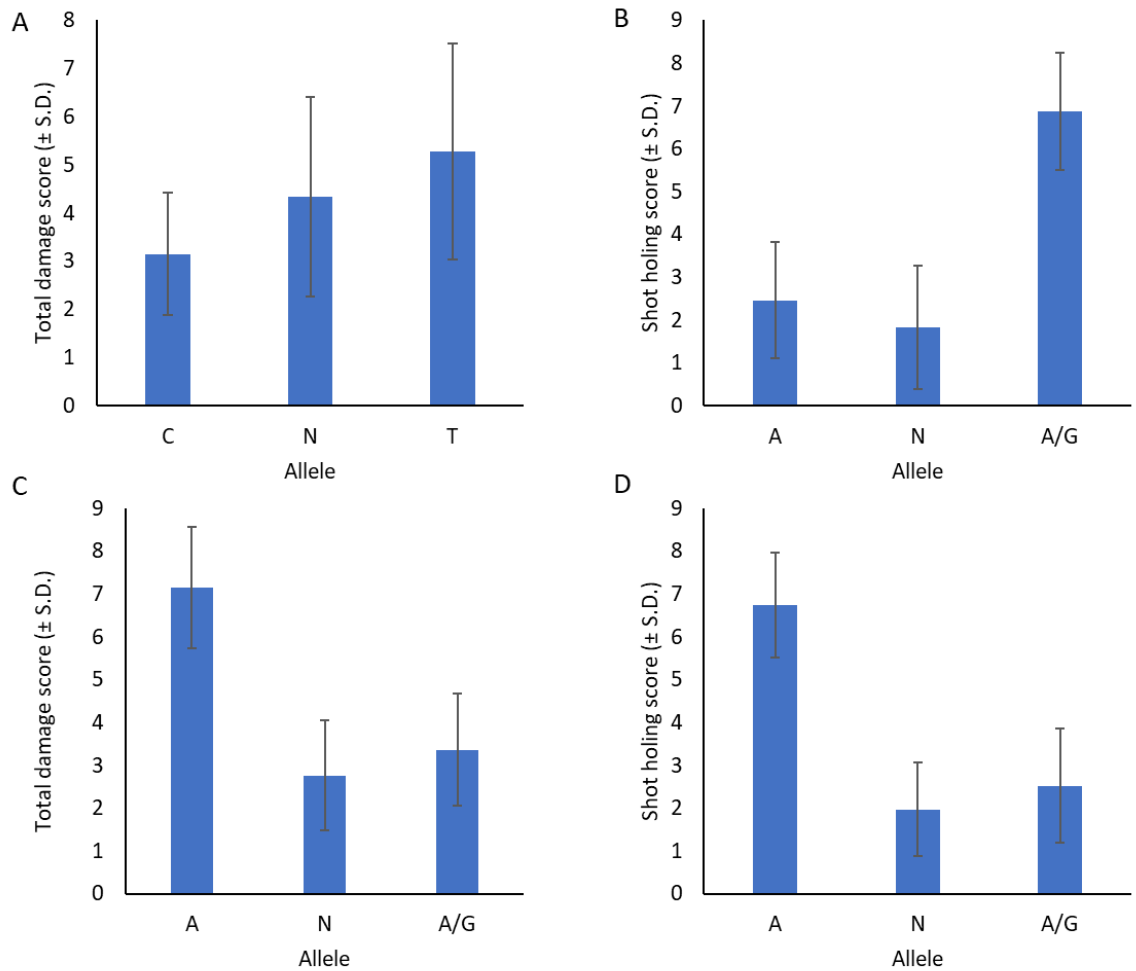
Figure 3.15. CSFB total and shot holing phenotypes and their allele calls for A: JCVI_10519:503 and total CSFB damage, B: JCVI_8639:406 and shot holing CSFB damage, C: JCVI_1507:55 for total CSFB damage and D: JCVI_1507:55 for shot holing CSFB damage. Note that allele call of ''N'' denotes no allele call.

Whilst these three markers from the top of the association peaks provide valuable information on genetic variation linked to CSFB herbivory, it is possible they are not causing the observed variation. It is possible that other nearby markers may be the causal genes linked to these most prominent SNP markers. To explore this further, each of the three peak top markers were tested for Linkage Disequilibrium (LD) against all other markers on the chromosome and the size of any resulting LD determined.

Both JCVI_10519:503 (the most highly associated SNP for total CSFB damage on chromosome A02) and JCVI_8639:406 (the mostly highly associated SNP for shot holing CSFB damage on chromosome A02) demonstrated LD with r^2 over 0.15 (Figure 3.16A and B, respectively), indicating these are likely to be regions where the causal gene is located.

Exploring LD observed for JCVI_10519:503 on chromosome A02 for total CSFB damage, an LD representing 2.51Mbp and covering 902 genes (Figure 3.16A) was identified. Of interest was the neighbouring downstream gene by 3142bp, was JCVI_26996, a gene encoding a kinase superfamily protein. Another two genes of interest were CV544662, upstream by 23Kbp encoding IQ-domain 2 and JCVI_3949, upstream by 21Kbp encoding a ribosomal protein S12/S23 family protein.

Focusing on JCVI_8639:406 on chromosome A02 identified as associated with shot holing CSFB herbivory, we observed an LD of 2.86Mbp covering 998 genes. The neighbouring upstream gene by 4850bp was of interest, identified as EX135729, a gene encoding WRKY DNA-binding protein 62. Another upstream SNP of interest by 40Kbp was JCVI_16679, another gene encoding a kinase superfamily protein. Finally, JCVI_25468 was observed 47Kbp upstream of the focal SNP on A02 for shot holing, a gene encoding serine/threonine protein kinase 1.

For the marker JCVI_1507:55, the most highly associated SNP for total and shot holing herbivory on chromosome A03, no LD was observed. However, it was observed that one of the most significant GEM hits for these feeding traits, marker JCVI_17077, was only 12Kbp upstream of this SNP and thus it remains a likely candidate gene for further exploration.
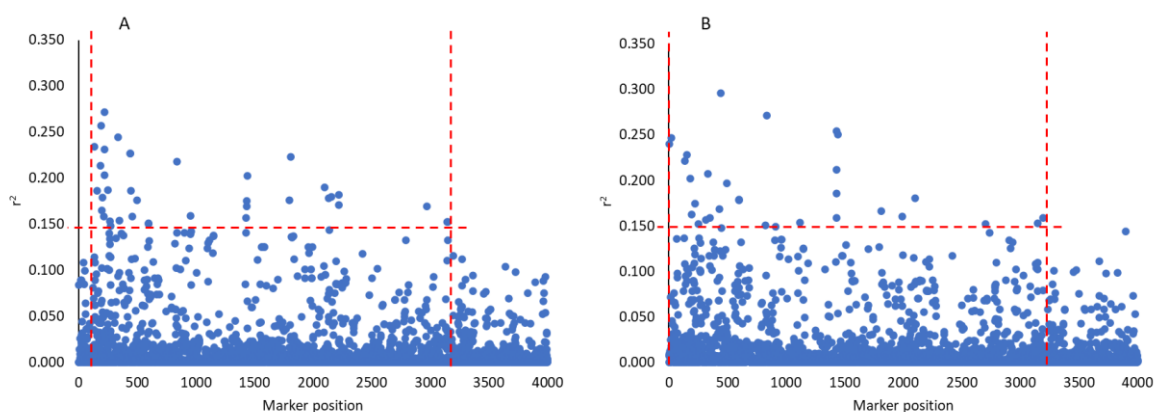
Figure 3.16. Linkage disequilibrium on chromosome A02 for A: marker JCVI_10519:503 for total CSFB feeding damage demonstrating a LD span of 2.51Mbp and B: marker JCVI_8639:406 for CSFB shot holing feeding damage demonstrating a LD span of 2.86Mbp. The horizontal red dashed line indicates a r^2 threshold of 0.15 for LD and the vertical red dashed lines indicate the boundaries of LD.

3.3.4 Arabidopsis mutant assays identified variation in CSFB herbivory

After putting CSFB feeding phenotypic datasets through an association transcriptomics pipeline and exploring the resulting SNP peaks, we selected a candidate gene for further phenotypic investigation, IQ-Domain 2 (IQD2). IQD2 was selected as although it not was not identified as a top marker linked to CSFB feeding damage itself, it was found to be in LD with a top SNP marker on chromosome A02, 20Kbp upstream of JCVI_10519. Furthermore, IQD1 (a closely related gene to IQD2), has been implicated as a plant defence gene and overexpression linked to reduction in insect herbivory (Levy et al., 2005; Barda & Levy, 2022). Therefore, IQD2 and IQD1 knockout mutants were selected for testing in CSFB herbivory assays. Here the hypothesis was that IQD2 and IQD1 mutants would receive higher levels of CSFB herbivory than their wild-type controls.

To test this hypothesis, non-choice assays were conducted with four *Arabidopsis thaliana* lines. Firstly IQD2-2, a knock-out mutant for IQD2 in a wild-type background of Columbia (Col-0). Secondly, ABB8, a loss-of-function of IQD1 mutant in a wild-type Wassilewskija (Ws-0) background. Finally, two wild-type controls, Ws-0 and Col-0 were selected. The experiment involved scoring percentage eaten (damage) for six leaves per plant, with photographic examples of levels of damage demonstrated in 3.2.2 Methods. As with previous percentage data presented in this thesis, data has been LOGIT+ transformed for analysis. Data was analysed using a two-way ANOVA of Arabidopsis line and date of assay as a blocking factor, including an interaction term between the two.

The key observation from this analysis was a statistically significant effect of Arabidopsis line on CSFB herbivory levels (Table 3.9) (Figure 3.17). A Fisher's multiple comparisons test demonstrated mutant lines IQD2-2 and ABB8 received significantly higher levels of damage compared with both wild-type controls Ws-0 and Col-0 (Table 3.10). The two mutant lines and two control lines did not significantly differ from each other.

