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Investigating Barley Yellow Dwarf Virus epidemiology and insecticide resistance in the English grain aphid *Sitobion avenae* in post-neonicotinoid Ireland

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19208844

A PhD thesis submitted to University College Dublin in fulfilment of the requirements for the degree of Doctor of Philosophy (PhD)

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Abbreviations

AAP	Acquisition access period
ANOVA	Analysis of variance
BYDV	Barley yellow dwarf virus
cDNA	Complementary deoxyribonucleic acid
CYDV	Cereal yellow dwarf virus
DAFM	Department of Agriculture, Food and Marine Ireland
DSS	Decision support system
DST	Decision support tool
ELISA	Enzyme-linked immunosorbent assay
IAP	Inoculation access period
IPM	Integrated pest management
kdr	Knock-down resistance
LC50	Lethal concentration 50
SD	Standard deviation
VGSC	Voltage-gated sodium channel
YDV	Yellow dwarf virus
λ	Lambda (pyrethroid compound)

Abstract

The English grain aphid *Sitobion avenae* is a major agricultural pest in Europe. It damages crops directly by feeding on the grain and indirectly by transmitting different species of barley yellow dwarf viruses (e.g. BYDV-PAS and BYDV-MAV), resulting in yield losses of up to 80%. In recent years, aphid control in cereals has become more challenging due the emergence of resistance to pyrethroids in a single clonal lineage of *S. avenae* (SA3), caused by a knock-down mutation (*kdr*) in the voltage-gated sodium channel gene. Additionally, the 2018 EU-ban on neonicotinoid seed treatments for outdoor use leaves pyrethroid as the remaining chemical control option. Therefore, it has been hypothesised that BYDV pressure in Irish winter barley will rise, due to the favourable selection of insecticide resistant *S. avenae* after insecticide spray applications.

Given their enormous potential agricultural impact, this research thesis first investigates whether the incidence of insecticide resistant aphids in Ireland has risen, following the neonicotinoid ban in 2018. For this, *S. avenae* collected from a three-year study in over 150 winter barley fields were analysed for the presence of *kdr* and BYDV. The results indicated that both resistance and BYDV levels highly vary between sampling years, and that there was no evidence for linking an increased incidence of resistance in *S. avenae* to an overuse of pyrethroids. Additionally, the application of an insecticide was associated with an overall reduction in aphid numbers (including other cereal aphids) in the field. There was no evidence of the emergence of homozygous resistant clones throughout this study, and only heterozygous resistance was detected. However, caution is still required, to prevent the emergence of new resistances in the future. Continued aphid monitoring will be a cornerstone of ensuring that changes in insecticide resistance levels or mechanisms are captured early.

From 2019 onwards, a 12.2 m suction tower network, which continuously samples migrating aphids throughout the year, was installed in the Republic of Ireland. The aim of this network was to systematically sample and monitor key crop pests and virus vectors, particularly those that vector BYDV. Chapter 3 of this thesis analysed *S. avenae* migration patterns and investigated the influence of weather conditions. Additionally, it aimed to assess whether resistance and BYDV levels in migrating

S. avenae increased following the neonicotinoid seed treatment ban in 2018. The results showed that, although *S. avenae* migration correlated with weather factors such as temperature and wind, there was a high variation in aphid numbers, resistance and BYDV levels depending on the sampling year and location of the suction tower. Even though resistance levels were slightly higher in the suction tower in comparison to samples collected in the crop, there was no evidence that migrating *S. avenae* collected from the suction tower network showed increasing resistance levels throughout the three years of sampling.

In Chapter 4 of this thesis, the ability of the insecticide resistant *S. avenae* clone to transmit BYDV efficiently onto winter barley plants was investigated, following anecdotal evidence that resistant aphids could be responsible for local BYDV outbreaks, making it a "super-spreader". For this, the acquisition and inoculation access periods, required for efficient transmission were investigated using two BYDV species found in Irish barley (BYDV-PAS and BYDV-MAV), and resistant and susceptible *S. avenae* clones, as well as another cereal aphid (*Rhopalosiphum padi*). The results showed that the overall BYDV-PAS transmission rates were very low for all *S .avenae* clones. Also, there was no significant difference in BYDV-MAV transmission rates between the resistant *S. avenae* clone and susceptible clones. Furthermore, preliminary evidence indicated reduced virus transmission efficiency onto BYDV-tolerant winter barley plants, which could have an impact on BYDV management in the future.

The last research chapter (Chapter 5) investigates the ability of BYDV to manipulate the behaviour of its aphid vector, to enhance its own spread (virus-vector manipulation theory). Fertility and behavioural experiments were conducted with BYDV-MAV and BYDV-PAS carried by *S. avenae* and *R. padi*, respectively. The results showed that virus-carrying aphids, which were placed in a new, stimuli-free environment, showed a significantly enhanced dispersion, velocity and initial movement time, in comparison to virus-free aphids. Additionally, behavioural manipulation in BYDV-PAS carrying aphids was significantly stronger than in BYDV-MAV carrying aphids, which may be a compensation mechanism by the poorly transmitted BYDV-PAS to enhance its own spread.

In conclusion, aphid monitoring on a local and landscape level, BYDV transmission experiments, and vector manipulation experiments showed that the importance of the insecticide resistant *S. avenae* for BYDV spread in Ireland is no greater than the spread by susceptible *S. avenae*. Furthermore, the results highlighted the complexity of the tripartite interactions between aphids, viruses, and plants, which all impact BYDV epidemiology and should be taken into consideration when developing next generation of decision support tools for Ireland and abroad.

Statement of Original Authorship

I hereby certify that the submitted work is my own work, was completed while registered as a candidate for the degree stated on the title page, and I have not obtained a degree elsewhere on the basis of the research presented in this submitted work.

Signed: ____

Date: ____________21/03/2024______________________________

List of Publications

Byrne, S., **Schughart, M.**, Ballandras, V., Carolan, J.C., Sheppard, L., and McNamara, L. 2024. A first survey using high-throughput sequencing of cereal and barley yellow dwarf viruses in Irish spring and winter barley crops (*in press; Irish Journal of Agricultural and Food Research*).

Author contribution: Design, management and collection of samples from field sites, ELISA testing, RNA extractions, writing and corrections.

Impact: Identifying BYDV species through high-throughput sequencing played a major contribution to this thesis, as previous knowledge of BYDV species in Ireland was based on serological methods which were limited in their range and accuracy. The above publication played a crucial role throughout all chapters, especially Chapter 4, as it set the base of knowledge on BYDV species present in Ireland, underpinning the importance and relevance of the research carried out in this thesis.

Byrne, S., **Schughart, M.**, Carolan, J.C., Gaffney, M., Thorpe, P., Malloch, G., Wilkinson, T. and McNamara, L., 2022. Genome sequence of the English grain aphid, *Sitobion avenae* and its endosymbiont *Buchnera aphidicola*. G3, 12(3), p.jkab418.

Author contribution: Sample collection, resistance testing, DNA and RNA extractions (including protocol optimisation), writing and corrections.

Impact: The full genome sequencing of the insecticide resistant *S. avenae* was a major milestone, which set the base for follow-up transcriptomic work (mentioned in Chapter 2.2.1.2). In these experiments, insecticide resistant and susceptible *S. avenae* were exposed to insecticides at different doses and timings, in order to identify potential molecular mechanisms of insecticide resistance, similar to those described in Bass and Nauen (2023). By the date of submission of this thesis, all exposure experiments, RNA extractions and RNA sequencing steps were finished, with data analysis still needed to be carried out. Thus, in this thesis, the publication is not fully covered.

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I dedicate this thesis to my uncle Dieter Schughart-Scheyer, a great lover of nature, who has tragically left us too early.

1

General Introduction

1.1 Introduction

In 2022, the human population on earth reached 8 billion people and is expected to continue to grow to 9.7 billion people in 2050, and 10.4 billion people by 2100 (UN, 2022). With an increasing population, there is also an increasing demand for food production, and feeding everyone in need has become the second most important goal (after the end of poverty) for the sustainable development of humanity in the future (Sachs et al., 2022). The largest agricultural crop type that contributes to food production worldwide are cereals (including wheat, barley, maize and rice), which grow on approximately half of the global cropland available and provide around 63% of calories and 56% of protein consumed by humans (Hunter et al., 2017).

To deal with food and market demand in 2050, it is estimated that the global demand for cereals will need to increase by 25-70% of the current production (Hunter et al., 2017). In the last century, the so-called "Green Revolution" lead to increased crop yields, due to an intensification and optimisation in land-use, modern plant breeding programs, as well as the development of fertilisers, fungicides, herbicides and pesticides (Evenson & Gollin, 2003). In developing countries for example, the average wheat yield increased by over 200% between 1960 and 2000 (Pingali, 2012). Within in the last two decades the term "Gene Revolution" has become popular, as biotechnological advances (e.g. by gene editing to create genetically modified plants) have become a rapidly growing field of research, to further increase crop yields (Hamdan et al., 2022).

Despite technological advantages that have the potential to increase overall yields, modern agriculture faces significant future challenges, including climate change. Increased temperatures are estimated to have a negative impact on yields in countries around the equator, but could also have a positive impact in countries with a temperate climate, as growing periods will be extended (Jägermeyr et al., 2021; OECD et al., 2023). However, warmer temperatures and shorter winters are directly linked to an increased metabolic rate, population growth and higher overwintering survival rates of insects, including agricultural pest species, which can cause yield losses of up to 80% throughout all types of crops worldwide (Oerke, 2006). In cereals, which are predominantly grown in temperate climates, insect pests are considered to cause yield losses between 5% to 20%; however, yield losses are

expected to further increase by 10% to 25% for each additional degree Celsius global temperature in the future (Deutsch et al., 2018).

Insect crop pests like aphids can also cause indirect damage to crops, by transmitting plant diseases such as Barley Yellow Dwarf Viruses (BYDV) (see Chapter 1.2) (Poehling et al., 2017; Riedell et al., 2003). For example, it has been shown that high percentages of BYDV infected plants in a field can lead to significant reduction in yields, though there was no correlation found between a high number of aphids and high BYDV percentages (Kennedy & Connery, 2001). This underpins that a low number of aphids can already cause significant damages in crops, if they carry the virus (Kennedy & Connery, 2005). Since the "Green Revolution" and the development of chemical control options, applying insecticides like pyrethroids to the field can achieve control of insect pests, which has significantly helped to prevent yield losses in the recent decades (Aktar et al., 2009; Liu et al., 2015; Tudi et al., 2021). However, overuse of the same active ingredient can lead to strong selection pressure and has already caused insecticide resistance in many insect crop pests such as aphids, fruit flies, blow flies and beetles (Bass & Nauen, 2023; Chen et al., 2023; Dong et al., 2014; Ffrench-Constant, 2013; Venkatesan et al., 2022).

1.1.1 Barley production and disease pressure in Ireland

In Ireland, the tillage sector is an important agricultural sector, contributing over €1.3bn per year to Irish economic output, employing over 11,000 full-time jobs on 9400 farms across the country (Wallace, 2020). In 2022, cereals grew on 285,700 ha of Irish farmland, with the predominant crop being barley (190,300 ha), followed by wheat (67,200 ha) and oats (28,200 ha), contributing to the malting barley, milling wheat, and oats industries, as well as to animal feed and seed production (CSO, 2022). The area of tillage crops in Ireland has declined by 42% within the last 40 years and declined by 15% between 2008 and 2018, but the overall cereal yield remains stable at around 2.3 m tonnes, which is thought to be due to agronomic improvements (Wallace, 2020). Although this output only contributes to 1% of the total crop production in Europe, Irish cereals have one of the highest yields

in the world (e.g. Irish winter barley yields in between 7.2 – 10.7 t/ha) (Schils et al., 2018).

Only 15% of the barley in Ireland is grown for human consumption (brewing/distilling), with the majority being produced for livestock feed. Barley produced for human consumption can be either used as malting barley or roasting barley, but there are significant price differences, at 23 €/t for malting barley and only 10 €/t for roasting barley, depending on the quality of the grain (Wallace, 2020). Many agronomic, environmental, and biological factors can influence grain quality and cause yield differences. First, agronomic (agriculture + economic) factors describe methods of managing crops from pre-planting until harvest, including the selection of barley varieties, planting date, sowing mechanics, irrigation, nutrient supply and disease control during growth (Hennessey, 2011). Second, environmental factors, primarily climate and weather fluctuations in temperature and rain, as well as photoperiod and soil dynamics, can influence grain quality and yield. Lastly, biological factors like diseases and pests, can negatively affect both yields and grain quality too.

In barley, over 5 bacterial (primarily blight), 36 fungal (e.g. septoria, rust and rot), and at least 26 viral (e.g. yellow dwarf viruses or mosaic viruses) diseases have been described (Dean et al., 2012; Fernando et al., 2021; Mathre & Mathre, 1997; Peters et al., 2022). While bacterial and fungal diseases are primarily transmitted through the air or soil, most viral diseases are dependent on insect vectors like aphids, thrips, beetles, leafhoppers, or whiteflies (Gray & Banerjee, 1999). In barley, yellow dwarf viruses (YDVs), which are transmitted by cereal aphids including the English grain aphid (*Sitobion avenae* F.), the bird cherry-oat aphid (*Rhopalosiphum padi* L.), and the rose-grain aphid (*Metopolophium dirhodum* W.), can cause total yield losses of up to 80% (Poehling et al., 2017; Riedell et al., 2003). Typical symptoms of YDV infection in barley include yellowing of leaves, delayed maturity, chlorosis, stunted growth, reduced root mass, and reduced grain size, which leads to a loss of grain quality and yield (Herbert Jr et al., 1999; Leybourne, 2024). BYDV infections also lead to reduced grain weight and grain plumpness but increase wort protein, diastatic power, and total protein count, all of which negatively affect grain quality (Edwards et al., 2001).

1.2 Barley yellow dwarf viruses cause yield losses

1.2.1 Taxonomic classification of yellow dwarf viruses

Yellow dwarf viruses (YDVs) are split into two genera: barley yellow dwarf viruses (BYDV) and cereal yellow dwarf viruses (CYDV) (Miller & Lozier, 2022). There are seven species of single-stranded RNA+ BYDVs identified/recognised (BYDV-PAV, BYDV-MAV, BYDV-PAS, BYDV-GAV, BYDV-SGV, BYDV-kerII, and BYDV-kerIII), all located within the genus *Luteovirus* in the taxonomic family Tombusviridae (Miller & Lozier, 2022). Five species of CYDVs have been identified (CYDV-RPV, CYDV-RPS, MYDV-RMV, MaYMV, BVG, and WYDV-GPV) in the genus Poleroviruses in the taxonomic family Solemoviridae. Both Luteoviruses and Poleoviruses were considered to belong to the same family of Luteoviridae, but recent taxonomic analyses by the International Committee on Taxonomic Issues, based on the gene encoding RNA-dependent RNA polymerase, led to a reclassification between both families (Miller & Lozier, 2022). With over 100 Poaceae species, including perennial grasses and crops like barley (*Hordeum vulgare* L.) and wheat (*Triticum aestivum* L.), BYDVs and CYDV are highly versatile in their host range (Irwin & Thresh, 1990). However, host range and severity of symptoms, and therefore potential yield losses, vary greatly between YDV species (Baltenberger et al., 1987; Griesbach et al., 1990).