However, the analysis also revealed a significant effect of block and a significant interaction of block with Arabidopsis line, indicating lines behaved differently in different blocks (Table 3.11). A Fisher's multiple comparisons test revealed significant differences between three pairs of blocks; Four and one, three and one and two and one. This indicates that feeding levels were different in block one compared with the others.

Table 3.9. Summary output of a two-way ANOVA assessing differences between Arabidopsis line, block and their interaction for CSFB feeding damage.

	df	Sum of Squares	Mean Square	F value	p value
Line	3	9.135	3.045	3.32	0.024
Block	3	15.191	5.064	5.52	0.002
Line:Block	9	19.861	2.207	2.41	0.018
Residuals	80	73.405	0.918		

Table 3.10. Summary output from a Fisher's multiple comparisons test demonstrating differences between Arabidopsis lines for CSFB feeding damage.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Ws-0 – Col-0	-0.054	-0.605	0.496	0.8450
Ws-0 – IQD2-2	-0.635	-1.186	-0.085	0.0242
Ws-0 – ABB8	-0.650	-1.201	-0.100	0.0211
Col-0 – IQD2-2	-0.581	-1.131	-0.031	0.0388
Col-0 – ABB8	-0.596	-1.146	-0.046	0.0341
IQD2-2 – ABB8	-0.015	-0.565	0.535	0.9565
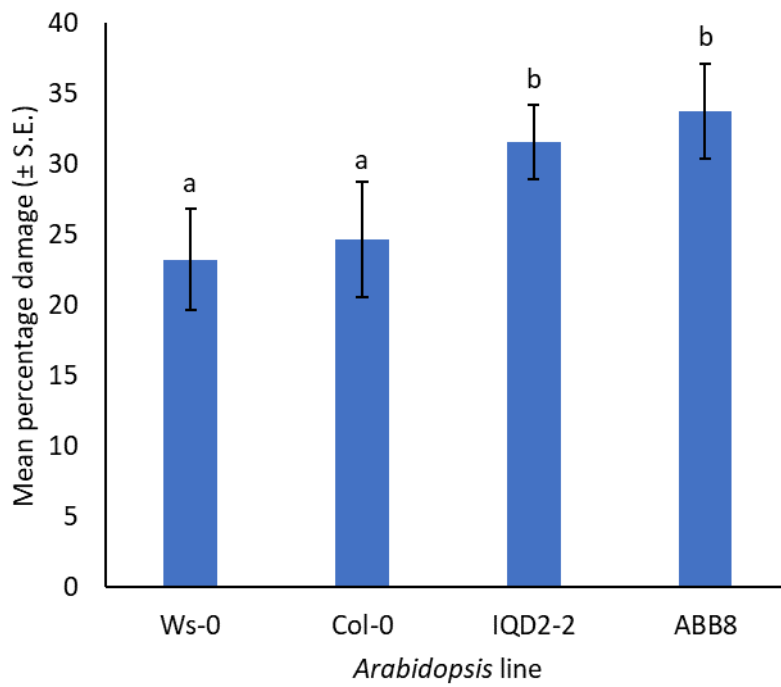
Figure 3.17. Mean percentage CSFB feeding damage for four Arabidopsis lines, two controls (Ws-0 and Col-0) and two mutants (IQD2-2 and ABB8) (± Standard Error). n = 4.

Table 3.11. Summary output from a Fisher's multiple comparisons test demonstrating differences between blocks for CSFB feeding damage.

	Mean difference	95% C.I.		p value
		Lower	Upper	
Four – Three	-0.440	-0.990	0.110	0.1155
Four – Two	-0.504	-1.054	0.049	0.0723
Four – One	-1.117	-1.667	-0.566	0.0001
Three – Two	-0.064	-0.614	0.489	0.8189
Three – One	-0.677	-1.227	-0.126	0.0166
Two – One	-0.613	-1.613	-0.063	0.0294

Therefore, we observed variation in CSFB feeding damage between Arabidopsis mutants for IQD2 and IQD1 and their controls. Specifically, the mutant lines received higher levels of damage compared with wild-type controls, thus this did support our hypothesis.

3.4 Discussion

In total, the expression of 82 GEMs were correlated with CSFB total, shot holing herbivory traits at an FDR significance level of 0.05. Of particular interest was Lorelei Like Protein (LLP), identified as a top GEM for both total and shot holing herbivory traits on A03. LLP has been demonstrated to have a role in Pattern Recognition Receptor signalling (Shen et al., 2017) and accumulation of immune response proteins in the plasma membrane (Chen et al., 2022). Another gene demonstrating significant expression variation for total and shot holing CSFB herbivory on chromosome A05 was Cinnamate 4-hydroxylase (C4H), a gene involved in phenylpropanoid biosynthesis and development (Kim et al., 2021). This gene has also been indicated to have an immune function in response to Light Leaf Spot (LLS) infection in recent research by Fell *et al*. (2023). Finally, Alpha-crystallin domain 31.2 (ACD32.1), a small heat shock protein, on chromosome C08 was of interest for total and shot holing damage traits. This gene has a role in circadian rhythm (Chandler & Melzer, 2004) but has been demonstrated to be strongly repressed in Poplar trees when exposed to insect herbivory (Ralph et al., 2008). As gene expression for all these genes demonstrated a positive correlation with CSFB damage, future work should consider testing knockout mutants in Arabidopsis for levels of herbivory.

Looking to top SNP markers, no markers were recorded to be significant with an FDR < 0.05. This is unsurprising as collection of quantitative pest herbivory data is notoriously difficult. However, a total of 55 SNPs were identified to be associated with CSFB herbivory traits at a significance threshold of log10p > 4. Three were selected at the tops of peaks on chromosomes A03 and A02 for further exploration.

For the total CSFB herbivory trait, PPR7, a gene involved in circadian rhythm, was identified as the top SNP marker at the peak of A02. Examination of allelic effects demonstrated that those *B. napus* varieties with C alleles received less herbivory than those with T alleles. This gene itself does not appear to be particularly linked to herbivory or defense responses, but was in LD with surrounding genes and is thus the likely chromatic region conferring the phenotypic differences observed in total CSFB herbivory. CDL1 was identified to be in LD with the top SNP on A02, a gene that regulates brassinosteroid signalling and plant growth but has also been implicated to be involved in immune responses to pathogens (Rao et al., 2018). IQD2 was also observed in this region, a calmodulin binding gene involved in mediating calcium signals. IQD2 was of interest due to a closely related gene, IQD1, being involved in plant defense against insect herbivory (Levy *et al*., 2005; Barda & Levy, 2022).

LD was also observed for the top SNP marker on chromosome A02 for shot holing damage, identified as UBC30, a gene involved in ubiquination of proteins. For this gene, *B. napus* varieties carrying an A allele were associated with lower levels of feeding. WRKY DNA-binding protein 62 (WRKY62) was highlighted in this region as interesting as it has a clear, established role in plant defense responses (Kim et al., 2008; Jeong et al., 2011). Other plant defense genes were also identified in this region, such as a gene encoding serine/threonine protein kinase 1 (CIPK14) which is demonstrated to have a role in Reactive Oxygen Species (ROS) responses and Salicylic Acid accumulation (SA) (Ma et al., 2021).

Another point of note is that throughout these AT analyses clearer peaks were observed for shot holing compared to total CSFB damage. This indicates that shot holing is a more precise scoring methodology. Furthermore, shot holing as a metric is easier to quantify than total damage scores. The other two feeding traits analysed, grazing and stem damage, whilst interesting, are more minor forms of herbivory and perhaps useful for understanding feeding behaviours but not so useful for investigating underlying causal genetics. Therefore, future studies aiming to investigate the genetics behind CSFB feeding phenotypic data may want to consider a shot hole scoring method rather than total damage or other minor herbivory traits.

Although there was no significant LD observed on the A03 peak for total and shot holing herbivory traits, we do observe peaks and one of the top SNP markers is notably LLP. All of the mentioned genes here warrant further investigation as potential candidates conferring the causal phenotypic variation observed in CSFB feeding traits. In particular, LLP was found to be both a top SNP marker but also a top GEM hit, and is linked to defense responses. Future work may consider investigating variation between *B. napus* varieties genome sequences. Additionally, further characterising herbivory phenotypic data with Arabidopsis knockout feeding assays would be beneficial for any genes of interest.

As SNP data first was examined first, IQD2 was selected as a potential candidate gene and follow-up assays utilising Arabidopsis loss-of-function mutants for IQD2 and IQD1 were conducted, due to potential links to responses to insect herbivory (Levy *et al*., 2005; Barda & Levy, 2022). In retrospect, if GEMs had been examined before assays were conducted, LLP may have also been selected for further CSFB herbivory assays. Nonetheless, novel Arabidopsis assays were successfully designed for testing differences in CSFB feeding levels. One issue experienced in this experiment was using ImageJ software to capture changes in greenness scores and thus herbivory. This is challenging as during the 48 hours of the assay Arabidopsis seedlings grew significantly, and the group is continuing to work on engineering a ImageJ pipeline to account for this.

The results from this experiment supported the hypothesis of IQD2 and IQD1 receiving more CSFB damage than their controls. Future work may benefit from comparing IQD2 sequences between our more resistant *B. napus* variety, Apex-93_5 X Ginyou_3 DH Line and more susceptible variety Altasweet. Additionally, running Arabidopsis feeding experiments with an increased panel of mutants would be beneficial.

4. General Discussion & Conclusion

This thesis describes a series of novel adult CSFB feeding assays, which ultimately resulted in successful identification of a more resistant variety and more susceptible variety. Developing an effective assay system was challenging, particularly due to the large panel of *B. napus* varieties to screen. Nonetheless, high-throughput six-way choice assays still enabled selection of more resistant and more susceptible *B. napus* varieties for further testing, particularly Apex-93_5 X Ginyou_3 DH Line and Altasweet. An additional challenge was maintaining an active population of adult CSFB that were feeding due to the aestivation period shortly after emergence. To combat this large populations needed to be maintained and beetles screen prior to inclusion in assays.

The project successfully tested feeding differences between Altasweet, Apex-93_5 X Ginyou_3 DH Line and an F1 cross using these parental lines in choice and non-choice assays. These experiments revealed that the F1 cross received similar levels of damage to Apex-93_5 X Ginyou_3 DH Line rather than Altasweet. This work could be taken further by generating an F2 population and testing herbivory levels of these plants, either in laboratory or field conditions.

There are many other experimental approaches that could be utilised to better understand CSFB interactions with plants. One such approach maybe to conduct Y-tube experiments, where beetles are offered a choice of food material down two different paths. This may help elucidate how CSFB use volatiles to make feeding decisions. Another approach that could help improve understanding of feeding decisions and herbivory behaviour is to record feeding over time, either in real time or recording. Software such as BORIS (Behavioural Observation Research Interactive Software, https://www.boris.unito.it/) could be used to score when and where beetles feed over time – for example, do beetles taste all food options before settling on something most palatable, or do they settle on the first food source they locate? Comparison of metabolites, such as glucosinolates (that work by Bartlet et al., (1994), Kuzina et al., (2011), Bohinc et al., (2013) and Ahn et al. (2019) have focused on) between different *B. napus* varieties continues to be an important avenue to explore. Finally, utilising microscopy approaches to examine differences in anatomy in more detail between *B. napus* varieties could be beneficial, for plant traits such as trichomes and leaf waxiness that have been demonstrated to influence insect feeding (Soroka et al., (2013) and Xuan et al. (2020)).

Two field trials were conducted between 2019 and 2020-2021 to better understand how selected resistant and susceptible *B. napus* varieties did in agricultural conditions. The 2019 trial ended prematurely and is exemplary of how challenging field experiments can be. In this year conditions were not optimal when sowing with warm and dry weather, along with immediate heavy herbivory from pests, including CSFB. Nonetheless, we managed to conduct a much more successful trial the following year, proving support for our conclusions made from laboratory experiments.

Phenotypic CSFB feeding data successfully obtained from six-way choice assays was also suitable for use in an AT pipeline, allowing further exploration of genes and gene expression linked to CSFB herbivory. A number of GEMs and SNPs (with two markers showing significant LD) were successfully identified to be associated with CSFB total and shot holing herbivory. The research group is now continuing to explore candidate genes and look for sequence variation between *B. napus* varieties.

Working with a tetraploid species with a hexaploidy ancestry, such as *B. napus*, presents certain difficulties. For example, the presence of multiple sets of homologous chromosomes giving multiple copies of genes can result in masking of phenotypic effects, complicating the process of identifying gene functions. Furthermore, allelic interactions can result in differential effects from homoeologous loci such that causal alleles can not be specifically identified, such as in this project (Section 3.3.3 and Figure 3.15).

In this project, selection of IQD2 as a candidate gene for further investigation was possible. Predictable differences for were observed in Arabidopsis mutants, providing support that this candidate gene may be associated with CSFB herbivory. Further investigation of IQD2 is required, but it could prove useful for future breeding efforts. Whilst ImageJ pipelines require further adaptation to account for plant growth during experiments, a successful assay system was developed, suitable for using entire Arabidopsis plants for CSFB damage. This method could be used in future research to phenotypically quantify CSFB herbivory of other Arabidopsis mutants for other candidate genes.

Identification of genes and gene expression linked to beneficial traits such as reduced susceptibility to pests is becoming increasingly important for agriculture. The plant growing industry is moving away from a reliance on chemical controls, due to increasing understanding of the detrimental impacts on the environment (Goulson (2013)). As such, more modernised approaches utilising exploration of crop genetics, such as research conducted in this project, are becoming more important for crop protection.

CRISPR-Cas9 Gene Editing, which enables precise targeting and knockdown of specific genes via introduction of mutations or regulatory changes, is a potentially promising technology that is making advancements in crop improvement. CRISPR-Cas9 can be utilised to generate combined Arabidopsis mutants, which could be used to further investigate CSFB herbivory preferences and associations with underlying genes. Another advancing technology is RNA interference (RNAi). RNAi uses double-stranded RNA to degrade messenger RNA and thus silence gene expression.

During this project there were also attempts to assess variation in CSFB larval success on the 96 *B. napus* panel (data not presented). Unfortunately, this experiment failed for a number of reasons. It was a large, time-consuming experiment which was set to span across two years. CSFB eggs would

be applied to bagged *B. napus* plants and resulting adult emergence recorded. However, the COVID-19 pandemic, and resulting lockdown restrictions, significantly set back this experiment. Furthermore, we observed large amounts of variation in egg viability, and the experiment was terminated during the pandemic. The research group has continued to develop this protocol using hatched larvae instead of eggs to remove the egg viability confounding variable. However, they are still experiencing large levels of variation between replicates and thus experiments with larval CSFB require further optimisation. The next experiment being considered to combat this is conducting a destructive time-series, where larvae are applied to plants and those plants dissected at different time points to better understand when and what is happening to larvae before they make it to pupation.

4.1 Conclusion

For future crop and environmental sustainability, there needs to be a move away from reliance on chemical controls and adoption of a more integrated pest management approach. In this study, novel assay systems were developed for screening *B. napus* varieties and Arabidopsis mutants for adult CSFB herbivory. This led to the discovery of potential resistant and susceptible varieties, and also provided data for genetic exploration, setting the groundwork for breeding/crop improvement.

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Appendices

Appendix 1.

Appendix 1. Table describing full name of *Brassica napus* genotypes from the BnaDFFS, RIPR or elsewhere, and their crop types. *Denotes seed obtained for Rachel Wells.

Genotype	BnaDFFS and/or RIPR	Crop type
Abukuma Natane	BnaDFFS & RIPR	Winter OSR
Altasweet	BnaDFFS & RIPR	Swede
Amber X Commanche DH Line	BnaDFFS & RIPR	Winter OSR
Apex	BnaDFFS & RIPR	Winter OSR
Apex-93_5 X Ginyou_3 DH Line	BnaDFFS & RIPR	Winter OSR
Baltia	BnaDFFS & RIPR	Winter OSR
Bienvenu DH4	BnaDFFS & RIPR	Winter OSR
Bolko	Unknown*	Winter OSR
Brauner Schnittkohl	BnaDFFS & RIPR	Siberian Kale
NK Bravour	RIPR	Winter OSR
Bronowski DH1	BnaDFFS & RIPR	Spring OSR
Cabernet	RIPR	Winter OSR
Cabriolet	RIPR	Winter OSR
Canard	BnaDFFS & RIPR	Winter forage rape
Canberra X Courage DH Line	BnaDFFS & RIPR	Winter OSR
Capitol	RIPR	Winter OSR
Capitol X Mohican DH Line	BnaDFFS	Winter OSR
Castille	RIPR	Winter OSR
Catana	RIPR	Winter OSR
Ceska Krajova	RIPR	Spring OSR
Chuanyou 2	RIPR	Chinese
Columbus X Nickel DH line	BnaDFFS	Winter OSR
Coriander	BnaDFFS & RIPR	Winter OSR
Couve Nabica	BnaDFFS & RIPR	Leafy vegetable
Darmor	BnaDFFS	Winter OSR
Dimension	RIPR	Winter OSR
Dippes	BnaDFFS & RIPR	Winter OSR
Drakkar	BnaDFFS & RIPR	Spring OSR
Duplo	BnaDFFS & RIPR	Spring OSR
Dwarf Essex	BnaDFFS & RIPR	Forage rape
English Giant	BnaDFFS & RIPR	Winter Fodder
Erglu	BnaDFFS & RIPR	Spring OSR
Eurol	BnaDFFS & RIPR	Winter OSR
Excalibur	BnaDFFS	Winter OSR
Expert	DEKALB*	Winter OSR
Flash	RIPR	Winter OSR

Groene Groninger Snijmoes	BnaDFFS & RIPR	Siberian Kale
Hanna	BnaDFFS	Spring OSR
Hansen X Gaspard DH Line	BnaDFFS & RIPR	Winter OSR
Huguenot	BnaDFFS & RIPR	Swede
Huron X Navajo DH Line	BnaDFFS & RIPR	Winter OSR
Inca X Contact DH Line	BnaDFFS & RIPR	Winter OSR
Janetzkis Schlesischer	BnaDFFS & RIPR	Winter OSR
Jaune A Collet Vert	BnaDFFS & RIPR	Swede
Jet-Neuf	BnaDFFS & RIPR	Winter OSR
Karoo-057DH	BnaDFFS & RIPR	Spring OSR
Kromerska	BnaDFFS & RIPR	Winter OSR
Lembkes Malchower (Lenora)	BnaDFFS & RIPR	Winter OSR
Lesira	BnaDFFS & RIPR	Winter OSR
Licrown X Express DH Line	BnaDFFS & RIPR	Winter OSR
Liho	BnaDFFS & RIPR	Spring fodder
Madrigal X Recital DH Line	BnaDFFS & RIPR	Winter OSR
Major DH	BnaDFFS	Winter OSR
Matador	BnaDFFS & RIPR	Winter OSR
Moana, Moana Rape	BnaDFFS & RIPR	Fodder rape
Monty-028DH	BnaDFFS & RIPR	Spring OSR
N01D-1330	BnaDFFS & RIPR	Spring OSR
N02D-1952	BnaDFFS & RIPR	Spring OSR
Ningyou 7	BnaDFFS & RIPR	Chinese
Norin	BnaDFFS & RIPR	Winter OSR
Palmedor	RIPR	Winter OSR
Palu	BnaDFFS	Winter fodder
POH 285, Bolko	BnaDFFS & RIPR	Winter OSR
Primor	BnaDFFS	Winter OSR
Q100	BnaDFFS & RIPR	synthetic
Quinta	BnaDFFS & RIPR	Winter OSR
Rafal DH1 Line	BnaDFFS & RIPR	Winter OSR
Ragged Jack	BnaDFFS & RIPR	Rape kale
Ramses	BnaDFFS & RIPR	Winter OSR
Rapid Cycling Rape (CrGC5-1)	BnaDFFS & RIPR	Spring OSR
Regent	Unknown*	Spring OSR
Rocket	RIPR	Winter OSR
Rocket (PST) X Lizard DH Line	BnaDFFS	Winter OSR
Samourai	BnaDFFS & RIPR	Winter OSR
Sensation NZ	BnaDFFS & RIPR	Swede
Shannon X Winner DH Line	BnaDFFS & RIPR	Winter OSR
Shengliyoucai	RIPR	Chinese
Siberische Boerenkool	BnaDFFS & RIPR	Siberian kale
Slapska, Slapy	BnaDFFS & RIPR	Winter OSR
Slovenska Krajova	BnaDFFS & RIPR	Winter OSR
Stellar DH	BnaDFFS & RIPR	Spring OSR