Throughout all crops and grasses worldwide, BYDV-PAV is considered the most prevalent and damaging YDV species, whereas BYDV-PAS and its main vector *R. padi* are considered to be the most widespread in winter barley (Aradottir & Crespo-Herrera, 2021; Choudhury et al., 2017). In Ireland though, previous studies found that the most prevalent YDV species in winter and spring barley is BYDV-MAV (named BYDV F-type), followed by only a few findings of BYDV-PAV and CYDV-RPV (named BYDV B-type) (Kennedy & Connery, 2001, 2005). Through recent sequencing of winter barley, BYDV-MAV, BYDV-PAS, and BYDV-PAV were found to be the predominant BYDV species in Ireland, whereas CYDVs were uncommon; thus CYDVs are not considered relevant for Irish YDV epidemiology in this thesis (Appendix Table A2, Byrne et al. (2024)).

1.2.2 Barley yellow dwarf viruses

All BYDVs in the Luteoviridae family are icosahedral (T = 3) virus particles with diameters ranging from 25 to 30 nm (Fig. 1.1). They consist of two ~22 kDa and ~52 kDa coat proteins, which encapsulate the single-stranded +RNA virus genome ranging from 5.6 kb to 6.0 kb in size (King et al., 2011). The first genome sequence of a BYDV was published in 1988, revealing that BYDVs consist of six open reading frames that encode genes for viral replication and the production of coat, movement, and cell-regulatory proteins (Miller et al., 1988). Upon entering the plant, BYDVs first replicate in neighbouring phloem cells (e.g. phloem parenchyma cells, companion cells, and sieve tubes), before being released and traveling the whole plant through the vascular tubes (Choudhury et al., 2017; Irwin & Thresh, 1990). The first symptoms of BYDV infection can occur four to twelve days post-inoculation, as the massively replicated virions start to interfere with biochemical and physiological processes in the plant (Jensen & D’Arcy, 1995).

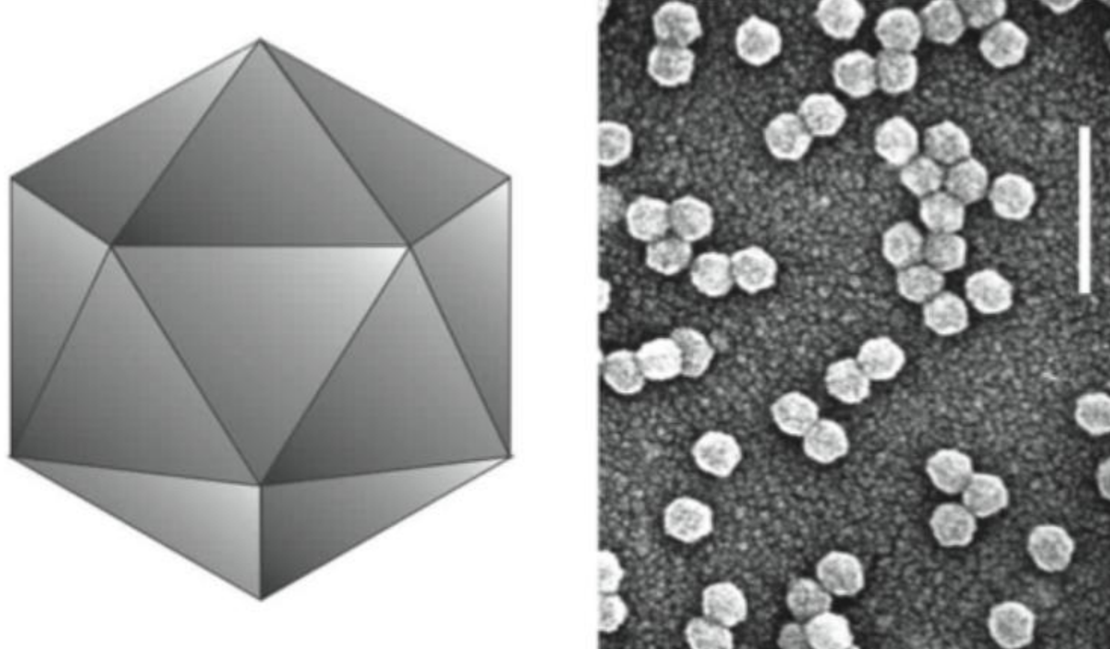

Figure 1.1: Diagram of the proposed structure of Luteovirus particles (left); negative contrast electron micrograph of particles of barley yellow dwarf virus-PAV (BYDV-PAV) (right). Bars represent 100 nm. Images taken from International Committee on Taxonomy of Viruses (ICTV); https://ictv.global/report_9th/RNApos/Luteoviridae.

The main symptoms associated with BYDV infection are dwarfism and colour changes of leaf blades, especially leaf tips, which vary in intensity depending on the growth stage of the plant at inoculation, interactions between the plant and the virus,

and abiotic stressors (Choudhury et al., 2017; Mc Namara et al., 2020). Typical visible BYDV symptoms in barley are leaves turning bright yellow starting at the leaf tip, whereas oat leaves turn orange or red, and wheat turns yellow or red. BYDV infection has also been shown to reduce root length, distance from the seminal root tip to the nearest lateral root, and root to shoot ratio (reviewed in Choudhury et al. (2017)).

1.2.3 Aphids as crop pests

Aphids (Aphididae), the most common vectors of BYDV, are known for their high plasticity in life-history traits, which can change depending on their environment (Dai et al., 2016). This phenotypic plasticity enables them to be one of the major pest species of cereals and other Poaceae, where they feed on phloem sap, preferably from the upper leaves or the ears of grains as soon as they emerge (Poehling et al., 2017; Wratten, 1978). Feeding can cause direct and indirect damage to the plant, which results in an overall loss of total yield. Direct damage is caused by the feeding process, which reduces phloem flow and therefore nutrient transport. In addition, plant stress reactions are caused by enzymes in the aphid saliva (Zhang et al., 2017). Aphids also excrete sugar-rich honeydew, which blocks the stomata on the leaves and favours the growth of sooty mould fungi, leading to a reduced photosynthetic capacity (Chomnunti et al., 2014; Rossing, 1991). Plant viruses cause indirect damage to grains, with BYDV being one of the most severe threats to yield reduction and damages in cereals (Herbert Jr et al., 1999). The virus, which is predominantly vectored by the three cereal aphids *S. avenae, R. padi,* and *M. dirhodum,* negatively influences the growth of roots and shoots, and limits photosynthesis leading to reduced grain size and decreased grain weight (Fabre et al., 2005; Poehling et al., 2017; Riedell et al., 2003).

1.2.3.1 Plant selection and feeding mechanism

Aphids choose their hosts and feeding spots based on visual and olfactory/chemical queues. A few examples are specific landing responses based on the colour of the host leaf, acceptance or rejection of specific plants based on plant volatiles, and identification of favourable feeding sites without other aphid competition or

parasitoids (Döring & Chittka, 2007; Ninkovic et al., 2013; Nottingham et al., 1991; Pettersson et al., 1995). After successful host selection, the aphid starts to feed in multiple phases, using their highly adapted stylet (Fig. 1.2). The flexible stylet is formed by a bundle of two mandibular and two maxillary stylets, and has two canals, one for food uptake and one for salivation, which fuse at the tip of the stylet (Pollard, 1973).

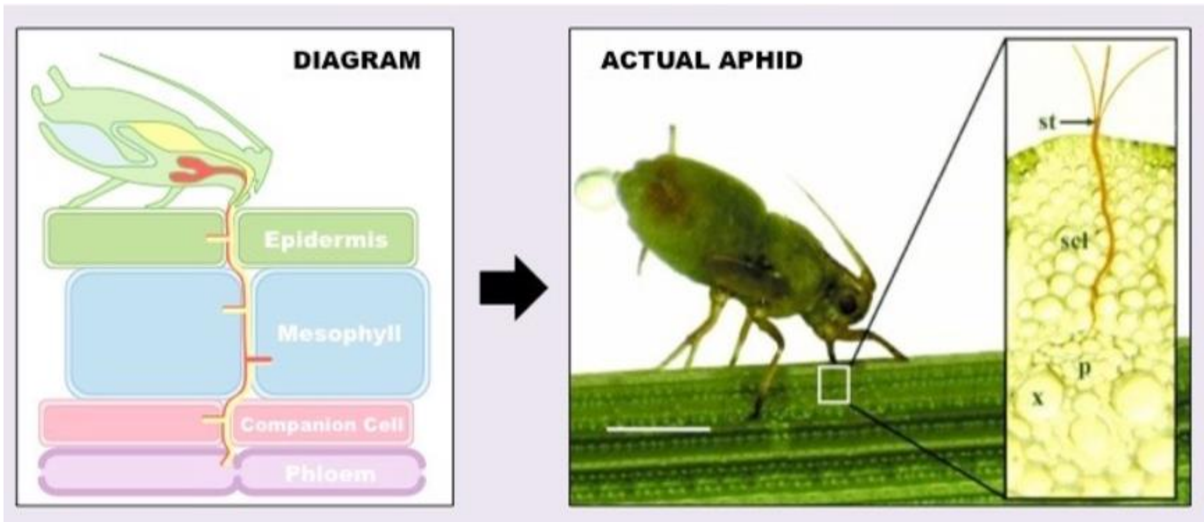

Figure 1.2: Diagram (left) and photograph (right) of an aphid feeding on a leaf (Image from D. Fischer, Reproduced from Plants in Action, http://plantsinaction.science.uq.edu.au, published by the Australian Society of Plant Scientists. modified by https://old-ib.bioninja.com.au/_Media/aphid-stylet_med.jpeg)

The feeding process starts with the "pathway" sap-probing phase, during which the stylet reaches intercellular towards the sieve elements (phloem) of the host plant, while taking intracellular sap samples on the way (Powell et al., 2006; Tjallingii & Esch, 1993). During the entire feeding process, the sap is analysed by the gustatory organ and taste cells for structural and chemical information of the host plant, which leads to either acceptance or rejection of the host plant (Wensler & Filshie, 1969). Simultaneously, different types of saliva are injected, to support and protect the stylet by lubrication or by forming a sheath, to overcome host defence mechanisms with effector proteins, or to enhance water flow (Bos et al., 2010; Miles, 1999; Pettersson et al., 2017; Zhang et al., 2017). The phloem-feeding phase starts with the successful acceptance of the host and can last up to multiple hours of nutrient-rich phloem feeding (Prado & Tjallingii, 1994). Aphids can also feed on nutritionally poor xylem sap, but this is thought to only occur occasionally for water uptake to compensate for dehydration (Kuhlmann et al., 2013; Spiller et al., 1990).

1.2.3.2 BYDV transmission by aphids

To spread from plant to plant, BYDVs and other plant viruses are dependent on insect vectors, such as aphids (Gray & Banerjee, 1999; Xavier & Whitfield, 2023).

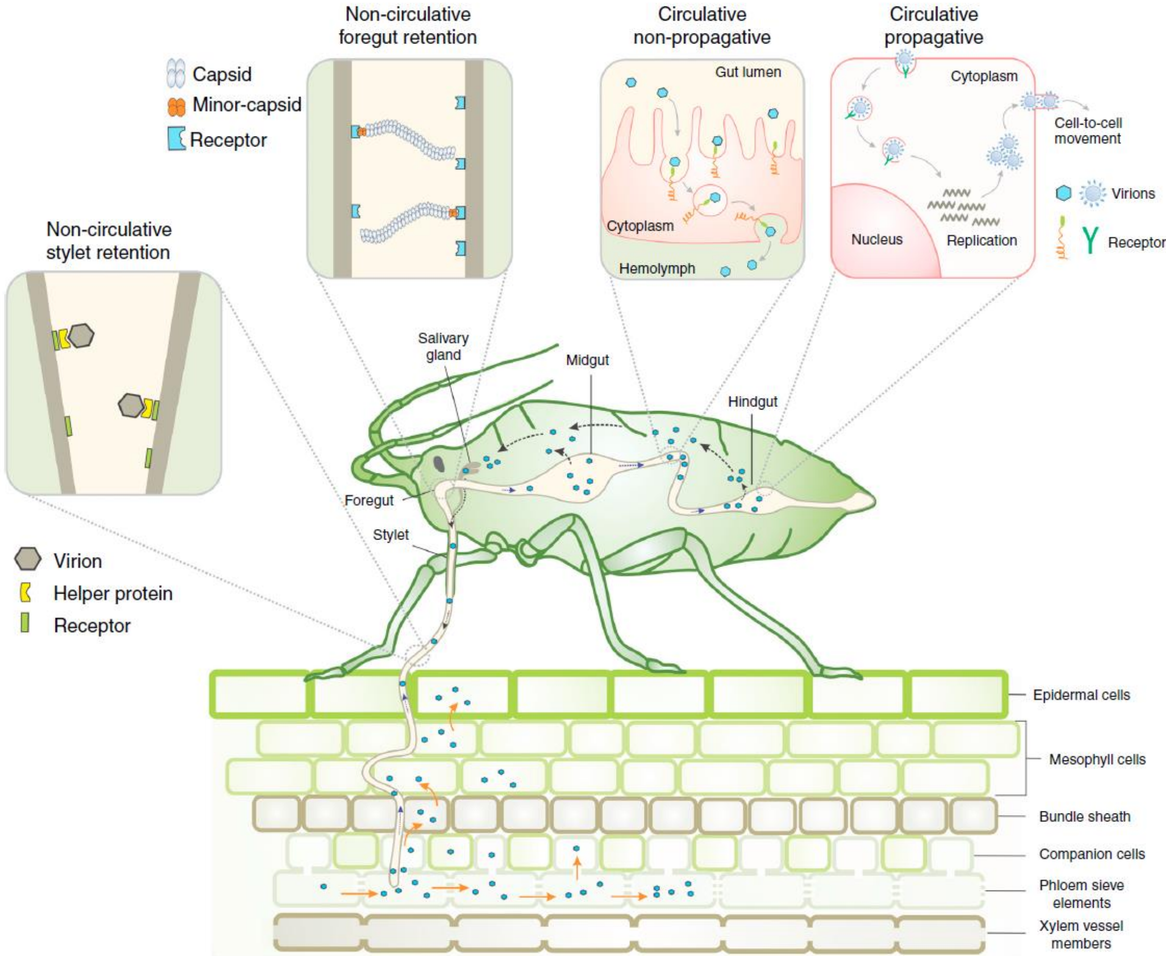

Figure 1.3: Plant virus transmission by aphids. Plant viruses are either transmitted through a circulative (plant viruses need to travel through the aphid) or a non-circulative manner and can be either propagative (replication inside the vector) or non-propagative. BYDVs are circulative and non-propagative plant viruses. Image taken from Xavier and Whitfield (2023).