Surpass 400-024DH	BnaDFFS & RIPR	Spring OSR
Taisetsu	BnaDFFS & RIPR	Winter OSR
Tapidor DH	BnaDFFS & RIPR	Winter OSR
Temple	BnaDFFS & RIPR	Winter OSR
Tequilla X Aragon DH Line	BnaDFFS	Swede
Tina	BnaDFFS & RIPR	Swede
Topas	BnaDFFS & RIPR	Spring OSR
Verona	RIPR	Winter OSR
Victor	BnaDFFS	Winter OSR
Vige DH1	BnaDFFS & RIPR	DH Swede
Vision	RIPR	Winter OSR
Westar DH10	BnaDFFS & RIPR	Spring OSR
Wilhelmsburger; Reform	BnaDFFS & RIPR	Swede
York	BnaDFFS & RIPR	Swede
Yudal	BnaDFFS & RIPR	Spring OSR
Xiangyou_15	RIPR	Chinese
Zhongshuang_II	RIPR	Chinese

Appendix 2.

Appendix 2. Table listing full application schedule, type and rate for 2019 field trials, provided by Darryl Playford.

Application date	Product	Active	Type	Rate
12/09/2019	Sulphan (26%N; 38% SO3)	Nitrogen & Sulphur	Fertiliser	275kg/ha
16/09/2019	Falcon	Propaquizafop	Herbicide	0.5L/ha
16/09/2019	Nutriphyte PGA	Phosphoric acid	Nutrition	0.375L/ha
16/09/2019	Shadow	Dimethenamid-P, Metazachlor, Quinmerac	Herbicide	1.25L/ha
10/10/2019	Nurriphyte PGA	Phosphoric acid	Nutrition	0.75L/ha
28/10/2019	Omex 3X	Micronutrients	Nutrition	3.0L/ha
28/10/2019	Omex NA13	Micronutrients	Adjuvant	0.2L/ha

Appendix 3.

Appendix 3. Table listing full application schedule, type and rate for 2020 to 2021 field trials, provided by Darryl Playford. *Denotes applications made only to the pesticide treated part of the trial.

Application date	Product	Active	Type	Rate
07/09/2020	Hallmark	Lambda-cyhalothrin	Insecticide*	75ml/ha
07/09/2020	Shadow	Dimethenamid-P, Metazachlor, Quinmerac	Herbicide	1.25L/ha
15/09/2020	Hallmark	Lambda-cyhalothrin	Insecticide*	75ml/ha
15/09/2020	Nutriphyte PGA	Phosphoric acid	Nutrition	0.375L/ha
22/09/2020	Belkar	Halauxifen-methyl and Picloram	Herbicide	0.25L/ha
22/09/2020	Falcon	Propaquizafop	Herbicide	0.5L/ha
22/09/2020	Nutriphyte PGA	Phosphoric acid	Nutrition	0.375L/ha
02/10/2020	Hallmark	Lambda-cyhalothrin	Insecticide*	75ml/ha
19/10/2020	Karis	Lambda-cyhalothrin	Insecticide*	75ml/ha
19/10/2020	Biscaya	Thiacloprid	Insecticide*	300ml/ha
27/10/2020	Astrokerb	Aminopyralid and Propyzamide	Herbicide	1.7L/ha
27/10/2020	Proline	Prothioconazole	Fungicide	0.32L/ha
27/10/2020	Photrel	Micronutrients	Nutrition	3.0kg/ha
25/11/2020	Proline	Prothioconazole	Fungicide	0.32L/ha
25/11/2020	Photrel	Micronutrients	Nutrition	3.0ka/ha
19/03/2021	Photrel	Micronutrients	Nutrition	2.0kg/ha
19/03/2021	Tesoro	Tebuconazole	Fungicide	0.5L/ha
14/04/2021	Mavrik	Tau-fluvalinate	Insecticide*	0.2L/ha
14/04/2021	Pictor	Boscalid and Dimoxystrobin	Fungicide	0.5L/ha
14/04/2021	Bortrac	Boron	Nutrition	1.5L/ha
14/04/2021	Magnesium sulphate	Magnesium	Nutrition	5kg/ha
30/04/2021	Mavrik	Tau-fluvalinate	Insecticide*	0.2L/ha
30/04/2021	Aviator Xpro	Bixafen and Prothioconazole	Fungicide	1.0L/ha
30/04/2021	Magnesium sulphate	Magnesium	Nutrition	5kg/ha

| 21/06/2021 | Kyleo | 2,4-D and Glyphosate | Herbicide | 5.0L/ha |
| 21/06/2021 | Activator 90 | Non-ionic wetting agent | Adjuvant | 200ml/ha |

Appendix 4.

Appendix 4. Summary of output from a Fisher's multiple comparisons test demonstrating differences between *B. napus* varieties for CSFB grazing damage.

	Mean difference	95% C.I.		*p* value
		Lower	Upper	
Altasweet - Quinta	-0.4863	-0.9701	-0.0026	0.0488
Altasweet - ERGLU	-0.4945	-0.9796	-0.0094	0.0457
Altasweet - POH 285, Bolko	-0.4981	-0.9829	-0.0133	0.0441
Altasweet - Karoo-057DH	-0.5001	-0.9854	-0.0148	0.0434
Altasweet - Madrigal X Recital DH Line	-0.5106	-0.9928	-0.0283	0.038
Altasweet - Canard	-0.516	-0.9913	-0.0408	0.0334
Altasweet - Moana, Moana Rape	-0.5189	-1.0023	-0.0354	0.0355
Altasweet - Baltia	-0.5228	-1.0066	-0.039	0.0342
Altasweet - Licrown X Express DH Line	-0.5252	-1.0084	-0.0421	0.0332
Altasweet - Topas	-0.5272	-1.0115	-0.0429	0.0329
Altasweet - Palmedor	-0.5289	-1.0134	-0.0444	0.0324
Altasweet - Shannon X Winner DH Line	-0.5369	-1.026	-0.0478	0.0315
Altasweet - Ramses	-0.5389	-1.0185	-0.0594	0.0277
Altasweet - Braunder Schnittkohl	-0.539	-1.0225	-0.0555	0.0289
Altasweet - Temple	-0.5399	-1.0241	-0.0556	0.029
Altasweet - Flash	-0.5401	-1.0222	-0.058	0.0282
Altasweet - Zhongshuang II	-0.5608	-1.0499	-0.0717	0.0247
Altasweet - Norin	-0.5663	-1.051	-0.0816	0.0221
Altasweet - Hansen X Gaspard DH Line	-0.5719	-1.0561	-0.0878	0.0207
Altasweet - Matador	-0.5753	-0.9369	-0.2137	0.0019
Altasweet - Capitol X Mohican DH Line	-0.5853	-1.0685	-0.1022	0.0177
Altasweet - Slovenska Krajova	-0.5949	-1.0801	-0.1097	0.0163
Altasweet - Victor	-0.6062	-1.0973	-0.115	0.0157
Altasweet - Ningyou 7	-0.7138	-1.2011	-0.2264	0.0042
Altasweet - Palu	-0.775	-1.2598	-0.2901	0.0018
Altasweet - Cabriolet	-0.8704	-1.3539	-0.3869	0.0004
Altasweet - Dippes	-0.9666	-1.4496	-0.4837	0.0001
Ragged Jack - Palmedor	-0.4554	-0.91	-0.0008	0.0496
Ragged Jack - Ramses	-0.4655	-0.9293	-0.0017	0.0492
Ragged Jack - Braunder Schnittkohl	-0.4656	-0.9289	-0.0022	0.0489

Ragged Jack - Temple	-0.4664	-0.921	-0.0118	0.0444
Ragged Jack - Flash	-0.4666	-0.9292	-0.0041	0.048
Ragged Jack - Zhongshuang II	-0.4873	-0.9503	-0.0244	0.0391
Ragged Jack - Norin	-0.4928	-0.9605	-0.0252	0.0389
Ragged Jack - Hansen X Gaspard DH Line	-0.4985	-0.9623	-0.0347	0.0352
Ragged Jack - Matador	-0.5018	-0.8347	-0.169	0.0032
Ragged Jack - Capitol X Mohican DH Line	-0.5119	-0.9743	-0.0494	0.0301
Ragged Jack - Slovenska Krajova	-0.5215	-0.9847	-0.0582	0.0274
Ragged Jack - Victor	-0.5327	-0.997	-0.0684	0.0246
Ragged Jack - Ningyou 7	-0.6403	-1.1043	-0.1764	0.0069
Ragged Jack - Palu	-0.7015	-1.1653	-0.2376	0.0031
Ragged Jack - Cabriolet	-0.797	-1.2542	-0.3397	0.0007
Ragged Jack - Dippes	-0.8932	-1.3562	-0.4302	0.0002
Excalibur - Zhongshuang II	-0.4664	-0.9304	-0.0024	0.0489
Excalibur - Norin	-0.4719	-0.9354	-0.0083	0.046
Excalibur - Hansen X Gaspard DH Line	-0.4775	-0.9409	-0.0141	0.0434
Excalibur - Matador	-0.4809	-0.8133	-0.1484	0.0047
Excalibur - Capitol X Mohican DH Line	-0.4909	-0.9574	-0.0245	0.0392
Excalibur - Slovenska Krajova	-0.5005	-0.9635	-0.0376	0.0341
Excalibur - Victor	-0.5117	-0.9747	-0.0487	0.0304
Excalibur - Ningyou 7	-0.6194	-1.0829	-0.1559	0.0089
Excalibur - Palu	-0.6805	-1.1436	-0.2175	0.004
Excalibur - Cabriolet	-0.776	-1.2387	-0.3133	0.0011
Excalibur - Dippes	-0.8722	-1.3351	-0.4094	0.0002
Kromerska - Hansen X Gaspard DH Line	-0.464	-0.9262	-0.0019	0.0491
Kromerska - Matador	-0.4674	-0.7989	-0.1359	0.0058
Kromerska - Capitol X Mohican DH Line	-0.4775	-0.9383	-0.0166	0.0423
Kromerska - Slovenska Krajova	-0.487	-0.9487	-0.0254	0.0387
Kromerska - Victor	-0.4983	-0.964	-0.0325	0.0361
Kromerska - Ningyou 7	-0.6059	-1.0684	-0.1434	0.0103
Kromerska - Palu	-0.6671	-1.1334	-0.2007	0.0051
Kromerska - Cabriolet	-0.7625	-1.2273	-0.2977	0.0013
Kromerska - Dippes	-0.8587	-1.3194	-0.3981	0.0003
Jaune A Collet Vert - Matador	-0.4617	-0.794	-0.1294	0.0066

Jaune A Collet Vert - Capitol X Mohican DH Line	-0.4718	-0.9335	-0.01	0.0453
Jaune A Collet Vert - Slovenska Krajova	-0.4814	-0.9465	-0.0162	0.0425
Jaune A Collet Vert - Victor	-0.4926	-0.9552	-0.03	0.0369
Jaune A Collet Vert - Ningyou 7	-0.6002	-1.0631	-0.1373	0.0111
Jaune A Collet Vert - Palu	-0.6614	-1.1281	-0.1946	0.0056
Jaune A Collet Vert - Cabriolet	-0.7568	-1.2186	-0.295	0.0014
Jaune A Collet Vert - Dippes	-0.853	-1.3183	-0.3878	0.0003
Ceska Krajova - Matador	-0.4503	-0.7835	-0.1172	0.0082
Ceska Krajova - Slovenska Krajova	-0.47	-0.9336	-0.0065	0.0469
Ceska Krajova - Victor	-0.4812	-0.9479	-0.0145	0.0433
Ceska Krajova - Ningyou 7	-0.5888	-1.056	-0.1217	0.0136
Ceska Krajova - Palu	-0.65	-1.1144	-0.1856	0.0062
Ceska Krajova - Cabriolet	-0.7455	-1.2001	-0.2909	0.0014
Ceska Krajova - Dippes	-0.8417	-1.3054	-0.378	0.0004
Apex-93_5 X Ginyou_3 DH Line - Matador	-0.4451	-0.7784	-0.1118	0.009
Apex-93_5 X Ginyou_3 DH Line - Capitol X Mohican DH Line	-0.4551	-0.9099	-0.0004	0.0498
Apex-93_5 X Ginyou_3 DH Line - Slovenska Krajova	-0.4647	-0.9183	-0.0112	0.0446
Apex-93_5 X Ginyou_3 DH Line - Victor	-0.476	-0.9404	-0.0115	0.0446
Apex-93_5 X Ginyou_3 DH Line - Ningyou 7	-0.5836	-1.0477	-0.1194	0.0138
Apex-93_5 X Ginyou_3 DH Line - Palu	-0.6448	-1.1086	-0.1809	0.0065
Apex-93_5 X Ginyou_3 DH Line - Cabriolet	-0.7402	-1.2042	-0.2763	0.0018
Apex-93_5 X Ginyou_3 DH Line - Dippes	-0.8364	-1.2988	-0.3741	0.0004
English Giant - Matador	-0.439	-0.8	-0.0781	0.0172
English Giant - Ningyou 7	-0.5776	-1.0625	-0.0926	0.0197
English Giant - Palu	-0.6387	-1.1229	-0.1545	0.0098
English Giant - Cabriolet	-0.7342	-1.2173	-0.2511	0.003
English Giant - Dippes	-0.8304	-1.3072	-0.3536	0.0007
Stellar DH - Matador	-0.418	-0.7503	-0.0857	0.0138
Stellar DH - Ningyou 7	-0.5565	-1.011	-0.102	0.0165
Stellar DH - Palu	-0.6177	-1.0811	-0.1543	0.0091
Stellar DH - Cabriolet	-0.7131	-1.1785	-0.2478	0.0027

Stellar DH - Dippes	-0.8094	-1.2729	-0.3458	0.0007
Catana - Matador	-0.4128	-0.7445	-0.0811	0.0148
Catana - Ningyou 7	-0.5513	-1.0155	-0.0871	0.02
Catana - Palu	-0.6125	-1.0759	-0.1491	0.0097
Catana - Cabriolet	-0.7079	-1.1615	-0.2543	0.0023
Catana - Dippes	-0.8041	-1.2686	-0.3397	0.0007
Taisetsu - Matador	-0.407	-0.7398	-0.0742	0.0166
Taisetsu - Ningyou 7	-0.5455	-1.0107	-0.0803	0.0216
Taisetsu - Palu	-0.6067	-1.0698	-0.1435	0.0103
Taisetsu - Cabriolet	-0.7021	-1.1645	-0.2397	0.003
Taisetsu - Dippes	-0.7983	-1.2638	-0.3328	0.0008
Rapid Cycling Rape (CrGC5) - Matador	-0.3898	-0.7508	-0.0289	0.0343
Rapid Cycling Rape (CrGC5) - Ningyou 7	-0.5284	-1.0135	-0.0433	0.0328
Rapid Cycling Rape (CrGC5) - Palu	-0.5895	-1.0647	-0.1144	0.0151
Rapid Cycling Rape (CrGC5) - Cabriolet	-0.685	-1.1714	-0.1986	0.0059
Rapid Cycling Rape (CrGC5) - Dippes	-0.7812	-1.2647	-0.2977	0.0016
Bronowski - Matador	-0.389	-0.7213	-0.0567	0.0219
Bronowski - Ningyou 7	-0.5275	-0.9907	-0.0643	0.0257
Bronowski - Palu	-0.5887	-1.0514	-0.126	0.0127
Bronowski - Cabriolet	-0.6841	-1.1466	-0.2217	0.0038
Bronowski - Dippes	-0.7804	-1.246	-0.3147	0.0011
Apex - Matador	-0.3887	-0.7211	-0.0563	0.022
Apex - Ningyou 7	-0.5272	-0.9908	-0.0636	0.0259
Apex - Palu	-0.5884	-1.0508	-0.126	0.0127
Apex - Cabriolet	-0.6838	-1.1461	-0.2216	0.0038
Apex - Dippes	-0.7801	-1.2337	-0.3265	0.0008
JetNeuf - Matador	-0.3812	-0.7136	-0.0489	0.0246
JetNeuf - Ningyou 7	-0.5197	-0.9857	-0.0538	0.0289
JetNeuf - Palu	-0.5809	-1.0436	-0.1182	0.014
JetNeuf - Cabriolet	-0.6764	-1.1392	-0.2136	0.0043
JetNeuf - Dippes	-0.7726	-1.2422	-0.303	0.0013
Abukuma Natane - Matador	-0.3579	-0.6906	-0.0252	0.035
Abukuma Natane - Ningyou 7	-0.4964	-0.9603	-0.0326	0.036
Abukuma Natane - Palu	-0.5576	-1.0116	-0.1036	0.0162
Abukuma Natane - Cabriolet	-0.653	-1.1201	-0.186	0.0062

Abukuma Natane - Dippes	-0.7493	-1.2117	-0.2868	0.0015
Tina - Matador	-0.3569	-0.6889	-0.025	0.0351
Tina - Ningyou 7	-0.4954	-0.9587	-0.0321	0.0361
Tina - Palu	-0.5566	-1.0139	-0.0993	0.0171
Tina - Cabriolet	-0.6521	-1.1177	-0.1864	0.0061
Tina - Dippes	-0.7483	-1.2101	-0.2865	0.0015
N01D-1330 - Matador	-0.3526	-0.6853	-0.0198	0.0379
N01D-1330 - Ningyou 7	-0.4911	-0.9544	-0.0278	0.0378
N01D-1330 - Palu	-0.5522	-1.0172	-0.0872	0.02
N01D-1330 - Cabriolet	-0.6477	-1.1104	-0.185	0.0062
N01D-1330 - Dippes	-0.7439	-1.2052	-0.2827	0.0016
Darmor - Matador	-0.3519	-0.6846	-0.0191	0.0383
Darmor - Ningyou 7	-0.4904	-0.9537	-0.027	0.0381
Darmor - Palu	-0.5515	-1.019	-0.0841	0.0208
Darmor - Cabriolet	-0.647	-1.1098	-0.1842	0.0062
Darmor - Dippes	-0.7432	-1.2053	-0.2811	0.0017
Major - Ningyou 7	-0.4891	-0.973	-0.0052	0.0476
Major - Palu	-0.5503	-1.0334	-0.0671	0.0257
Major - Cabriolet	-0.6457	-1.1289	-0.1626	0.0089
Major - Dippes	-0.7419	-1.2244	-0.2595	0.0026
Lembkes Malchower (Lenora) - Matador	-0.3468	-0.6783	-0.0153	0.0403
Lembkes Malchower (Lenora) - Ningyou 7	-0.4853	-0.9396	-0.0311	0.0363
Lembkes Malchower (Lenora) - Palu	-0.5465	-1.0106	-0.0824	0.0211
Lembkes Malchower (Lenora) - Cabriolet	-0.642	-1.1053	-0.1786	0.0067
Lembkes Malchower (Lenora) - Dippes	-0.7382	-1.2047	-0.2717	0.002
Columbus X Nickel DH Line - Matador	-0.3441	-0.6766	-0.0116	0.0426
Columbus X Nickel DH Line - Ningyou 7	-0.4826	-0.9456	-0.0196	0.0411
Columbus X Nickel DH Line - Palu	-0.5438	-1.0059	-0.0816	0.0212
Columbus X Nickel DH Line - Cabriolet	-0.6392	-1.1054	-0.173	0.0073
Columbus X Nickel DH Line - Dippes	-0.7354	-1.1965	-0.2744	0.0018
Slapska, Slapy - Matador	-0.3426	-0.6763	-0.009	0.0442
Slapska, Slapy - Ningyou 7	-0.4811	-0.9482	-0.0141	0.0435
Slapska, Slapy - Palu	-0.5423	-1.0084	-0.0762	0.0227
Slapska, Slapy - Cabriolet	-0.6378	-1.101	-0.1746	0.0071
Slapska, Slapy - Dippes	-0.734	-1.1967	-0.2713	0.0019