The duration of plant access plays an important role in plant virus transmission, as viruses can be obtained in any plant tissue and during any of the feeding phases (Fig. 1.3). Although, depending on the plant virus and aphid species, modes of transmission vary and are split into three categories: non-persistent (virus acquired within seconds and retained in the aphid for a few minutes), semi-persistent (virus acquired within minutes to hours and retained for several hours), and persistent

(virus acquired within minutes to hours and retained for a very long period, sometimes up to the aphids' death) (Blanc, 2008; Brault et al., 2010). Furthermore, the non-persistent and semi-persistent plant viruses are classified as non-circulative, as they only reside on the aphids' stylet and foregut without entering the haemolymph. Persistent plant viruses are circulative, meaning that they have to travel through the aphids' hindgut, haemolymph and towards the salivary glands before being able to be passed back to a host plant again (Fig. 1.3) (Kennedy et al., 1962). Circulative viruses can be propagative, meaning that they reproduce inside the vector, but most plant viruses are non-propagative (Stevens & Lacomme, 2017). BYDVs and other Luteoviruses are phloem-limited and belong to the group of persistent, circulative, non-propagative plant viruses, suiting phloem-feeding aphids such as the English grain aphid *Sitobion avenae* as vectors (Brault et al., 2010).

	Resistance status	BYDV			CYDV		
Aphid Species		**MAV**	**PAV**	**PAS**	**RPV**	**RMV**	**RPS**
Sitobion avenae **(English grain aphid)**	susceptible	+++	++	?	-----	-----	
	resistant	?	?	?			
Rhopalosiphum padi **(Bird-cherry oat aphid)**	n/a	-----	+++	?	+++	+	?
Metopolophium dirhodum **(Rose-grain aphid)**	n/a	+++	++	?	-----		

Figure 1.4 Overview of insecticide resistant status and BYDV transmission efficiencies of three cereal aphids (*S. avenae*, *R. padi*, and *M. dirhodum*). +++: Very efficient BYDV transmission; ++ efficient BYDV transmission; + poor BYDV transmission; ----- no BYDV transmission; ? unknown BYDV transmission. Transmission efficiencies taken from Van den Eynde et al. (2020).

It is important to note that aphids transmit different BYDV species with varying efficiencies. For example, it has been shown that *S. avenae* is a "very efficient" transmitter of BYDV-MAV (75-100% transmission rate), an "efficient" transmitter of BYDV-PAV (25-75% transmission rate), and that it can transmit BYDV-PAS, but the transmission rates are unknown (Fig. 1.4) (Van den Eynde et al., 2020).

1.3 The English grain aphid (*Sitobion avenae*) - Biology

1.3.1 Aphid phylogeny

The English grain aphid *Sitobion avenae* (Hemiptera; Aphididae) belongs to the major group Aphididae, which consists of over 5000 phytophagous insects, of which approximately 100 species are considered economically important because of their specialisation in agricultural crops (Foottit et al., 2008; Van Emden & Harrington, 2017). Aphid variety (morphological, behavioural, and genetic) comprises of multiple traits such as sexual and asexual lifecycle, rapid reproduction rates by asexual cloning, phenotypic plasticity, co-evolution with their host plants, endosymbionts and predators, or fast adaptation and high ecological flexibility (Loxdale et al., 2017). In the northern temperate region, the subfamily *Aphidinae* is the most predominant with over 2900 different species. Ancestral *Aphidinae* fed on woody plants but aphids further evolved into either alternating between woody and herbaceous plants (about 15% of *Aphidinae*) or specialising exclusively on herbaceous plants (about 85% of *Aphidinae*), following human plant agriculture (Blackman & Eastop, 2007). *S. avenae* belongs to the latter and colonize a large variety of Poaceae*,* from cereals to pasture grasses. They are one of the most common aphid species and are distributed worldwide from Europe, Asia, Africa, the Middle East, North America and South America (Blackman & Eastop, 2000).

All aphids are soft-bodied and can vary in colours from pink to green, yellow, brown, black, or slightly translucent. Despite a great variation in appearance, they share common morphological characteristics like the siphunculi (secretory organs), the cauda ("tail" to flick honeydew droplets away from the anus), five to six segmented antennas consisting of two basal segments, and a flagellum of three to four segments (Blackman & Eastop, 2007). The high morphological variation in aphids

entailed taxonomic issues with the identification of single aphid species in the past (Coeur d'acier et al., 2014; Via, 1999). Today, aphid phylogeny and speciation based on microscopic identification are complemented by the use of molecular methods such as DNA barcoding, which has led to a renewed aphid phylogeny (Coeur d'acier et al., 2014; Foottit et al., 2008; Lee et al., 2014). However, despite the identification of most aphid species, there are still many open cases, underlining the high genetic variation in aphids. For example, the taxonomy and relationship between the grain aphids *Sitobion avenae* from Europe and *Sitobion miscanthi* from East Asia (mostly China) was thought to be understood but is still discussed today (Morales-Hojas et al., 2020).

1.3.2 Genetic and endosymbiont diversity in *Sitobion avenae*

Due to its life cycle (explained in Chapter 1.3.3.), *S. avenae* populations (and most other aphid species) consist of clonal lineages (or genotypes), that can originate from a single aphid that produces identical clones, which establish in a high abundance on a landscape level (Loxdale et al., 2017). Genotyping *S. avenae* from multiple countries using molecular markers has shown that there are hundreds of different clonal lineages in *S. avenae* (Loxdale, 2008; Simon et al., 1999; Wang et al., 2020; Zepeda-Paulo & Lavandero, 2021). It was also found that there are regional differences in the relative abundance of the same aphid genotype, depending on the reproductive mode, with anholocyclic reproduction leading to a higher proportion of the same genotype in regions where holocyclic reproduction was less favoured (see Chapter 1.3.3) (Simon et al., 1999). It is also possible, yet uncommon, that a single clonal lineage within a population can become predominantly abundant in the aphid population (also referred to as a superclone), if the environmental conditions are favourable (Loxdale et al., 2017). For example, it has been shown that the partially insecticide resistant *S. avenae* clonal lineage SA3, has become predominant within aphid populations in the UK and in Ireland, most likely due to its ability to survive insecticide spray applications (see Chapter 1.5.2) (Walsh, Schmidt, et al., 2020).

Most aphid populations though, including *S. avenae*, can be described as a "mixture" of genetically and phenotypically distinct aphid clones that have

established over months or years (Dedryver et al., 2005). Typically, each clone shows differences in life-history traits such as fecundity, development time or plant specialisation (Dai et al., 2016), but it was also found that genotypic differences can lead to differences in the transmission efficiencies of plant viruses such as BYDV (see Chapter 1.2.3.2). For example Dedryver et al. (2005) showed that BYDV-PAV transmission efficiencies highly vary (3% to 92%) between different *S. avenae* clones, while in another study, the BYDV-PAV transmission rate across *S. avenae* clones was found to be low. In contrast BYDV-PAV transmission rates in *R. padi* was highly variable (Leybourne et al., 2024). Three potential biological mechanisms have been proposed that could cause these differences in virus transmission between aphid clones. Besides genetic differences between the aphid clones causing the presence or absence of virion-interacting proteins (by "vectoring alleles") in the hindgut or salivary glands, there are two other potential mechanisms involving either the obligate aphid endosymbiont *Buchnera aphidicola* or other facultative endosymbionts (Leybourne, 2024).

Buchnera aphidicola is an obligate (essential) bacterial endosymbiont which is present in all aphid species and has been transmitted maternally for over 100 million years (Chong et al., 2019). *B. aphidicola* provides the aphid with supplementary nutrients and amino acids, which are not present in the phloem sap (Douglas & Prosser, 1992; Moran, 2021), and it has also been shown that it can induce heat tolerance to the aphid (Dunbar et al., 2007). More recently, it has been shown that *B. aphidicola* might also be involved in BYDV transmission, as higher efficiencies of virus transmission were linked to higher symbiont titres and therefore higher presence of symbiont-derived proteins such as chaperonins, which facilitate virus acquisition (Leybourne, 2024). Similarly, the presence of different facultative (non-essential) aphid symbionts might have an impact on BYDV transmission as it has been shown that aphid feeding in the presence of the facultative endosymbiont *Hamiltonella defensa* enhances aphid feeding, which might positively impact virus acquisition (Leybourne et al., 2020). However, a recent study suggests that aphid genotype and presence of vectoring alleles are more likely to explain differences in virus transmission between clones, rather than obligate or facultative endosymbionts (Leybourne et al., 2024).

1.3.3 *Sitobion avenae* life cycle

S. avenae are monoecious, meaning they live, feed, and reproduce exclusively on herbaceous Poaceae hosts all year long, including crops like barley, wheat, and oats (Blackman, 2010). Different lineages of the non-host-alternating *S. avenae* show a variation of reproduction strategies throughout the year (Fig. 1.5). Genetically identical, but different phenotypic morphs specialise in either reproduction (sexually/asexually), migration and dispersal, or survival during unfavourable conditions (polyphenism). These polyphonic traits play an important role in the life cycle of *S. avenae* and are key to the success of rapid spreading. During spring and summer, parthenogenetic, wingless clones are produced when the population density is low and the food quality is high (Hardie, 2017; Müller et al., 2001). When the population overcrowds or the nutritional quality of the feeding plant decreases, pheromone signals trigger the production of winged parthenogenetic females, which then migrate to new food sources (Watt & Dixon, 1981). In autumn, there are three additional reproductive strategies in *S. avenae* in response to lower temperatures and shorter day lengths (Dedryver et al., 1998). Lineages can then either continuously produce parthenogenetic females (anholocyclic life cycle), continuously produce males and parthenogenetic females (androcyclic), produce mating males and females that lay eggs for overwintering (holocyclic), or produce offspring in all of the above ways (intermediate).

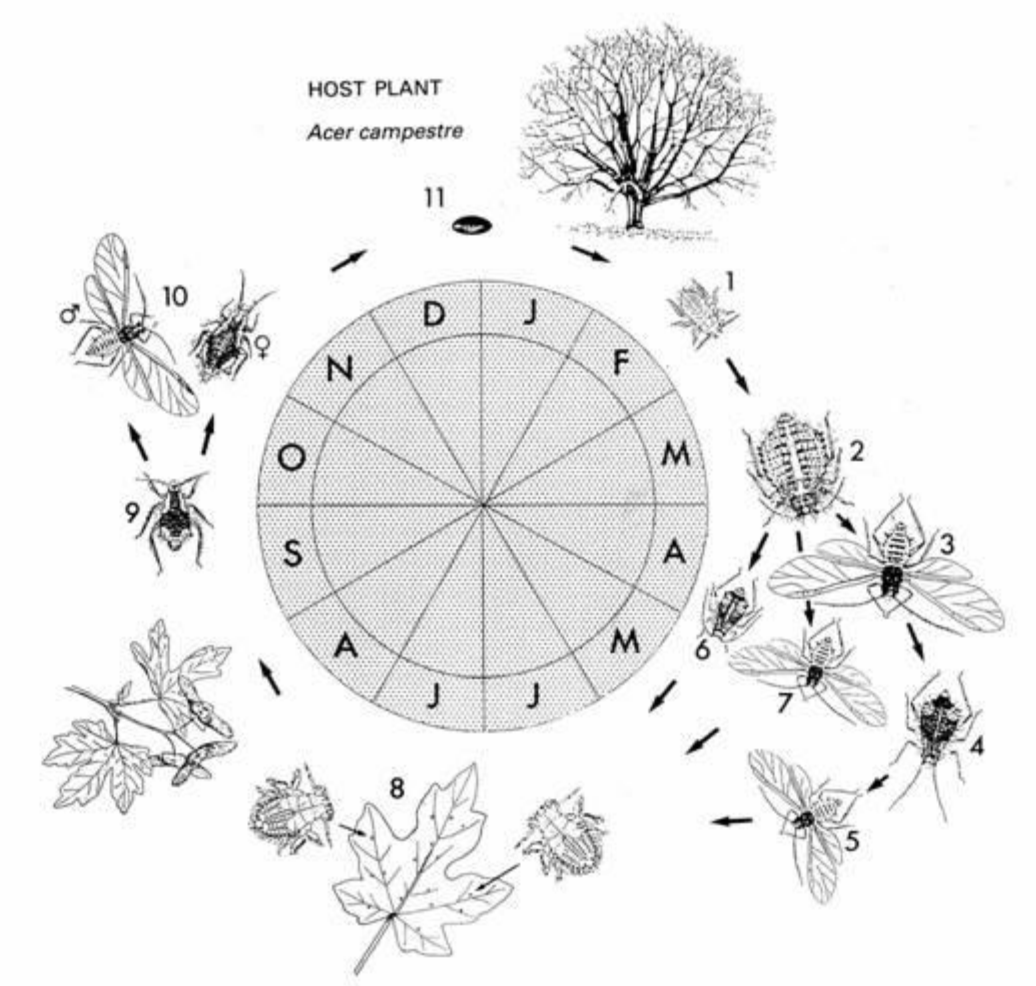

Figure 1.5: Example of an aphid life cycle (*Periphyllus testudinaceus*). Image taken from Utah Blackman and Eastop (2000)

The life-cycle variation is linked with the local climate, as studies from the UK and France have shown a predominance of anholocyclic *S. avenae* in warmer regions and holocyclic *S. avenae* in colder regions (Dedryver et al., 2001; Llewellyn et al., 2003; Simon et al., 1999). This may have a significant ecological impact in the future, as winters in Ireland are predicted to be warmer due to climate change (García et al., 2020). If anholocyclic reproduction is favoured in warmer climates, parthenogenetic cloning is also favoured year-long, which might lead to high numbers of aphids before natural predators are present in the field.

1.3.3.1 The adult grain aphid

The adult unwinged (aptera) English grain aphid (*Sitobion avenae*) reaches between 1.3-3.3 mm, while the winged (alate) adult reaches between 1.6-2.9 mm (Fig. 1.6)

(Blackman & Eastop, 2008). The colour of *S. avenae* varies between light green, green, red, and brown, and depends on genetic or environmental factors, which cause differently pigmented tissues or different sclerotization of the cuticle (Hardie, 2017). While little is known about the genetic mechanisms causing this polyphenism, it has been found that different-coloured *S. avenae* are produced under a changing photoperiod. For example, during short days, red *S. avenae* produce more green offspring, whereas under reverse conditions, more red offspring are produced (Markkula & Rautapäa, 1967). However, under strong UV radiation, brown aphids were found to be better protected against damaging rays than green aphids (Thieme, 1998). All adult *S. avenae* can be identified by their long, black siphunculi, which are approximately a quarter longer than the cauda. Their antennae are six-segmented and uniformly black or dark brown, and their legs show tiger-like black and green patterns (Blackman & Eastop, 2008). The mean survival time of an adult *S. avenae* is 20 days, and within that period, it produces an average of eight nymphs per day, once it has reached the adult life stage (Watt, 1979).