N02D-19Huron X Navajo DH Line - Matador	-0.3414	-0.6726	-0.0101	0.0434
N02D-19Huron X Navajo DH Line - Ningyou 7	-0.4799	-0.9428	-0.017	0.0422
N02D-19Huron X Navajo DH Line - Palu	-0.5411	-1.0031	-0.079	0.0218
N02D-19Huron X Navajo DH Line - Cabriolet	-0.6365	-1.0985	-0.1745	0.007
N02D-19Huron X Navajo DH Line - Dippes	-0.7327	-1.1943	-0.2712	0.0019
Surpass400-024DH - Matador	-0.3343	-0.6662	-0.0024	0.0484
Surpass400-024DH - Ningyou 7	-0.4728	-0.936	-0.0096	0.0455
Surpass400-024DH - Palu	-0.534	-0.9973	-0.0706	0.024
Surpass400-024DH - Cabriolet	-0.6294	-1.0922	-0.1666	0.0078
Surpass400-024DH - Dippes	-0.7256	-1.1916	-0.2596	0.0023
Sensation NZ - Ningyou 7	-0.4704	-0.9332	-0.0076	0.0463
Sensation NZ - Palu	-0.5316	-0.9941	-0.0691	0.0243
Sensation NZ - Cabriolet	-0.6271	-1.0809	-0.1733	0.0069
Sensation NZ - Dippes	-0.7233	-1.1845	-0.2621	0.0022
Coriander - Ningyou 7	-0.4681	-0.9316	-0.0046	0.0478
Coriander - Palu	-0.5292	-0.9927	-0.0657	0.0253
Coriander - Cabriolet	-0.6247	-1.0878	-0.1617	0.0083
Coriander - Dippes	-0.7209	-1.1824	-0.2595	0.0023
York - Palu	-0.5218	-0.9763	-0.0672	0.0245
York - Cabriolet	-0.6172	-1.0832	-0.1512	0.0095
York - Dippes	-0.7135	-1.1773	-0.2496	0.0026
Bolko - Palu	-0.5201	-1.0029	-0.0374	0.0348
Bolko - Cabriolet	-0.6156	-1.0986	-0.1325	0.0126
Bolko - Dippes	-0.7118	-1.1942	-0.2294	0.0039
Capitol - Palu	-0.5144	-0.9769	-0.0518	0.0294
Capitol - Cabriolet	-0.6099	-1.0718	-0.1479	0.0098
Capitol - Dippes	-0.7061	-1.1676	-0.2445	0.0028
Tequilla X Aragon DH Line - Palu	-0.5091	-0.9637	-0.0546	0.0282
Tequilla X Aragon DH Line - Cabriolet	-0.6046	-1.0666	-0.1426	0.0104
Tequilla X Aragon DH Line - Dippes	-0.7008	-1.1643	-0.2374	0.0031
Siberische Boerenkool - Cabriolet	-0.5929	-1.1058	-0.0799	0.0236
Siberische Boerenkool - Dippes	-0.6891	-1.2017	-0.1765	0.0085

Duplo - Palu	-0.4923	-0.9561	-0.0285	0.0375
Duplo - Cabriolet	-0.5878	-1.0451	-0.1305	0.0119
Duplo - Dippes	-0.684	-1.1462	-0.2218	0.0038
Vision - Palu	-0.4841	-0.9474	-0.0208	0.0406
Vision - Cabriolet	-0.5796	-1.0412	-0.1179	0.014
Vision - Dippes	-0.6758	-1.1366	-0.215	0.0041
Samourai - Cabriolet	-0.5662	-1.0504	-0.082	0.022
Samourai - Dippes	-0.6624	-1.1466	-0.1782	0.0074
Expert - Palu	-0.4638	-0.9179	-0.0098	0.0453
Expert - Cabriolet	-0.5593	-1.0227	-0.0959	0.0181
Expert - Dippes	-0.6555	-1.1181	-0.1929	0.0056
Cabernet - Palu	-0.459	-0.9119	-0.0061	0.047
Cabernet - Cabriolet	-0.5545	-1.0169	-0.092	0.0189
Cabernet - Dippes	-0.6507	-1.1166	-0.1848	0.0063
Inca X Contact DH Line - Cabriolet	-0.543	-1.0083	-0.0777	0.0223
Inca X Contact DH Line - Dippes	-0.6392	-1.1006	-0.1778	0.0067
Bravour - Cabriolet	-0.5394	-1.0014	-0.0773	0.0222
Bravour - Dippes	-0.6356	-1.0968	-0.1744	0.007
Xiangyou 15 - Cabriolet	-0.5353	-0.9986	-0.0719	0.0236
Xiangyou 15 - Dippes	-0.6315	-1.0948	-0.1682	0.0076
Eurol - Cabriolet	-0.5304	-0.9941	-0.0667	0.025
Eurol - Dippes	-0.6266	-1.0932	-0.1601	0.0086
Huron X Navajo DH Line - Cabriolet	-0.5279	-0.9914	-0.0644	0.0257
Huron X Navajo DH Line - Dippes	-0.6241	-1.0873	-0.1609	0.0084
Yudal - Cabriolet	-0.5277	-0.9915	-0.0639	0.0258
Yudal - Dippes	-0.6239	-1.0875	-0.1603	0.0084
Dimension - Cabriolet	-0.5099	-0.9725	-0.0473	0.0308
Dimension - Dippes	-0.6061	-1.0684	-0.1439	0.0103
Rocket (pst) x Lizard DH line - Cabriolet	-0.5082	-0.9708	-0.0456	0.0314
Rocket (pst) x Lizard DH line - Dippes	-0.6044	-1.0674	-0.1414	0.0106
Castille - Cabriolet	-0.507	-0.9612	-0.0528	0.0288
Castille - Dippes	-0.6032	-1.066	-0.1403	0.0107
Lesira - Cabriolet	-0.5004	-0.9623	-0.0385	0.0338
Lesira - Dippes	-0.5966	-1.059	-0.1343	0.0115
Rocket - Cabriolet	-0.4968	-0.9595	-0.0342	0.0354

Rocket - Dippes	-0.593	-1.0559	-0.1302	0.0121
Amber X Commanche DH Line - Cabriolet	-0.4873	-0.9502	-0.0245	0.0391
Amber X Commanche DH Line - Dippes	-0.5835	-1.0488	-0.1182	0.0141
Q100 - Cabriolet	-0.4865	-0.9511	-0.0219	0.0402
Q100 - Dippes	-0.5827	-1.0453	-0.1202	0.0136
Bienvenu DH4 - Cabriolet	-0.4768	-0.9395	-0.014	0.0435
Bienvenu DH4 - Dippes	-0.573	-1.0351	-0.1109	0.0152
Janetzkis Schlesischer - Cabriolet	-0.4753	-0.9436	-0.007	0.0467
Janetzkis Schlesischer - Dippes	-0.5715	-1.0371	-0.1059	0.0162
Chuanyou 2 - Cabriolet	-0.4741	-0.9371	-0.011	0.0448
Chuanyou 2 - Dippes	-0.5703	-1.0337	-0.1069	0.016
Wilhelmsburger DH - Cabriolet	-0.474	-0.9353	-0.0126	0.0441
Wilhelmsburger DH - Dippes	-0.5702	-1.0229	-0.1175	0.0137
Monty-028DH - Cabriolet	-0.4681	-0.9307	-0.0056	0.0473
Monty-028DH - Dippes	-0.5644	-1.0266	-0.1021	0.0168
Westar DH10 - Cabriolet	-0.4636	-0.9265	-0.0006	0.0497
Westar DH10 - Dippes	-0.5598	-1.0223	-0.0973	0.0178
Couve Nabica - Cabriolet	-0.4635	-0.9265	-0.0005	0.0498
Couve Nabica - Dippes	-0.5597	-1.025	-0.0944	0.0185
Drakkar - Cabriolet	-0.4616	-0.915	-0.0081	0.046
Drakkar - Dippes	-0.5578	-1.0197	-0.0959	0.018
Rafal DH1 - Dippes	-0.5566	-1.0108	-0.1023	0.0164
Dwarf Essex - Dippes	-0.5498	-1.0035	-0.096	0.0177
Canberra X Courage DH Line - Dippes	-0.5436	-0.9968	-0.0904	0.0188
Groene Groninger Snijmoes - Dippes	-0.5425	-1.0082	-0.0769	0.0225
Huguenot - Dippes	-0.5413	-1.0068	-0.0758	0.0227
Primor - Dippes	-0.5386	-0.9926	-0.0846	0.0201
Hannah - Dippes	-0.5306	-0.9922	-0.0689	0.0244
Vige - Dippes	-0.5258	-0.9931	-0.0585	0.0275
Verona - Dippes	-0.5045	-0.9677	-0.0413	0.0328
Regent - Dippes	-0.4876	-0.9499	-0.0252	0.0388
Quinta - Dippes	-0.4803	-0.942	-0.0185	0.0415
ERGLU - Dippes	-0.4721	-0.9357	-0.0085	0.0459
POH 285, Bolko - Dippes	-0.4686	-0.9319	-0.0052	0.0475
Karoo-057DH - Dippes	-0.4665	-0.9305	-0.0025	0.0488