Figure 1.6: A winged adult English grain aphid (*Sitobion avenae*) feeding on a Winter Barley plant. Photo taken on a Leica M125C stereoscope at 20 x magnification (Leica Microsystems GmbH, Wetzlar, Germany).

1.3.3.2 Nymphs

Due to parthenogenetic clonal reproduction, *S. avenae* nymphs are born alive rather than hatching from eggs (Fig. 1.7). They develop immediately after budding off the oocyte from the germarium and are then born alive as first-instar nymphs (Braendle et al., 2006). The nymphs carry embryo-nymphs during development inside the mother aphid. This guarantees high reproduction rates, as reproduction can already start seven to ten days after birth (Dixon, 2012). Aphids are hemimetabolous insects, and therefore do not undergo the pupal stage. To reach adulthood, nymphs must undergo four instars, in which they mould their outer cuticle in a process called ecdysis (Newton & Dixon, 1990). The exuviae are dropped near or within the colony, and it has been discovered that the decoy cuticle distracts parasitic wasps as they spend more time searching for a living aphid (Muratori et al., 2008). In comparison to the adult *S. avenae*, the first instar nymph has a smaller size, smaller antenna with one or two less segments, no cauda, and a comparatively longer rostum (Blackman & Eastop, 2000). For each nymphal stage, the nymphs progressively grow into adults; however, the growth rate slowly decelerates because of a decline in nutrient uptake in comparison to body weight (Newton & Dixon, 1990).

Figure 1.7: A grain aphid nymph (*S. avenae*) clinging to its mother. Photo taken on a Leica M125C stereoscope at 20x magnification (Leica Microsystems GmbH, Wetzlar, Germany).

1.3.3.3 Eggs and fundatrices

Throughout autumn, holocyclic reproduction may occur where, after mating with a male, ovioparous females lay fertilized eggs, which can survive in low temperatures over winter (Muller, 1977). After oviposition on the host plant, the 0.5-0.8 mm sized eggs undergo a 2 to 4 day long colour change, starting from golden yellow to green to a shiny black (Blackman, 1987). In colder climates, egg production seems to be the best survival strategy for aphids because overwintering eggs are capable of surviving lower temperatures than living individuals (Simon et al., 2002). However, even though approximately 90% of *S. avenae* clones produce sexual morphs in autumn, eggs are rarely found, suggesting that a more successful strategy for overwintering is parthenogenetic reproduction (Blackman, 2010; Dean, 1974b). In spring, highly fecund fundatrices (first-generation females) hatch from the eggs. Fundatrices produce more ovarioles and embryos than females of later generations, are round in shape, and have shorter antennae, legs, and siphunculi (Moran, 1992). The offspring of a fundatrix are less fecund fundatrigeniae (wingless females), which reproduce parthonegenetic again during spring and summer (Hardie, 2007).

1.3.4 Growth rates, development and population dynamics

1.3.4.1 Individual development

Due to the combination of viviparity (giving live birth) and parthenogenesis, female aphids can reach extreme reproduction rates in short generation times, leading to exponentially increasing populations. Under perfect growing conditions, aphids can reproduce approximately one week after birth and give birth to up to 95 nymphs per day (Watt, 1979). Individual growth rates (gain of size), development rates (time until maturity), and fecundity (number of offspring) are influenced by several extrinsic and intrinsic factors such as genotype and life-trait plasticity, temperature and season, host-plant effects, and stress (Leather & Dixon, 1984; Leather et al., 1995).

Different clonal lineages of *Sitobion* have been shown to perform differently when grown under the same conditions (Araya et al., 1996; Sunnucks et al., 1998). However, the same genotype of *S. avenae* grown under varying conditions can also show different life-history traits and developmental rates, suggesting that both genotypic and phenotypic plasticity play a role in the spatial heterogeneity of aphid

growth and development, depending on their environment (Dai et al., 2016; Dai et al., 2014).

Temperature is one of the most influential factors for aphid growth and development. It has been shown that most aphid species have a strong linear relationship with an increased growth being linked to increasing temperatures ranging from 7 °C to 25 °C, whereas a strong decline in growth rates was observed at temperatures below or above this range (Acreman & Dixon, 1989; Campbell et al., 1974; Dean, 1974a). Treatments of *S. avenae* with laboratory-simulated heat waves resulted in a significant reduction in fecundity, prolonged developmental rates, reduced nymph birth weight, and lower survival rates (Jeffs & Leather, 2014). The same experiment was conducted with aphids being exposed to cold temperatures (-15°C for 1 h) and found prolonged developmental rates, but growth rates and fecundity remained at the same level as the control group. This might be due to a protective response mechanism against freezing temperatures, where insects, including aphids, can cold-harden (by producing anti-freezing proteins) to survive (Bale, 1996). In autumn, the acclimation process for aphids to cold-harden naturally takes several weeks, but rapid cold hardening has also been discovered, allowing a fast response to temperature fluctuations with almost no ecological deficits (Jeffs & Leather, 2014; Lee Jr et al., 1987; Powell & Bale, 2004). The supercooling point (the temperature at which the aphid spontaneously freezes to death) for *S. avenae* adults is around -25.5°C and for nymphs around -26.9°C, whereas aphid eggs can survive at temperatures around -30°C for up to a month (Bale, 1991; Strathdee et al., 1995). Longer exposure to cold temperatures (starting at -5°C for 6 h) continuously increases the negative effects on growth, development, and fecundity, and the median lethal temperatures (-7°C for adults and -10°C for nymphs) are significantly higher than the supercooling points (Bale, 1991; Parish & Bale, 1993). However, the survival of short periods of cold temperatures in the field is linked to the transmission of BYDV to winter and spring cereals, driving the spread of the virus continuously (Hutchinson & Bale, 1994).

Aphid growth rates may also be influenced by their host plant, as individual performance also relies on factors such as diet quality (Grüber & Dixon, 1988), plant nutrition and growth stage (Hosseini et al., 2010; Ma & Bechinski, 2009; Watt & Dixon, 1981), and host resistance (Dahlin & Ninkovic, 2013). Depending on which

part of the host plant *S. avenae* feeds also influences their development times and fecundity, achieving highest rates on the ears of the plants, followed by young leaves, flag leaves, lower leaves and senescent leaves (Watt, 1979).

1.3.4.2 Aphid population dynamics

Aphid populations can be modelled by collecting data on aphid abundance, growth rates, development rates, and fecundity. However, it was shown to be challenging to predict aphid populations on a landscape level, as environmental factors influence population growth as well (Ciss et al., 2014; Leather & Dixon, 1984). The population growth and abundance of aphids are mainly influenced by temperature and climate, (spring) migration and population density, predation and parasitism, host plant quality, and agricultural practises (Ciss et al., 2014; Dedryver et al., 2001; Llewellyn et al., 2003).

Climate, especially temperature and precipitation, influences aphid reproduction rates, genetic diversity, and the timing of spring migration (Llewellyn et al., 2003; Simon et al., 1999; Winder et al., 2014). The Rothamsted Insect Survey network has collected migrating aphids for over 55 years and has found that climate change favours earlier migration and a longer flight season (Bell et al., 2015). Future aphid outbreaks in Ireland might be greater, as warmer winters favour survival and anholocyclic parthenogenetic reproduction, and therefore earlier exponential growth (Walters & Dewar, 1986).

For *S. avenae*, population distribution in the field is spatially patchy on single plants, and population growth follows temperature and plant growth, with an exponential rise to a peak and a crash with increasing host age (Winder et al., 2014). During the exponential rise phase, a three or four day interruption of abundancy growth ("hiccup") was observed in *S. avenae* and other aphids, which is probably caused by a host plant change of some individuals (Leigh & Van Emden, 2017; Winder et al., 2014).

Aphid host plant changes are either inadvertent (e.g. by falling off the plant) or intentional (e.g. walking/flying off the plant) (Fereres et al., 2017). The intentional host plant change occurs at low nutritional quality, high individual density, or high predation pressure, and also leads to the production of winged aphids, which

migrate to different hosts (Müller et al., 2001; Watt & Dixon, 1981). Depending on the weather conditions, flights can range from reaching a neighbouring plant to landscape migration if the aphid is picked up and transported by the wind (Fereres et al., 2017). Therefore, a single migrating aphid might start a local population on an uninfested crop, and, if the aphid was infected with BYDV, spread the virus from there. However, despite the huge potential for landscape migration in *S. avenae*, most recurring outbreaks (outbreaks in the second year after the first infestation) seem to come from local overwintering aphids rather than flying migrants (Vialatte et al., 2007).

Aphid population growth and size also relies on the influence of predators and parasitoids, as consumption and infection rates can cause fluctuations in aphid abundance. Generally, with high rates of predation and parasitism, aphid population size shrinks, but modelling these rates to predict aphid populations is challenging because predators and parasitioids are highly diverse (Welch et al., 2012). For *S. avenae,* there are over 50 polyphagous predators and at least nine primary parasitoids, all of which influence aphid population size, depending on their presence or absence in the field (Traugott et al., 2008).

1.4 Control of aphids in crops

Cereal aphids, such as *S. avenae,* have become a major focus of current agricultural research because of their enormous potential impact as crop pests (Poehling et al., 2017). When aphid populations in winter barley fields increase, the risks of BYDV infections, which can cause high yield losses, also increase. Since the middle of the last century, the development of chemical pest control using insecticides has achieved great success in preventing aphid and BYDV outbreaks (Mc Namara et al., 2020). However, many insecticides have been banned, because of their negative impact on the environment (Dainese et al., 2019). For example, in Ireland, chemical aphid management of winter barley up to 2018 was achieved by neonicotinoid seed treatments and/or foliar applications of pyrethroids. Additionally, in 2021, sulfoxaflor (Isoclast™, Corteva, Indianapolis, US) was available for aphid control in winter barley in Ireland but was rarely applied (less than 1% of winter barley fields; personal communication with farmers and advisors). However, a

recent review by the EU led to it being revoked for outdoor use because of concerns about toxicity to bees (PAN-Europe, 2022). All three insecticides have different modes of actions (Walsh, Schmidt, et al., 2020), but due to European regulations in 2018 and 2022, pyrethroids remain the only product for chemical aphid management (Blake, 2018). Repeated use of insecticides has also led to the evolution of insecticide resistances in many (primarily) agricultural aphid pest species (Foster et al., 2017). To date, at least 20 important agricultural aphid pest species, such as *Myzus persicae*, *Aphis gossyphii, Schizaphis graminum,* and most recently the English grain aphid *Sitobion avenae,* are known to possess at least one, if not multiple, mechanisms of resistance (Bass & Nauen, 2023; Bass et al., 2014; Foster et al., 2014; Furk & Hines, 1993).

1.5 Insecticide resistance in aphids

1.5.1 Insecticide resistance mechanisms

Resistance to insecticides evolves when high selection pressure is applied on a population through repeated exposure (Foster et al., 2017). In aphids, over 1000 cases of insecticide resistances to multiple insecticide compounds have been reported worldwide, with the majority of cases (964) occurring in just ten aphid species that are agricultural pests (Bass & Nauen, 2023). The detection of new resistances usually begins with reports of spray failures by growers, followed by subsequent insecticide exposure bioassays of the suspected sample population. If there are behavioural or phenotypic responses showing high levels of survival after insecticide exposure, the molecular mechanism behind the resistance can be identified (Ffrench-Constant, 2013). Two main mechanisms confer resistance to insecticides in aphids (Fig. 1.8): 1) Metabolic resistance, which is caused by an increased enzymatic activity after an insecticide exposure. This leads to either a rapid degradation of the insecticide or a decreased uptake of insecticides, due to a strengthened cuticle by an over-expression of genes that encode cuticular proteins (Puinean et al., 2010). Or 2) Mutations in the target-site gene, which lead to reduced sensitivity due to a modified insecticide binding site (Bass & Nauen, 2023).

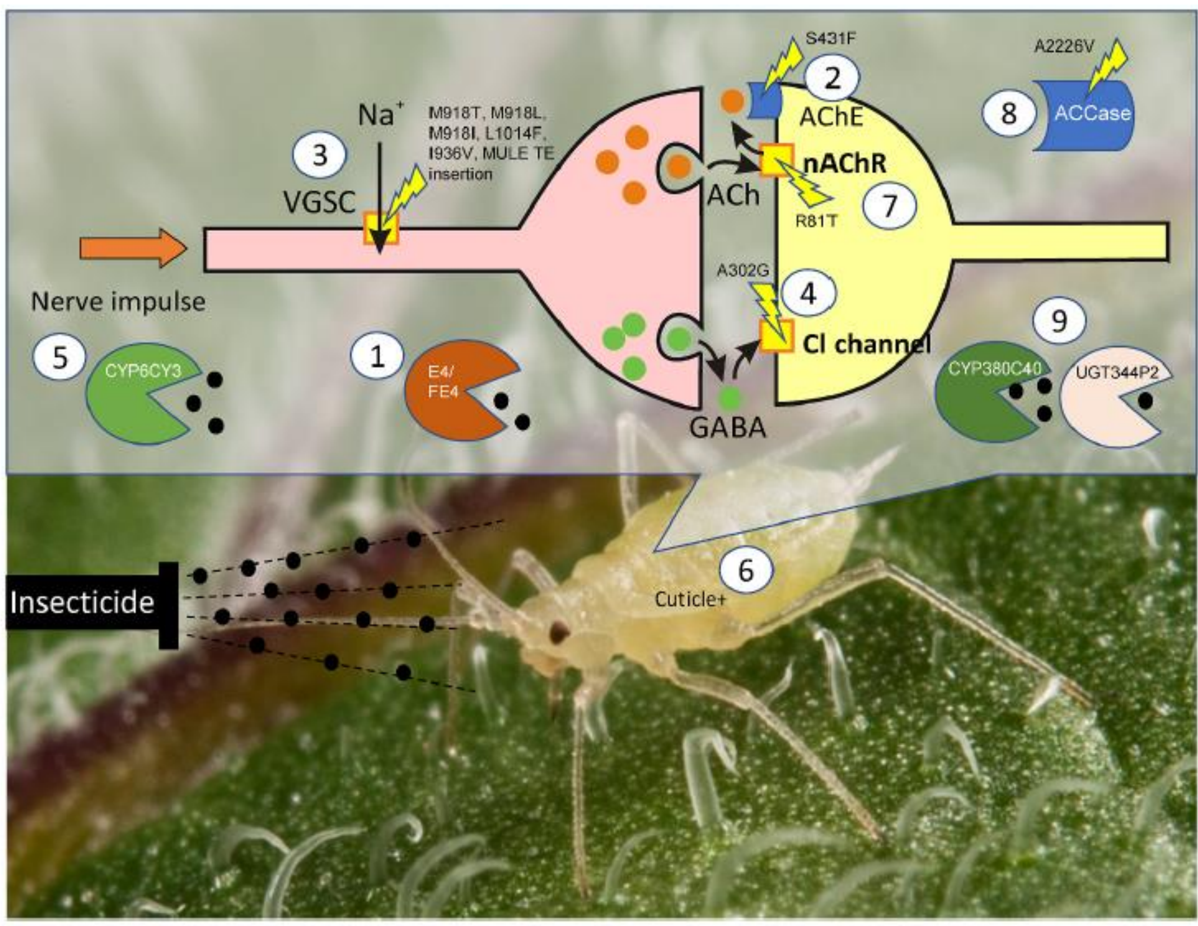

Figure 1.8: Example of multiple insecticide resistance mechanisms known in the Peach-potato aphid *Myzus persicae*. 1, 5 & 9 are resistance mechanisms through enhanced enzymatic expression and activity; 2, 3, 4, 7 & 8 depict resistance mechanisms through target-site mutations; 6 resistance through an enhanced cuticle; image taken from Bass and Nauen (2023).