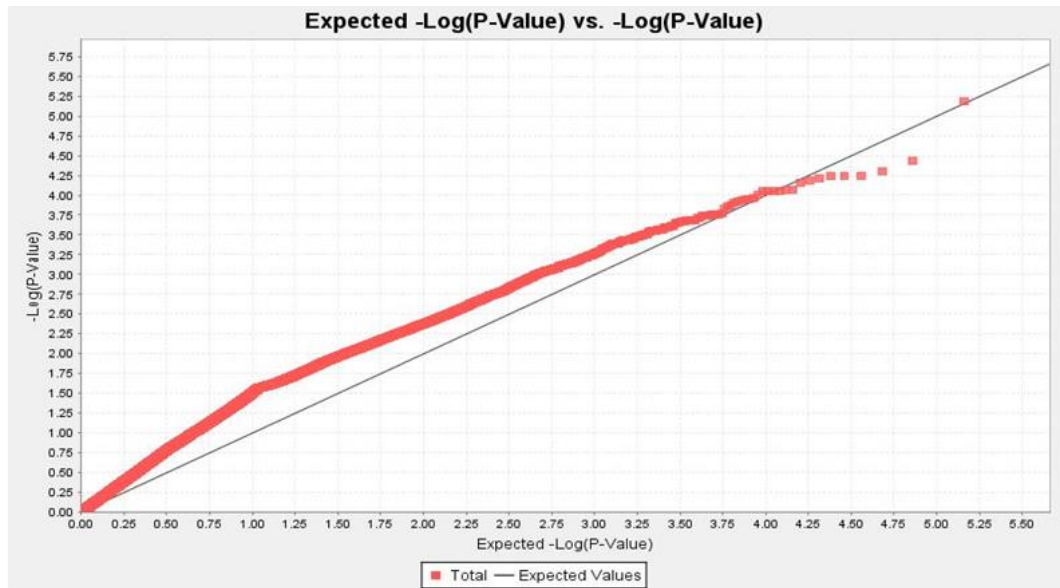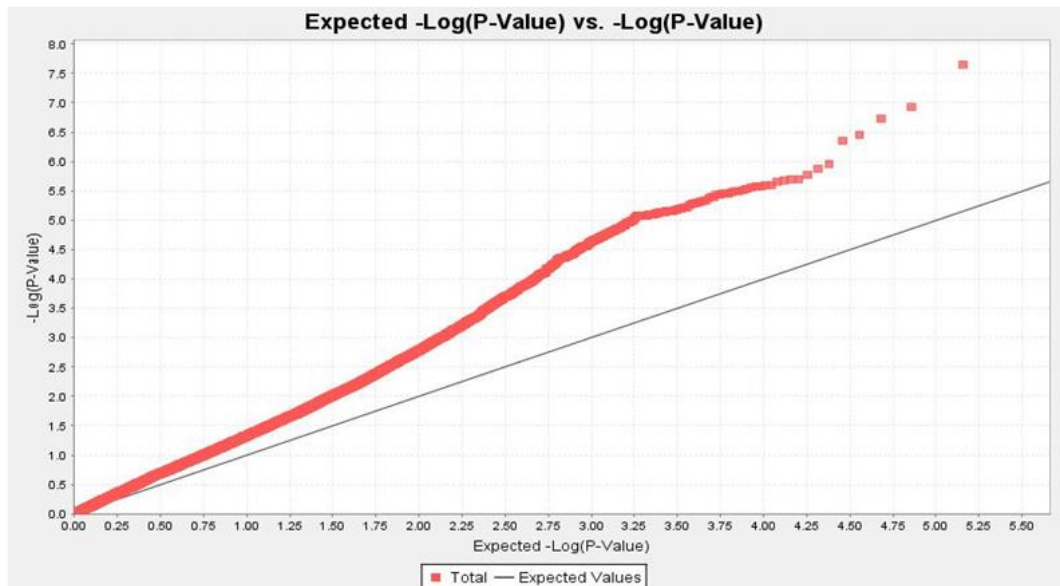|				
Matador - Dippes	-0.3914	-0.724	-0.0587	0.0212

Appendix 5.
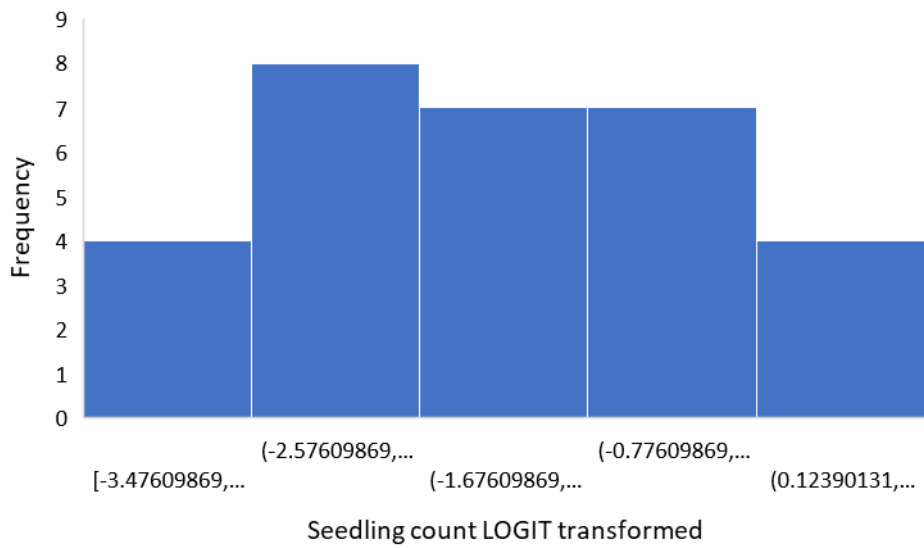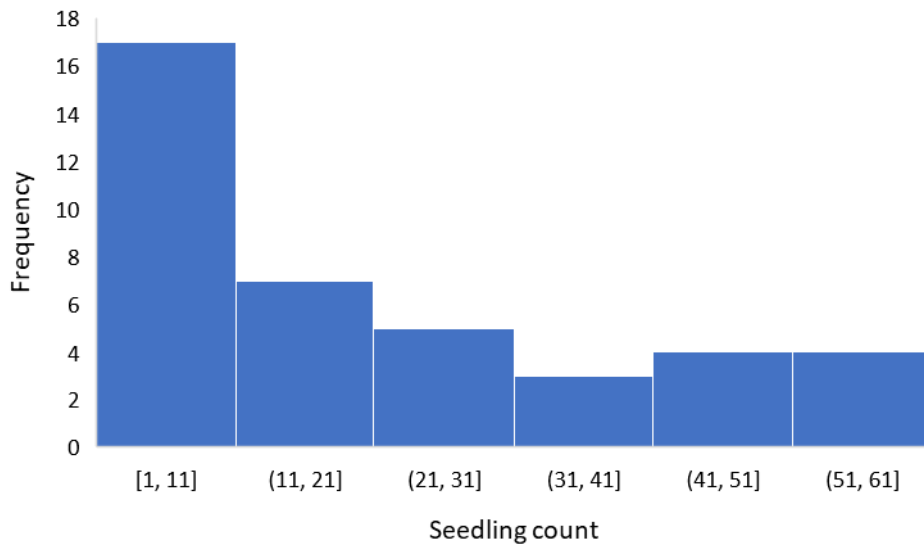
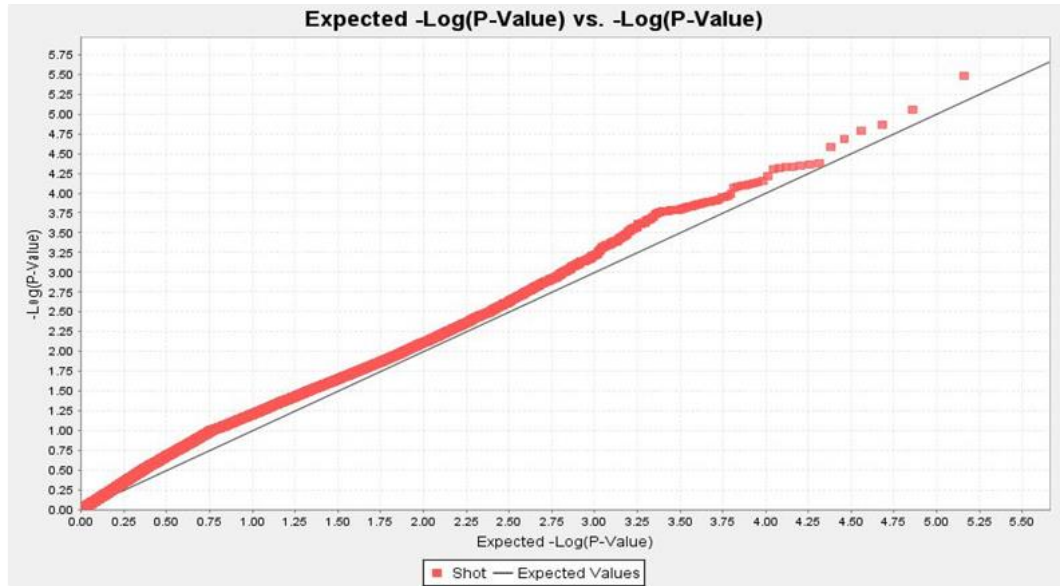Appendix 5. Figures of 2019 seedling count data before (top) and after (bottom) LOGIT transformation.
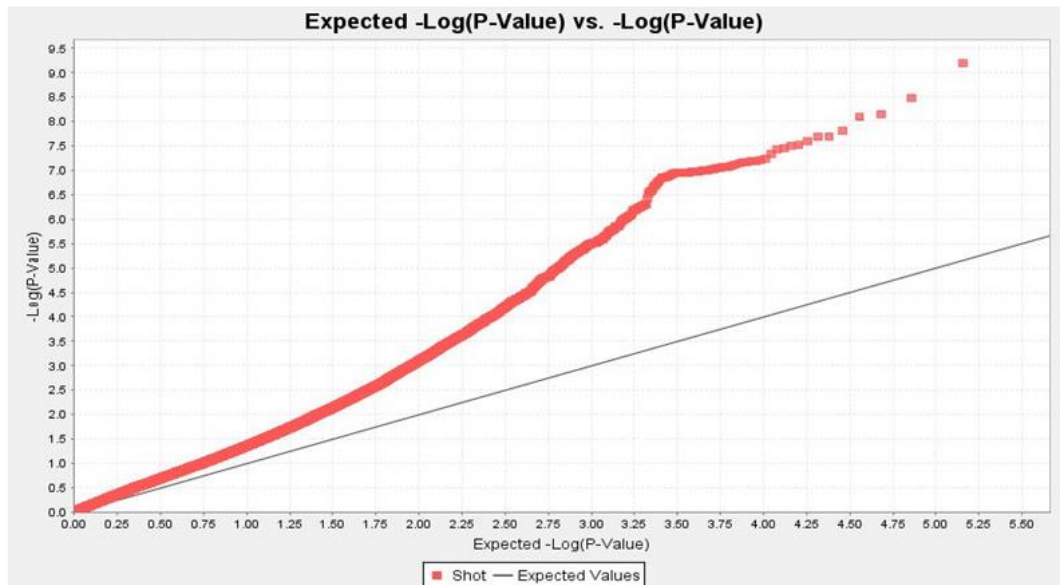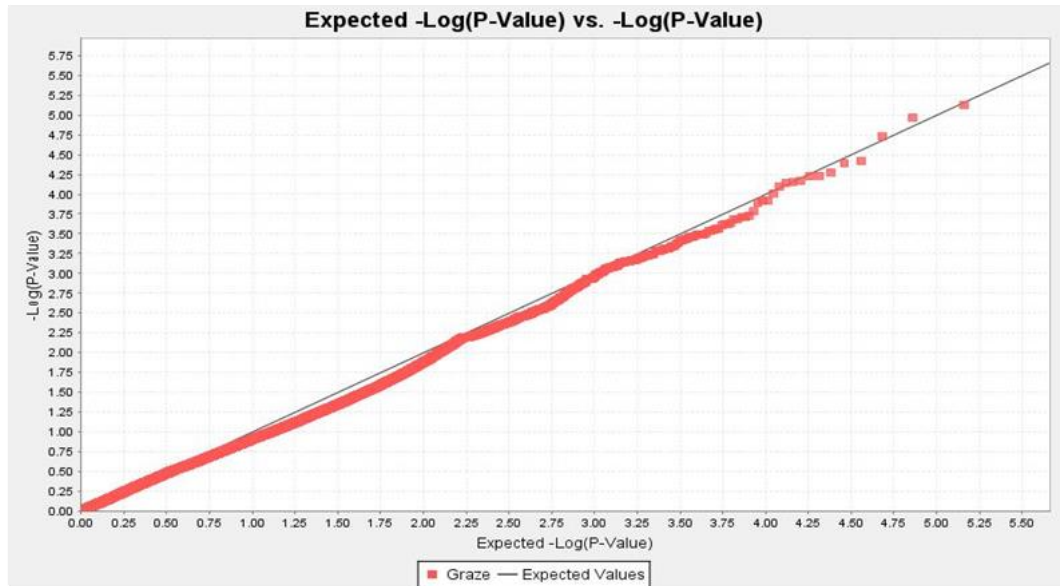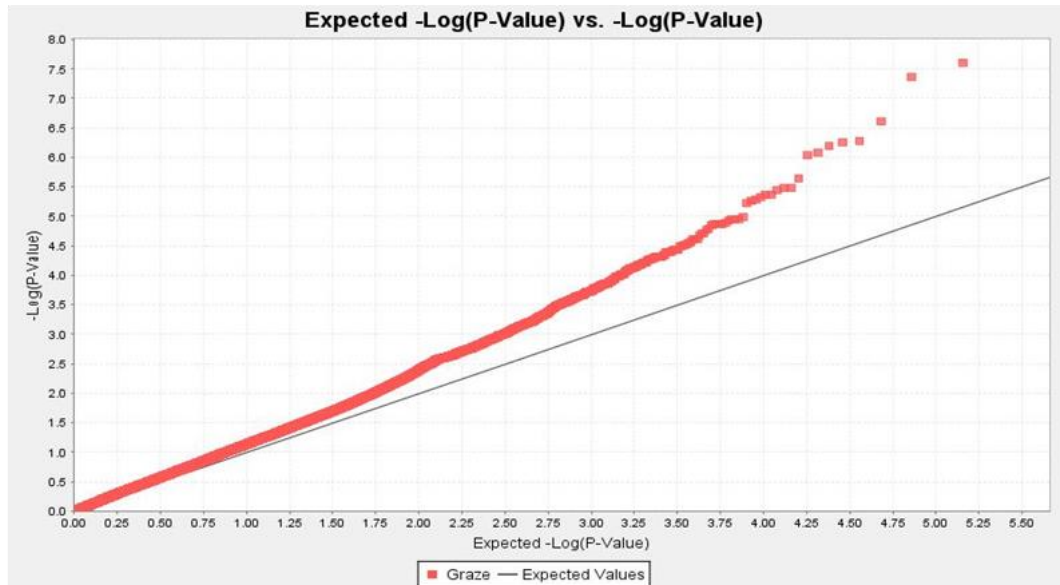
Appendix 6. QQ-plots of A: Mixed Linear Model and B: Generalised Linear Model, for total CSFB feeding data.

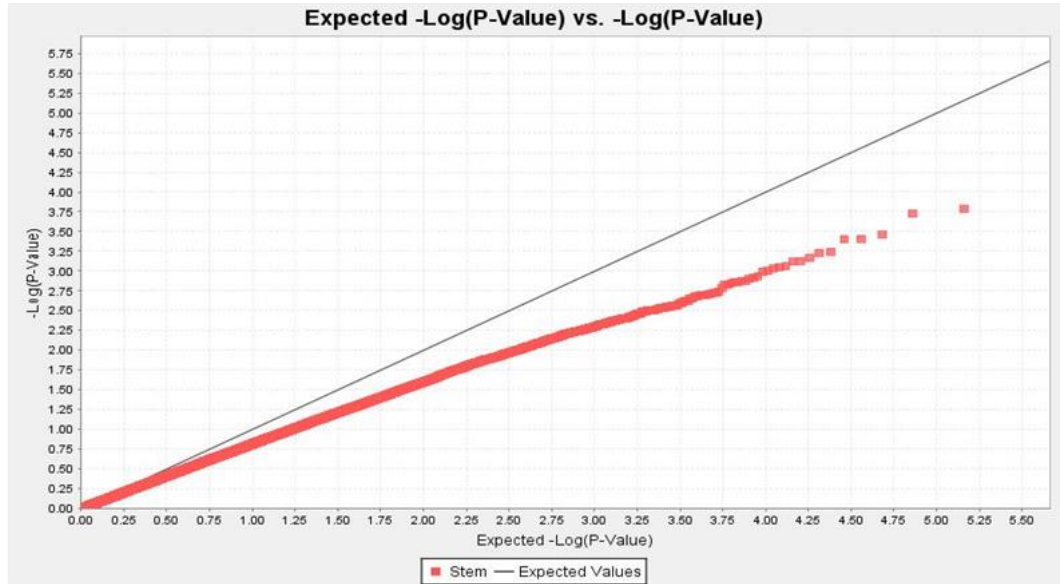Appendix 6. QQ-plots of A: Mixed Linear Model and B: Generalised Linear Model, for shot holing CSFB feeding data.

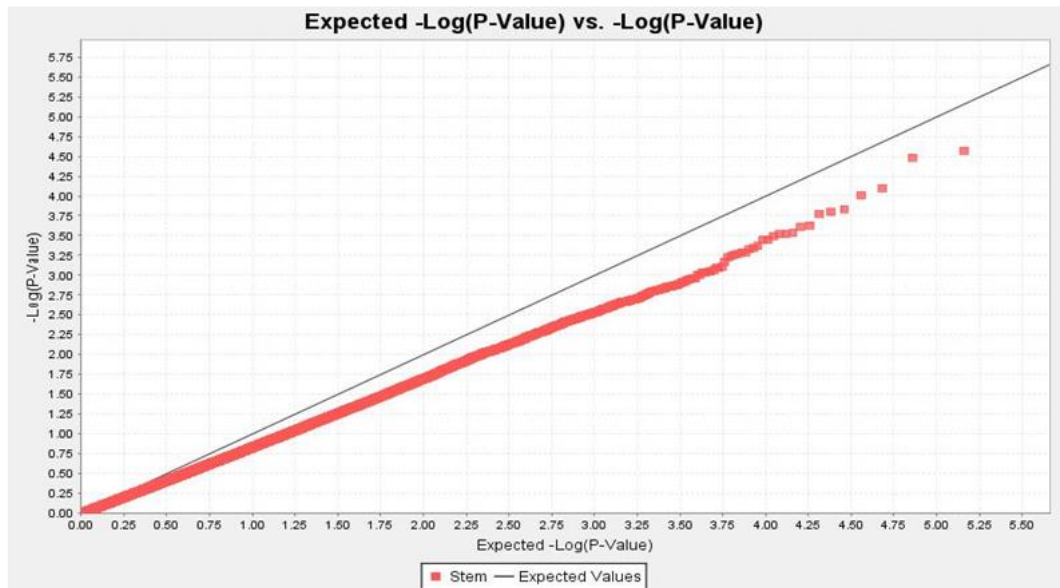Appendix 6. QQ-plots of A: Mixed Linear Model and B: Generalised Linear Model, for grazing CSFB feeding data.

Appendix 6. QQ-plots of A: Mixed Linear Model and B: Generalised Linear Model, for stem CSFB feeding data.