Metabolic resistance relies on increased enzymatic activity, which can be detected through measuring enzymatic activity (e.g. with immunoassays), or through gene expression studies (Devonshire et al., 1986; Puinean et al., 2010). Although, high metabolic resistance can also decrease over time, as the overexpression of detoxification enzymes can lead to a fitness trade-off if the aphids are not exposed to an insecticide for a longer period (Field et al., 1999; Kliot & Ghanim, 2012). In contrast, target-site mutations are genetically inherited and can be detected using PCR or sequencing (Foster et al., 2014). The most common target site resistance mechanisms are mutations in acetylcholinesterases which confer resistance to organophosphates and carbamates, and mutations in the voltage-gated sodium channel (VGSC) known as knock-down resistance (*kdr*), conferring resistance to pyrethroids (Foster et al., 2017). These *kdr* are widespread throughout many insect

pest species like houseflies and mosquitos, cockroaches, moths and aphids (Dong, 1997; Foster et al., 2017; Soderlund & Knipple, 2003).

1.5.2 Insecticide resistance in *Sitobion avenae*

After insecticide spray failures in the UK in 2012, a heterozygous *kdr* mutation conferring partial resistance to pyrethroids in the VGSC gene was found in a single clonal lineage (SA3) of *S. avenae* (Foster et al., 2014). The mutation in the SA3 clone is a single nucleotide substitution in the VGSC gene, causing an amino acid exchange (L1014F) that confers up to 40-fold resistance to pyrethroids (λ-cyhalothrin) (Fig. 1.9) (Burton et al., 2011; Foster et al., 2014).

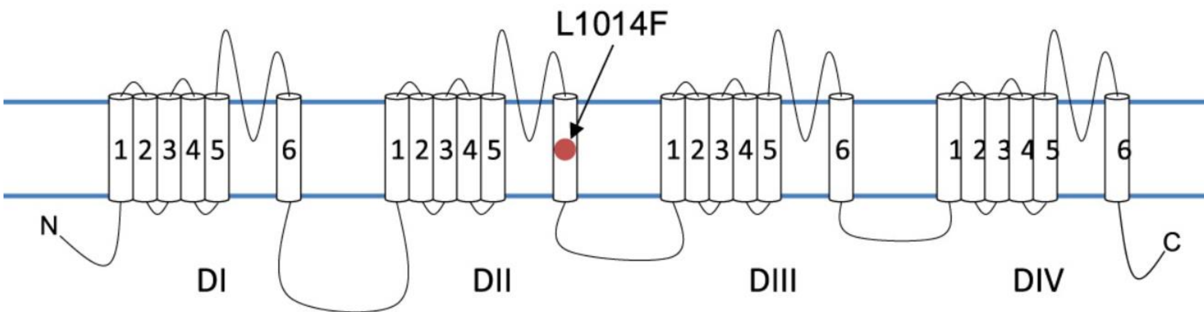

Figure 1.9: Simplified diagram of the voltage gated sodium channel protein with the L1014F mutation which confers resistance to pyrethroids. Image taken from Burton et al. (2011).

Within the last ten years, more discoveries of partially resistant SA3 have followed in Ireland in 2016 (Walsh et al., 2019), Germany and Belgium in 2017 (Poehling et al., 2017), China in 2021 (Gong et al., 2021) and most recently in France in 2022 (Fontaine et al., 2023). To date, no homozygous *kdr* has been found in *S. avenae*, but there are increased concerns because of the overuse of pyrethroids following the neonicotinoid ban, which adds additional pressure on the resistant clonal lineage to become fully resistant (Dewar & Foster, 2017). As a result of the recent emergence of insecticide resistance in *S. avenae*, there are increasing concerns that the grain aphid, which was previously considered a low-level problem, could become a major transmitter of BYDV because of its ability to survive insecticide applications (Poehling et al., 2017).

1.6 Integrated Pest Management

The emergence of insecticide resistance in *S. avenae* and the loss of chemical control options, in combination with EU goals to reduce the number of pesticides used by 50% in 2030, puts serious pressure on pest management strategies. One way to achieve these goals is through the utilisation of integrated pest management (IPM) practices, which are defined as "the careful consideration of all available pest control techniques and subsequent integration of appropriate measures that discourage the development of pest populations" (FAO, 2024a). This includes insect pest monitoring and forecasting, decision support tools (DST) for farmers, cultural control, and the development of resistant plant cultivars, but the correct implementation strongly varies between different crops, climates, and legislation worldwide (Deguine et al., 2021; FAO, 2024b).

In Ireland, the IPM method to manage *S. avenae* and BYDV in winter barley focuses predominantly on cultural control, biological and chemical pest control, and most recently, on planting BYDV tolerant/resistant barley cultivars (DAFM, 2024; Teagasc, 2023). In cultural control (or "pre-planting IPM") methods such as late sowing in autumn, ploughing techniques or minimizing the effects of green bridges after ploughing are implemented, all of which prevent or minimise the number of aphids from infecting and spreading during the emergence and establishment of the crop (Kennedy et al., 2010; Teagasc, 2023). Biological aphid control can be achieved by naturally occurring aphid predators like ladybird beetles, parasitoid wasps, or hoverfly larvae, which can help to reduce aphid populations in barley fields (Ramsden et al., 2017). When biological control is limited (e.g. during winter), chemical aphid control can assist in controlling aphids and BYDV (Teagasc, 2023). More recently, plant breeding companies introduced new winter barley varieties to the market which are claimed to be either BYDV-tolerant (cultivar Joyau, KWS Momont, France) or BYDV-resistant (cultivar Molly, Nordic, Denmark) (DAFM, 2024). While BYDV-tolerant varieties show little to no symptoms upon BYDV infections regardless of the virus titre, BYDV-resistant varieties are characterised by a reduced virus titre and restricted virus replication in the plant tissue, leading to almost no symptoms at all (Hu et al., 2019). Choosing a BYDV tolerant or resistant variety, where available, can therefore help to reduce BYDV pressure. However, these varieties were often only tested with one BYDV species (BYDV-PAV/PAS),

therefore it is unclear whether they would remain an efficient tool in Ireland, where other BYDV species under different climatic conditions are present as well (Hu et al., 2019; Kennedy & Connery, 2001).

1.7 Decision support tools

To identify whether a spray is necessary, growers can receive support using decision support tools (DST), which can provide guidance based on either weather and local climate data, or current aphid and disease pressure from surveillance networks (White et al., 2023). A DST can be developed as a simple treatment recommendation tool based on aphid thresholds in fields or temperature (e.g. degree-days), but research has shown that an accurate DST needs to be more complex, as the aphid population and disease pressure are dynamic (Norton et al., 1993). Over the last 30 years, many DST have been developed for BYDV and *S. avenae* management, based on aphid migration, aphid population, and prediction of crop infection and yield losses (summarized in White et al. (2023)). Over time, modelling BYDV and aphid pressure gained higher accuracies at the landscape level, but only a few DST were adapted and recommended for growers, as regional factors, crop management, limited processing power on computers, and time-intensive data collection all had an impact on the reliability of the DST (Rose et al., 2016). However, with the availability of smartphones and internet access, a new generation of DST based on real-time data is expected in the near future (Walls III et al., 2019). Most recently, ADAS (UK) have developed a decision support system (DSS) named ACroBAT that includes data on numbers of migrating aphids, daily temperature, crop-specific factors, BYDV levels in aphids, regional factors, and farm-specific factors (White et al., 2023). ACroBAT also includes three previously developed models, which consider the aphid population (number of aphids per plant), the disease epidemiology (proportion of infected plants), and analyses the data in a risk model, to predict BYDV risks and provide personalised spray recommendations (Kendall et al., 1992; Morgan, 2000; White et al., 2023).

Reliably applying these DST in Ireland is difficult, as Irish crop aphid populations and BYDV epidemiology, as well as the climate, differ from the UK or continental Europe, for which most DST were designed (Kennedy & Connery, 2001; Walls III et

al., 2019). For example, the ACroBAT decision support system (DSS) takes flight numbers of the bird cherry-oat aphid (*Rhopalosiphum padi*) into account, while the English grain aphid (*Sitobion avenae*) is thought to be the most predominant BYDV vector in Ireland (Kennedy & Connery, 2005). Irish IPM is also limited by the previous lack of an historical suction tower network, which provides essential data to identify migration patterns as well as virus and resistance levels in migrating aphids (Bell et al., 2015).

1.8 Objectives of this thesis

This PhD thesis investigates the English grain aphid (*Sitobion avenae* F), a major vector of BYDVs and its impact on Irish winter barley crops. After multiple control failures, a clonal lineage of *S. avenae,* in which a mutation causes partial resistance to pyrethroids, was first detected in the UK in 2012 and has spread to Ireland where it was found in 2016 (Foster et al., 2014; Walsh, Schmidt, et al., 2020). The impact of resistant *S. avenae* on winter barley production in post-neonicotinoid Ireland will be analysed in the next chapters. This includes:

1. Monitor resistance (*kdr*) and BYDV incidence in *S. avenae* in Ireland throughout a three-year winter barley field survey and across the Republic of Ireland suction tower network (Chapters 2 & 3).

2. Investigate the BYDV transmission efficiency of insecticide resistant *S. avenae* and its potential as a 'super spreader' of BYDV species found in Ireland (Chapter 4).

3. Investigate how different BYDV species can manipulate aphid fertility and vector behaviour, in order to enhance their own spread (Chapter 5).

Together, these studies are expected to help identify the impact of insecticide resistant *S. avenae* on Irish winter barley, as well as to investigate BYDV epidemiology in Ireland. The results of this thesis may contribute to improve the next generation of DST for Ireland.

2

Incidence of *kdr* and BYDV in post-neonicotinoid Ireland

2.1 Introduction

2.1.1 Overview of *kdr* in *Sitobion avenae*

Insecticides are an important and commonly used tool for controlling aphid pressure in crops to reduce yield losses caused by aphids and plant viruses, such as barley yellow dwarf viruses (BYDV) (Mc Namara et al., 2020). However, the repeated use of insecticides with the same chemistry has placed strong selection pressure on aphids in crops worldwide. This selection pressure over time has led to the evolution of multiple mechanisms that confer insecticide resistance to aphids (Foster et al., 2017). Today, at least 20 important agricultural aphid pest species including *Myzus persicae*, *Aphis gossyphii, Schizaphis graminum,* and most recently the English grain aphid *Sitobion avenae,* are known to possess at least one, if not multiple, mechanisms of resistance to insecticides (Bass & Nauen, 2023; Bass et al., 2014; Foster et al., 2014; Furk & Hines, 1993).

S. avenae is a major pest of cereals worldwide, including winter and spring barley (Dixon, 1987; Kennedy & Connery, 2005). For the last three decades in Europe, the availability of multiple insecticides (pyrethroid and neonicotinoid insecticides) that conferred reliable aphid control in fields led to the belief that *S. avenae* was a "low-level problem" (Mc Namara et al., 2020). However, in 2012, the detection of *S. avenae* surviving pyrethroid spray applications in the UK changed this situation. A heterozygous single point mutation in the voltage-gated sodium channel (VGSC) gene, known as knock-down resistance (*kdr*), causes partial resistance to insecticides based on pyrethroid compounds (e.g. λ-cyhalothrin) (Foster et al., 2014). This *kdr* mutation allows *S. avenae* to survive spray applications at recommended field rates, continue to reproduce parthenogenetically, and therefore, continue to cause damage by feeding on crops and transmitting plant viruses (Walsh et al., 2019). To date, heterozygous *kdr* has been predominantly found (with the exception of a single aphid in 2018) in only one *S. avenae* clonal lineage (SA3), which is thought to reproduce parthenogenetically, as there is currently no evidence of a fully resistant homozygous *kdr* (Malloch et al., 2016; Walsh, Schmidt, et al., 2020).

In insecticide exposure bioassays, SA3 showed up to 40-fold higher resistance to pyrethroids than other susceptible *S. avenae* clonal lineages, making it a potential threat to cereal production in Europe (Foster et al., 2014). After the first detection of

kdr-carrying *S. avenae* in the UK in 2012 (Foster et al., 2014), more discoveries of partially resistant SA3 have followed in Ireland in 2016 (Walsh et al., 2019), in Germany and Belgium in 2017 (Poehling et al., 2017), in China in 2021 (Gong et al., 2021) and most recently in France in 2022 (Fontaine et al., 2023).

2.1.2 Implications of loss of neonicotinoids in Europe

For decades, cereal aphid control in Europe has relied on two main insecticide components, neonicotinoids and pyrethroids, which successfully prevented large-scale BYDV infections and yield losses (Mc Namara et al., 2020). The emergence of resistance to pyrethroids in *S. avenae* was first discovered in 2012; however in 2016, over 50% of *S. avenae* caught were already partially pyrethroid-resistant SA3 clones, suggesting that this clonal lineage has become predominant in the *S. avenae* population in the UK, while both main insecticide components were still available (Malloch et al., 2016). The situation changed in 2018, when the European Union banned the outdoor use of neonicotinoid seed dressings because of their risk to pollinators, leaving pyrethroid spray applications (with the exception of one year with sulfoxaflor in 2021) as the only insecticide available to control aphid pests in cereal crops (European Commission, 2018). As sulfoxaflor has only been applied to less than 1% of Irish winter barley fields in 2021 (personal communication with farmers and advisors), the increased use of pyrethroids has been inevitable in recent years. Therefore, this overreliance on pyrethroids could be a reason for the favourable selection and increasing spread of pyrethroid resistant *S. avenae* (Dewar & Foster, 2017).

2.1.2 Implications of loss of neonicotinoids in Ireland for resistance

In winter and spring barley, the most relevant aphid species are the English grain aphid *Sitobion avenae*, the rose-grain aphid *Metopolophium dirhodum,* and the bird cherry-oat aphid *Rhopalosiphum padi,* with *S. avenae* being the predominant species in Ireland (Kennedy & Connery, 2005; Poehling et al., 2017). These aphids can transmit BYDVs, which cause yellow dwarf disease in barley, leading to yield losses of up to 80% in a severe outbreak. In order to investigate the incidence of *kdr*-carrying *S. avenae* while neonicotinoids were still available in Ireland, a field

study from 2016 to 2018 was conducted, with results indicating high but varying levels of resistance to pyrethroids between 54% and 20% (Walsh, Schmidt, et al., 2020). However, the emergence of partially pyrethroid resistant *S. avenae,* coupled with the loss of neonicotinoids in 2018, has raised new concerns about resistant aphids surviving spray applications, which could lead to a higher BYDV pressure and yield losses in crops (Blake, 2018; Jactel et al., 2019; Mc Namara et al., 2020). Therefore, monitoring *S. avenae* following the ban on outdoor use of neonicotinoids needs to be conducted, to improve our understanding of the incidence of insecticide resistance and BYDV epidemiology in Ireland, which will help to confirm and improve decision support tools for farmers.

2.1.3 Objectives

This research chapter contains three main objectives:

1) Confirm if *kdr* has persisted in an Irish *S. avenae* population.

2) Test the hypothesis that the incidence of insecticide resistant *S. avenae* has increased following the ban of neonicotinoid seed treatments in Ireland.

3) Determine if insecticide resistance in *S. avenae* can be linked with high BYDV pressure in winter barley crops.

For objective 1), *kdr*-carrying *S. avenae* were tested for their ability to survive insecticide exposure three and six years after they were caught in a field in 2017, to confirm *kdr* that persisted over time. For 2) and 3), fieldwork in a total of 155 winter barley fields over three consecutive years was carried out between 2021 and 2023. Aphids were collected in each field, to test, if virus and resistance levels in *S. avenae* have increased since the neonicotinoid ban in 2018. Additionally, each winter barley field was scored for the levels of BYDV symptoms, in order to determine the damage caused by the virus (virus pressure). Last, it was analysed whether a high BYDV score can be linked to a high aphid pressure, which could be caused by insecticide resistant aphids that continue to transmit BYDV after they survive insecticide spray applications.

2.2 Materials and Methods

2.2.1 Insecticide exposure assays

2.2.1.1 Aphid clones

The insecticide exposure assays were carried out with one insecticide resistant clonal lineage of the English grain aphid (*S. avenae*, SA3 clone), an insecticide susceptible *S. avenae* clone (SA27), and with a clonal lineage of the bird cherry-oat aphid (*R. padi*) (Table 2.1). To confirm the two *S. avenae* genotypes, genotyping was conducted as described in Chapter 4.2.2. All aphid clones were reared in colonies on winter barley (*Hordeum vulgare*), as described in chapter 4.2.2.

Table 2.1
S. avenae and *R. padi* clones used in the insecticide exposure experiments

Species	Clone	*kdr*	County / Country	Collection date	Crop
S. avenae	SA27	-	Carlow / Ireland	2021	Winter Barley
S. avenae	SA3	+	Carlow / Ireland	2017	Winter Wheat
R. padi	x	x	Carlow / Ireland	2020	Maize

2.2.1.2 Insecticide bioassays

In the first experiment carried out in mid-2020, insecticide resistant grain aphids (*S. avenae*, SA3 clone) and a clonal lineage of the bird cherry-oat aphid (*R. padi*) were tested for their survival rates after insecticide exposure (lethal concentration 50; LC50). For this, adult unwinged aphids were exposed to pyrethroids (λ-cyhalothrin) in a dose-response bioassay in glass vials at different concentrations between the recommended field rate for pyrethroids (at 5 g/ha active ingredient of λ-cyhalothrin) (Foster et al., 2014; George et al., 2022; Walsh et al., 2019). Concentrations chosen to estimate the LC 50 were 200% field rate (10 g/ha AI); 100% field rate (5 g/ha AI), 40% (2 g/ha AI), 20% (1 g/ha AI), 10% (0.5 g/ha AI), 5% (0.25 g/ha AI) and 0% (0 g/ha AI; acetone control). To prepare the solutions, the respective amounts of λ-cyhalothrin were diluted in acetone, and 500 µl of each concentration was pipetted into a cylindrical glass vial (38.56 cm^2 surface; Wheaton, Millville, US). Each vial was placed on a roller in a fume hood at room temperature

(RT) for 2 h, to allow acetone to evaporate and cover the inside with insecticide. To test the dose response, 15-17 adult apterous (wingless) aphids were placed into each vile for a 5 h exposure time. Afterwards, all aphids were placed onto a piece of tissue and scored under a microscope for their movement ability. Three scores were used: “mobile” (aphid can move coordinated), “affected” (aphid cannot move coordinated; considered as dead) and “dead” (aphid is dead). The experiment was carried out in three vials per concentration in three independent replicates, leading to a total of 945 *S. avenae* and 972 *R. padi* tested.

The second exposure experiment was conducted in 2023 to test whether the insecticide resistant *S avenae* clonal lineage SA3 has maintained its resistance, and to confirm the insecticide susceptibility of another non-*kdr* carrying clonal lineage SA27. However, this experiment was also part of another experiment which included the collection of transcriptomic data, and was therefore carried out slightly different to the first experiment. All *S. avenae* were exposed to insecticides in glass vials at different concentrations around the recommended field rate for pyrethroids at 5 g/ha active ingredient of λ-cyhalothrin. Concentrations chosen to test for survival were 100% field rate (5 g/ha AI), 40% (2 g/ha AI), 20% (1 g/ha AI), 10% (0.5 g/ha AI), 5% (0.25 g/ha AI) and 0% (0 g/ha AI; acetone control). For this, the respective amount of λ-cyhalothrin was diluted in acetone and 500 µl of each concentration was pipetted into a cylindrical glass vial (38.56 cm^2 surface), which was placed for 2 h on a roller in a fume hood at RT, to allow the acetone to evaporate and cover the inside of the vial with insecticide. To test the dose response, 15 adult apterous *S. avenae* were placed into each respective vile and visually checked for symptoms after 5 min, 10 min, 15 min, 20 min, 30 min, 45 min, 1 h, 2 h, 4 h, 24 h, and 48 h. After each respective time, the aphids were scored in five different behaviour types; “no symptoms” (aphid can move coordinated), “little symptoms” (aphid shows hints of uncoordinated movement), “strong symptoms” (aphid is moving uncoordinated), “barely moving” (aphid is on its back, not able to move) and “dead” (aphid is dead). The experiment was carried out in four vials with SA27 aphids at all concentrations and in four vials with SA3 aphids a field rate of 100%, leading to a total of 420 aphids tested.

2.2.2 Field sampling 2021 - 2023

2.2.2.1 Aphid sampling

Field sampling was carried out in 155 winter barley fields in eight Irish counties, sampled between May and June in 2021 (50 fields), 2022 (54 fields), and 2023 (51 fields). The fields were categorised as either treated (application of pyrethroids within the growing season) or untreated (no application of pyrethroids), with an equal number of fields selected per county.

Aphids were collected with a paintbrush from 50 winter barley tillers per field, which was split up into five sampling spots with 10 random tillers sampled per spot. The sample spots were lined up into two transects per field: one with three sampling spots and one with two sampling spots approximately 25 to 50 m apart, depending on the field size ("W-Method) (Walsh, Schmidt, et al., 2020). All collected aphids were placed into 90% Ethanol, identified in the laboratory under a microscope using Blackman's key, and stored long-term in 99% ethanol at -80 °C for later molecular analyses (Blackman & Eastop, 2000).

2.2.2.2 BYDV scoring and testing

Each winter barley field was scored for BYDV-symptomatic plants using either a 30x30 cm wooden frame in which the proportion of plants showing yellowing of the leaves was counted, or by walking three 20 m transects and estimating the proportion of symptomatic plants. Based on these estimates, fields were scored from 1-5 describing the severity of BYDV outbreaks per field (Score 1: 0-1% BYDV; Score 2: 1-10% BYDV; Score 3: 10-25% BYDV; Score 4: 25-50% BYDV; Score 5: >50% BYDV). For each field, 10-12 leaf samples showing symptoms of BYDV were randomly collected and stored at -20 °C. The presence of BYDV was later confirmed by ELISA (see Chapter 4.2.7), and a selection of samples was sent for sequencing to identify BYDV species (Appendix table A1, Byrne et al. (2024)).

2.2.3 *kdr* and BYDV testing

The collected aphids were tested for presence of *kdr*. For this, DNA was extracted from individual aphids using a sucrose buffer extraction method described in Louis (1997) followed by qPCR. As the aphids were stored in 90% ethanol, they were first

washed in 16x distilled water before being placed into single wells of a flat-bottom 96-well plate. 50 µl of 300 mM sucrose buffer (0.3 M sucrose, 0.3 M NaCL, 60 mM Tris HCL pH8) were added to each well and homogenised with a Burkhard 96-well homogeniser. The plate was covered with adhesive aluminium foil, placed in a 95 °C water bath for 9 min and spun down in a centrifuge for 2 min at 5000 x g. After resting the plate on ice for 5 min, 30 µl of debris-free DNA was transferred into a new 96-well plate. 1.5 µl of DNA from each extraction sample was analysed using a Nanodrop® ND-1000 Spectrophotometer (Labtech Int., Heathfield, UK). Afterwards, the 96-well sample plate was sealed with adhesive aluminium foil and stored at -20 °C.

Aphids were tested for the *kdr* mutation L1014F using an established TaqMan PCR protocol from Rothamsted Research, UK (Foster et al., 2014; Malloch et al., 2016). 1.5 µl of aphid DNA, 0.375 µl of a probe/primer master mix (0.135 µl forward primer, 0.135 µl reverse primer, 0.03 µl VIC probe targeting *kdr*, 0.03 µl FAM probe targeting the wild type, and 0.045 µl sterile water per reaction), 7.5 µl Faststarter, and 5.625 µl PCR-Water, for a total of 15 µl per reaction were pipetted onto a 96-Well PCR Plate (Roche, Basel, Switzerland) and sealed with optical film. The plates were spun down for 1 min at 5000 x g before being transferred into a Lightcycler 96® PCR machine (Roche, Basel, Switzerland) for amplification and fluorescence measurement. DNA was amplified using a three-stage program with 2 min at 50 °C, 10 min at 95 °C, and 40 cycles of 15 s at 95 °C and 45 s at 60 °C. SA3 aphids were used as positive controls for heterozygosity, SA27 aphids were used as negative controls, and water was used as a non-template control. Fluorescence values of both VIC and FAM probes were plotted on an X/Y-axis scatterplot for each sample after 40 PCR cycles and were manually analysed by setting threshold levels based on the readings of the positive and negative controls. Fluorescence values >1.0 were taken as significant readings, indicating a positive signal from the respective probe.

Additionally, all the extracted aphids were tested for the presence of BYDV, as described in Chapter 4.2.7 of this thesis.

2.2.4 Statistical analyses

The median lethal concentration LC50 and 95% confidence intervals from the insecticide exposure experiments were calculated in SPSS (IBM Corp. Released 2021. IBM SPSS Statistics for Windows, Version 28.0. Armonk, NY: IBM Corp). The LC 50 was calculated by probit analysis in a non-linearized concentration-response curve (Finney, 1978).

Data on aphid numbers, *kdr,* and virus presence in aphids were tested with a Shapiro-Wilkin test for normality and interaction between the variables (BYDV-score, year and insecticide application (treatment)) using the Scheirer-Ray-Hare-test in R (version 4.2.1) (Ihaka & Gentleman, 1996). All datasets were found to be non-normally distributed and were therefore further tested with a Kruskal-Wallis test followed by Dunn's test for multiple comparisons. Statistical significance was set at p-values < 0.05. All graphs were created using ggplot2 and the packages reshape2, scales, maps, mapdata, scatterpie, viridis, tidyverse, lubridate, bbplot, rcompanion, FSA, MASS, ggpubr, and ggnewscale (Wickham, 2011).

2.3 Results

2.3.1 Insecticide exposure assays

2.3.1.1 Insecticide resistance testing in 2020

Insecticide exposure bioassays were carried out in 2020, three years after the capture of the resistant *S. avenae* clonal lineage (SA3) from an Irish winter barley field (Walsh et al., 2019), and with a clonal lineage of *R. padi*, which was suspected to show an increased metabolic resistance (Walsh, Ferrari, et al., 2020). The results for *R. padi* in this experiment were part of another research project (see George et al. (2022)), and are therefore not further reported or discussed in this thesis chapter. For the resistant *S. avenae*, the LC50 was found to be at 3.65 g/ha AI (95% conf. limits at. 3.34 - 4.04 g/ha AI) (Fig. 2.1). Additionally, a ~35% chance of survival was found in aphids exposed for 5 h to the recommended field rates for pyrethroids (at 5 g/ha active ingredient of λ-cyhalothrin), confirming resistance in the SA3 clone.

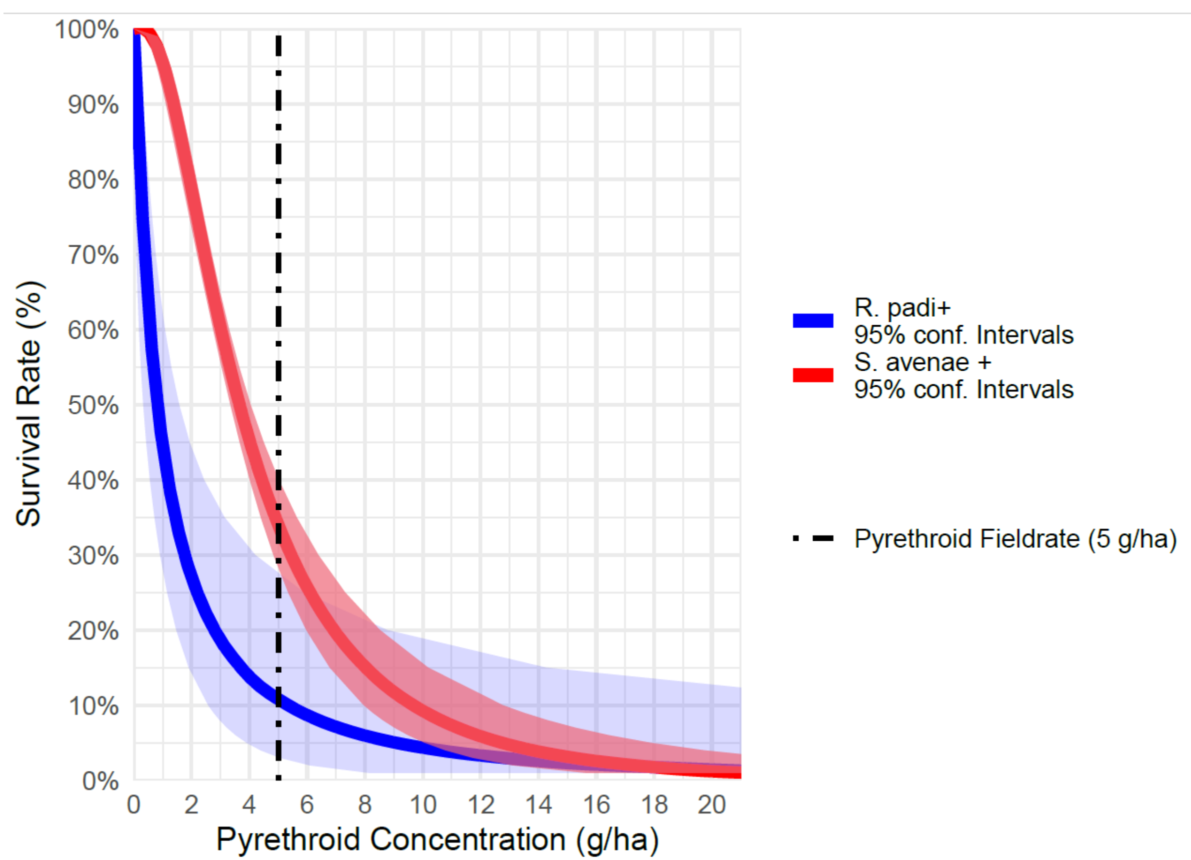

Figure 2.1: *S. avenae* and *R. padi* survival rates with increasing insecticide (pyrethroid) concentrations after 5 h direct exposure in a glass vial.

2.3.1.2 Insecticide resistance testing in 2023

A second insecticide bioassay experiment was carried out in 2023 with insecticide resistant and susceptible *S. avenae* six years after the last exposure. This experiment was a pre-experiment (for Chapter 4) to test the aphids' reaction to insecticides, and only their behaviour over time was visually studied, with no LC50 values calculated.

Table 2.1
Death rates (%) and severity of symptoms (colour coded) of insecticide susceptible aphids after being exposed to different concentrations of λ-Cyhalothrin over time; SA3 is the insecticide resistant clonal lineage and SA27 is susceptible to insecticides.

	% Death rate										
Insecticide Concentration (Clone)	5 min	10 min	15 min	20 min	30 min	45 min	1h	2h	4h	24h	48h
0% (SA27)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	11.67	40.00
5% (SA27)	0.00	0.00	1.67	1.67	1.67	1.67	1.67	8.33	18.33	60.00	78.33
10% (SA27)	1.67	1.67	1.67	3.33	3.33	3.33	3.33	16.67	16.67	68.33	73.33
20% (SA27)	0.00	0.00	0.00	1.67	1.67	5.00	13.33	20.00	21.67	76.67	96.67
40% (SA27)	0.00	0.00	1.67	1.67	1.67	15.00	15.00	23.33	21.67	86.67	96.67
100% (SA27)	3.33	3.33	3.33	5.00	5.00	5.00	25.00	40.00	48.33	90.00	100.00
100% (SA3)	0.00	1.67	1.67	1.67	1.67	1.67	1.67	5.00	10.00	50.00	91.67

no symptoms | strong symptoms | most aphids dead
little symptoms | barely moving anymore

The results (Tab. 2.1) showed that with increasing insecticide concentration, symptoms in susceptible aphids start to appear earlier and stronger. For example, at a 5% field rate, light symptoms of uncoordinated movement in susceptible *S. avenae* were only noticeable after 45 min of exposure, while at a 100% field rate, susceptible aphids were already affected after 5 min of exposure. The same applies to the severity of symptoms, which increases faster over time when aphids are exposed to higher insecticide concentrations. Aphids exposed to 100% field rate (5 g/ha AI) already showed strong symptoms after 10 min and severe symptoms after 1 h of exposure, while aphids exposed at 5% field rate only showed these symptoms after 2 h and 24 h respectively. The same pattern was also found in the percentage of dead aphids, which occurred faster with an increased insecticide concentration. Comparing the reaction of susceptible aphids exposed to 100% field rate against the susceptible *S. avenae* clone SA3, the results showed that SA3 aphids had a substantially less severe reaction to the full field rate exposure,

confirming their resistance. However, it must be noted that resistant aphids showed strong symptoms and 50% died after 24 h of consistent exposure. Additionally, after 48 h, over 90% of the resistant aphids died, showing that the insecticide can also strongly affect partially resistant (heterozygous) *kdr* aphids, as death rates were twice as high as in the control (40% death rate at 0% exposure after 48 h).

2.3.2 50 field sampling 2021-2023

2.3.2.1 Incidence of resistant *Sitobion avenae* in 2021

In 2021, 50 fields of winter barley in eight Irish counties were sampled (25 fields treated with insecticides and 25 fields untreated), and a total of 75 adult *S. avenae* were found across the country and tested for resistance. Table 2.2 shows the average number of aphids and resistance found in the treated and untreated fields per county in 2021.

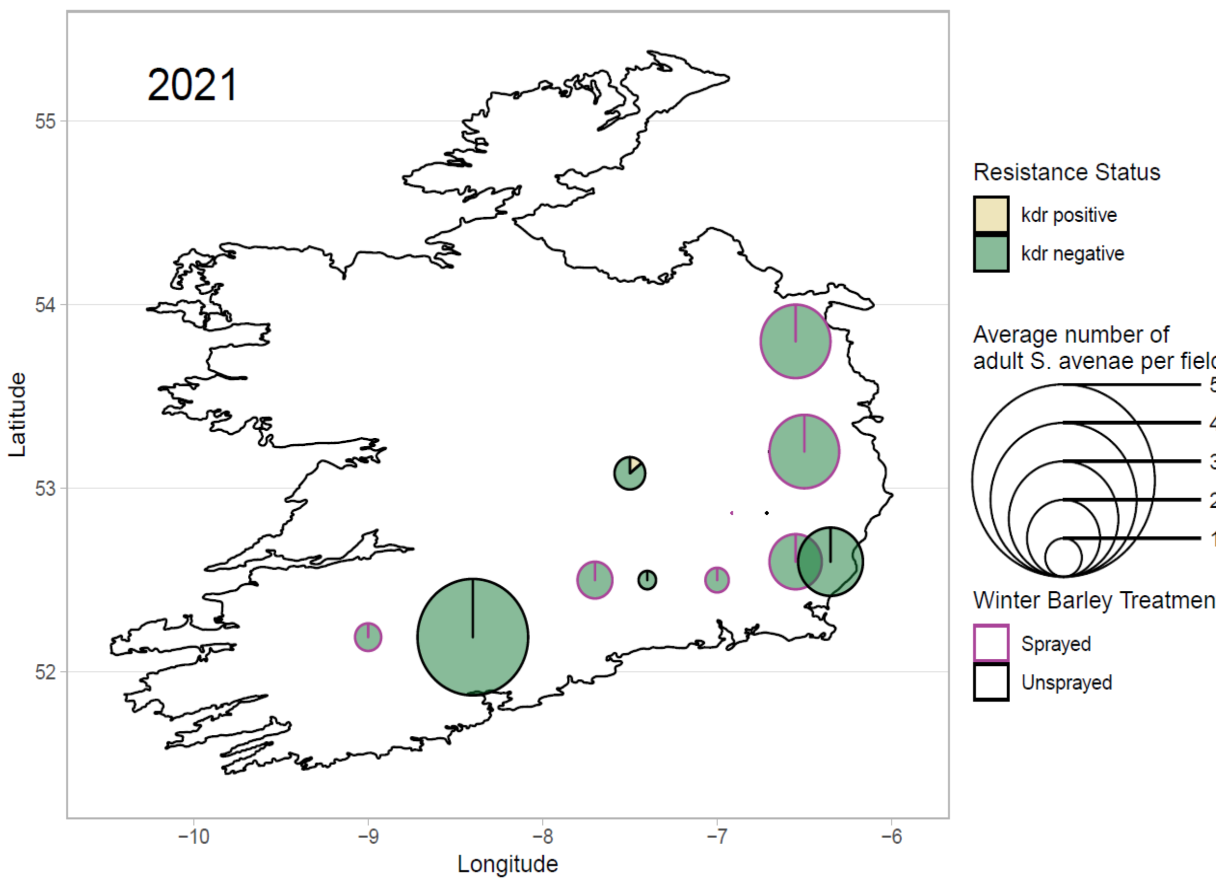

Figure 2.2: *kdr* incidence in 2021; 50 fields from eight counties over the major tillage growing regions in Ireland.

The highest numbers of *S. avenae* were found in untreated fields in county Cork with an average of 3.17 (SD +- 1.46) aphids per 50 tillers per field (Fig 2.2). Overall, there was no significant difference between the average number of *S. avenae* found in untreated fields (1.06, SD +-1.13), in comparison to insecticide treated fields (1.13, SD +-0.69) (chi-squared = 0.0049, df = 1, p-value = 0.9439). There was also only a single insecticide resistant aphid found in an untreated field in county Laois, showing that the overall resistance levels in treated and untreated fields across Ireland were very low in 2021 (1.17%).

Table 2.2
Fieldwork results 2021. SD: Standard deviation.

	Average grain aphid number per 50 tillers per field (+/- SD)		% Resistant aphids	
County	Treated	Untreated	Treated	Untreated
Carlow	0 (0) *(1 field)*	0 (0) *(1 field)*	0%	0%
Cork	0.75 (0.83) *(4 fields)*	3.17 (1.46) *(6 fields)*	0%	0%
Kildare	2 (1.51) *(7 fields)*	0 (0) *(1 field)*	0%	0%
Kilkenny	0.67 (0.47) *(3 fields)*	-	0%	-
Laois	-	0.88 (0.78) *(8 fields)*	-	14%
Meath	2 (1.10) *(5 fields)*	-	0%	-
Tipperary	1 (0) *(3 fields)*	0.5 (0.5) *(2 fields)*	0%	0%
Wicklow	1.5 (0.5) *(2 fields)*	1.86 (2.36) *(7 fields)*	0%	0%
all	1.13 (0.69) *(25 fields)*	1.06 (1.13) *(25 fields)*	0%	2.33%

2.3.2.2 Incidence of resistant *Sitobion avenae* in 2022

The fieldwork was conducted between May and June 2022 in 54 fields across the same eight Irish counties as of 2021. Of the 54 fields sampled, 27 were treated with an insecticide and 27 were untreated. Overall, a total number of 95 adult *S. avenae* were found and tested for resistance.

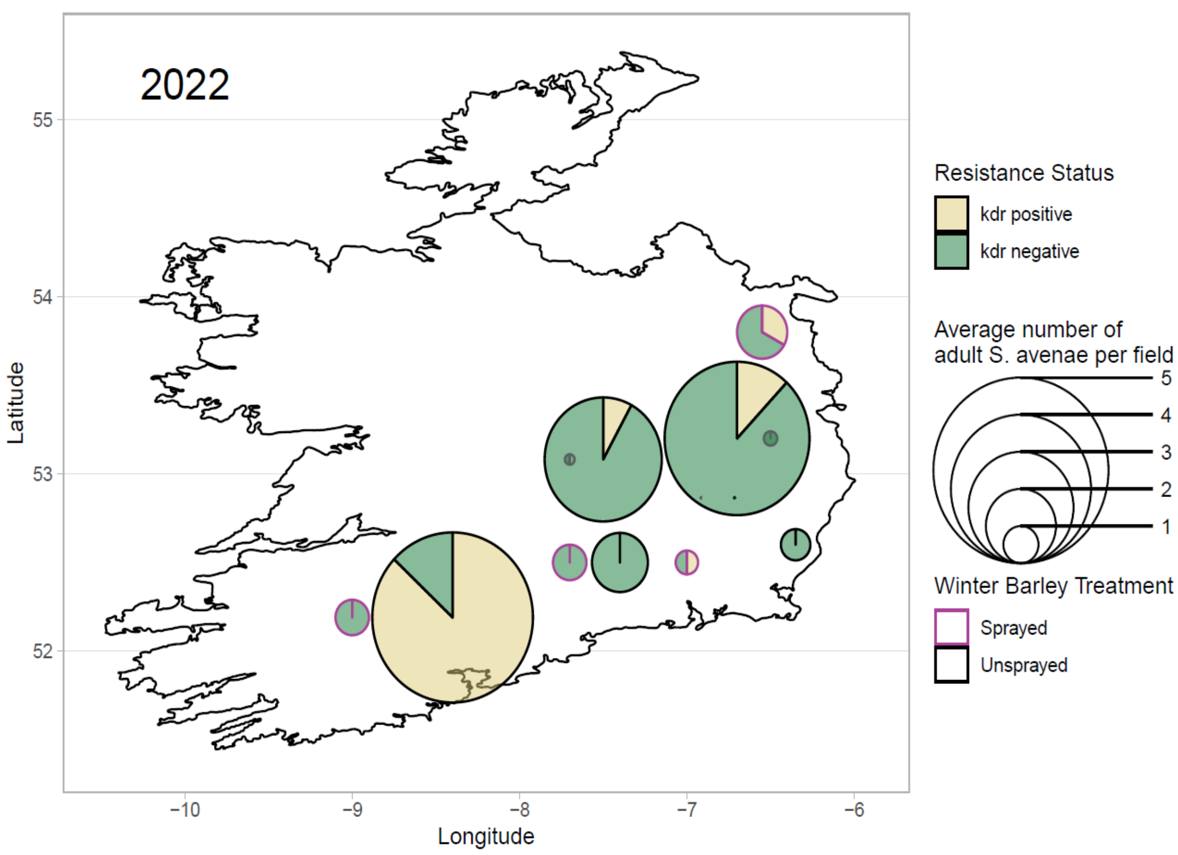

Figure 2.3: *kdr* incidence in 2022; 54 fields from eight counties over the major tillage growing regions in Ireland.

In 2022, the average number of *S. avenae* in insecticide treated fields across the country was 0.57 (SD +-0.49). This is a significantly lower number in comparison to untreated fields, where the average number of adult *S. avenae* was 4.81 times higher with an average of 2.74 (SD +-2.03) aphids found per field (Tab. 2.3) (chi-squared = 10.869, df = 1, p-value < 0.001). As in 2021, untreated fields in county Cork showed the highest average aphid numbers across the country (5.40, SD +-5.12), followed by county Kildare (5.00, SD +-5.94) and Laois (3.50, SD +-3.77). The resistance levels in *S. avenae* varied greatly in 2022, with no resistance found in many fields across the country, and the highest percentage (87%) was found in untreated fields in Cork (Fig. 2.3). The average percentage of resistant aphids in

insecticide treated fields across the country was 19.00%, and the resistance levels in untreated fields were almost identical at 17.83%. Therefore, the overall resistance level in 2022 was 18.42%.

Table 2.3:
Fieldwork results 2022. SD: Standard deviation.

	Average grain aphid number per 50 tillers per field (+/- SD)		% Resistant aphids	
County	Treated	Untreated	Treated	Untreated
Carlow	0 (0) *(1 field)*	0 (0) *(1 field)*	0%	0%
Cork	0.14 (0.35) *(7 fields)*	5.40 (5.12) *(5 fields)*	0%	87%
Kildare	0.40 (0.80) *(5 fields)*	5.00 (5.94) *(6 fields)*	0%	12%
Kilkenny	0.67 (0.47) *(3 fields)*	-	50%	-
Laois	0.29 (0.45) *(7 fields)*	3.50 (3.77) *(4 fields)*	50%	8%
Meath	1.50 (1.50) *(2 fields)*	-	33%	-
Tipperary	1 (1) *(2 fields)*	1.67 (1.25) *(3 fields)*	0%	0%
Wicklow	-	0.88 (1.05) *(8 fields)*	-	0%
all	0.57 (0.49) *(27 fields)*	2.74 (2.03) *(27 fields)*	19.00%	17.83%

2.3.2.3 Incidence of resistant *Sitobion avenae* in 2023

In 2023, 51 winter barley fields across eight Irish counties were sampled for *S. avenae* between May and June. Of the 51 fields, 18 were treated with an insecticide and 33 were untreated, and only 14 adult *S. avenae* were found and tested for resistance.

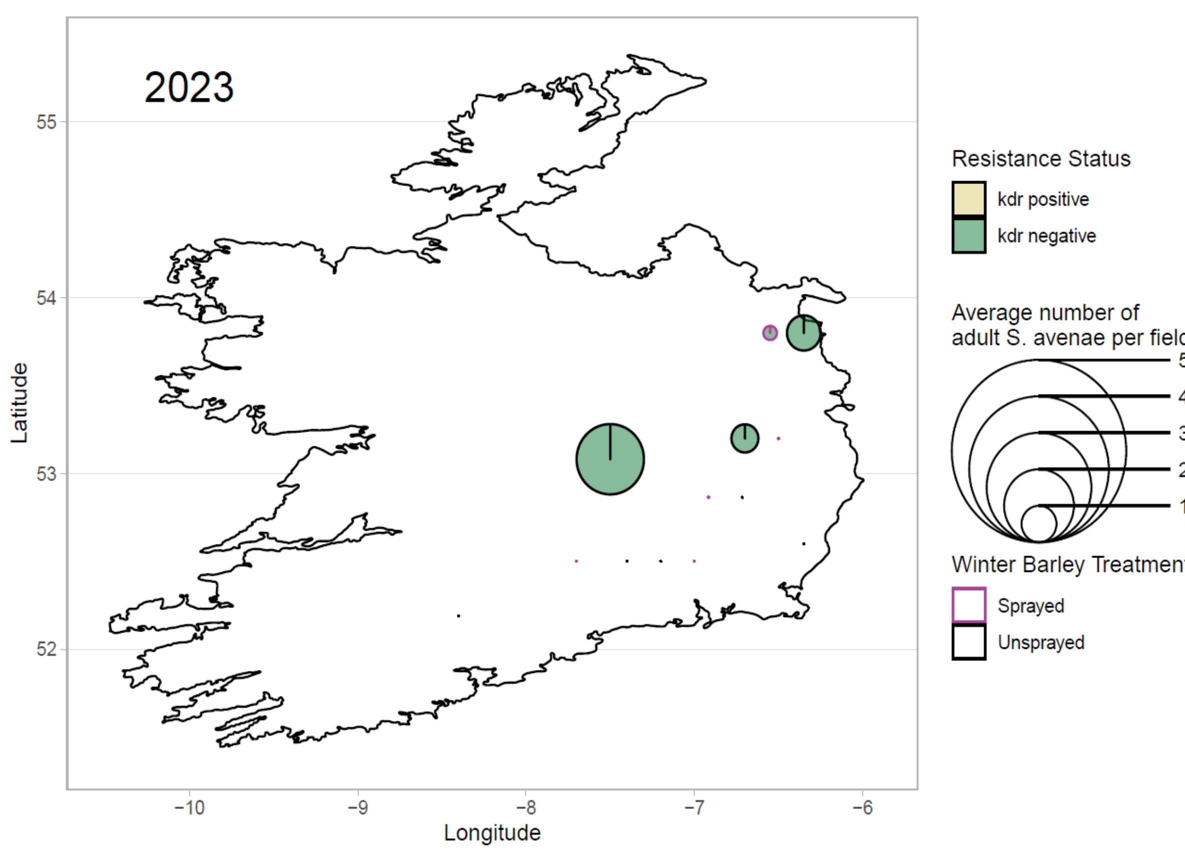

Figure 2.4: *kdr* incidence in 2023; 51 fields from eight counties over the major tillage growing regions in Ireland.

From the three years of field sampling, 2023 was the year with the lowest number of aphids found in both insecticide treated and untreated fields across the country (Fig. 2.4). The average aphid number in insecticide treated fields was 0.08 (SD +-0.16) and, even though the average number of aphids in untreated fields was 6 times higher at 0.48 (SD +-0.86) adult *S. avenae* per field found, there was no significant differences between the two treatments (chi-squared = 2.3966, df = 1, p-value = 0.1216). None of the 14 aphids tested positive for resistance, resulting in 0% resistance levels in 2023.

Table 2.4:
Fieldwork results 2023. SD: Standard deviation.

County	Average grain aphid numbers per 50 tillers per field (+/- SD)		% Resistant aphids	
	Treated	Untreated	Treated	Untreated
Carlow	0 (0) *(2 fields)*	0 (0) *(1 field)*	0%	0%
Cork	-	0 (0) *(13 fields)*	0%	0%
Kildare	0 (0) *(4 fields)*	0.8 (0.75) *(5 fields)*	0%	0%
Kilkenny	0 (0) *(3 fields)*	0 (0) *(2 fields)*	0%	-
Laois	-	2.00 (0.82) *(3 fields)*	0%	0%
Meath	0.40 (0.80) *(5 fields)*	1 (0) *(1 field)*	0%	-
Tipperary	0 (0) *(4 fields)*	0 (0) *(4 fields)*	0%	0%
Wicklow	-	0 (0) *(4 fields)*	0%	0%
all	0.08 (0.16) *(18 fields)*	0.48 (0.68) *(33 fields)*	0%	0%

2.3.2.4 *Sitobion avenae* – virus and resistance testing

A total of 53 out of 75 *S. avenae* caught in 2021, 81 out of 95 in 2022, and all *S. avenae* from 2023 were tested for the presence of BYDV (Tab. 2.5). The results show that, independent of the county, 13.2% of aphids collected in 2021 carried BYDV, with increasing proportions in 2022 (58.1%) and 2023 (71.4%), resulting in a three-year average of 47.57% (SD +/- 24.9%). In 2022, 19.8% of aphids tested were positive for both BYDV and *kdr*, whereas no aphids carrying both virus and resistance were found in 2021 and 2023. There was no significant correlation between aphid resistance and BYDV status (chi-squared = 3.4515, df = 1, p-value = 0.0632). The three-year average of resistance across all fields and counties was 11.33% (SD +/- 14.71).

Table 2.5
Percentages of BYDV and *kdr* carrying grain aphids (*S. avenae*) in Irish winter barley between 2021 and 2023. SD: Standard deviation.

	BYDV positive		BYDV negative	
Year (n aphids)	*kdr* pos.	*kdr* neg.	*kdr* pos.	*kdr* neg.
2021 (n=53)	0%	13.2%	1.9%	84.9%
2022 (n=81)	19.8%	38.3%	12.3%	29.6%
2023 (n=14)	0%	71.4%	0%	28.6%
average	6.60% (SD +/- 9.33%)	40.96% (SD +/-23.83%)	4.74% (SD +/- 5.41)	47.70% (SD +/- 26.31)

Figure 2.5 shows the resistance and virus status of each aphid caught per county and year. Most *S. avenae* carrying both BYDV and *kdr* were found in county Cork (13 aphids) in 2022, followed by county Kildare in 2022 (two aphids) and county Meath in 2022 (one aphid). However, it must be noted that the 13 BYDV and *kdr* aphids found in county Cork came from only three unsprayed fields that were heavily infected with BYDV (Fig. 2.5). Within all other fields sampled in 2022, and all fieldwork carried out in 2021 and 2023, there were no other *S. avenae* carrying both BYDV and *kdr*.

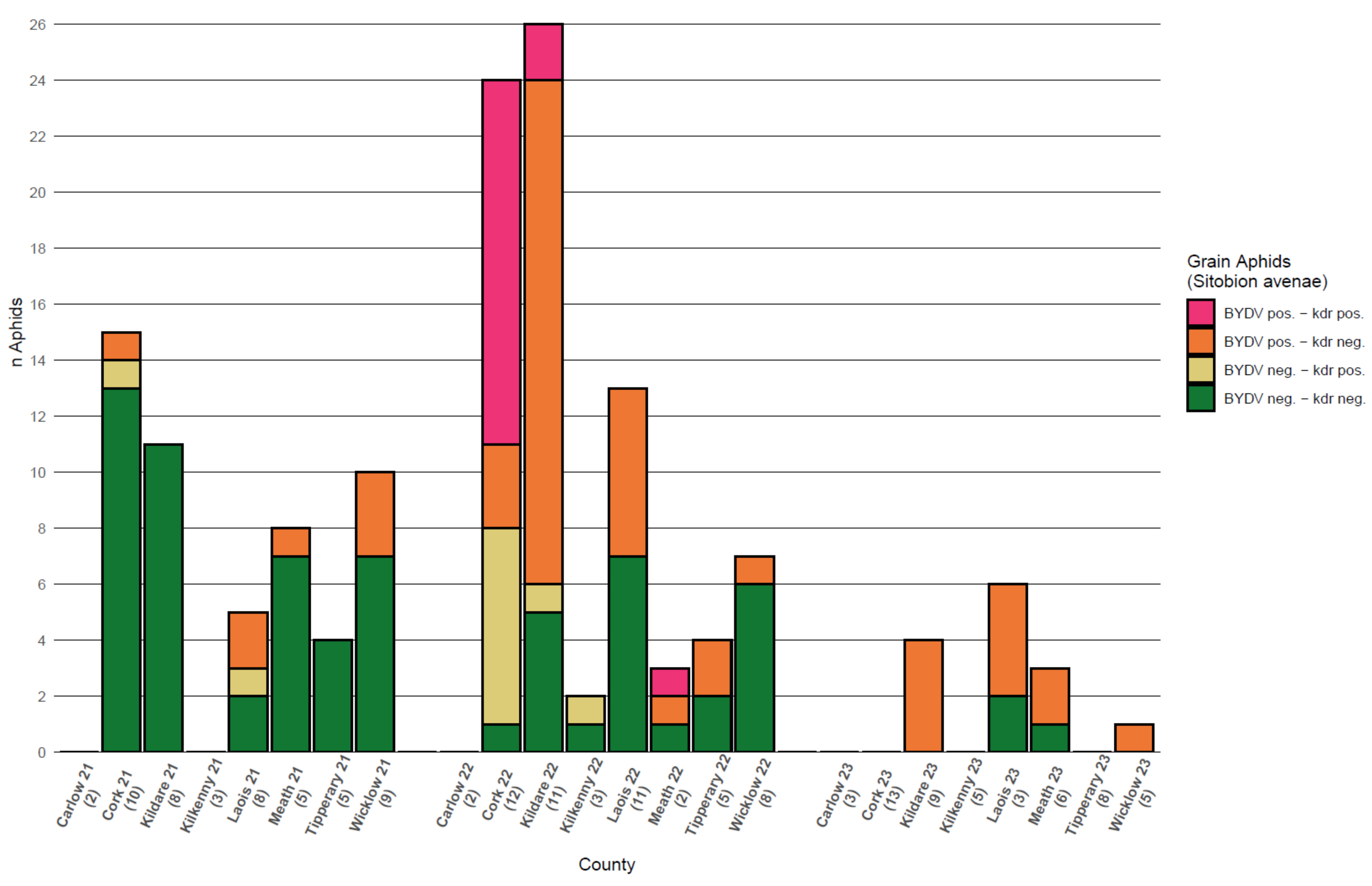

Figure 2.5: BYDV incidence and *kdr* in *S. avenae* caught in a total of 155 winter barley fields across eight Irish counties between 2021 and 2023

2.3.2.5 Incidence of BYDV between 2021 and 2023

During fieldwork between 2021 and 2023, all winter barley fields were additionally scored for the severity of BYDV infections, ranging from no infection (score 1) to severe BYDV outbreaks, with over 50% of plants showing symptoms of infection (score 5) (Fig. 2.6).

Table 2.6
BYDV scores (in percentage of winter barley fields) across Ireland between 2021 and 2023.

	BYDV score				
Year	1 (0-1% infected plants per field)	2 (1-10% infected plants per field)	3 (10-25% infected plants per field)	4 (25-50% infected plants per field)	5 (>50% infected plants per field)
2021	84.0%	16.0%	0%	0%	0%
2022	14.8%	59.3%	14.8%	7.4%	3.7%
2023	82.7%	7.7%	7.7%	0%	1.9%

The results (Tab. 2.6) show that BYDV scores were significantly different between the three sample years 2021 to 2023 (chi-squared = 62.283, df = 2, p-value < 0.001). In 2021, BYDV pressure was low, as 84.0% of fields across Ireland showed a BYDV score of 1, followed by 16.0% of fields with a score 2, and no higher scores were found. This is in contrast to 2022, where only 14.8% of the fields had a BYDV score of 1 and 85.2% of the fields scored 2 or higher, suggesting a very strong BYDV pressure with potentially high yield losses. Additionally, 3.7% of the fields were severely infected, with over 50% of the plants showing BYDV symptoms. In 2023, the BYDV score for most fields (82.7%), with only a few exceptions, was similar to 2021, suggesting that 2023 was a low-pressure year. Additionally, there was no significant impact of insecticide treatment on the BYDV score (chi-squared = 0.3817, df = 1, p-value = 0.5367) and no significant differences between the counties (chi-squared = 5.5675, df = 7, p-value = 0.5911).

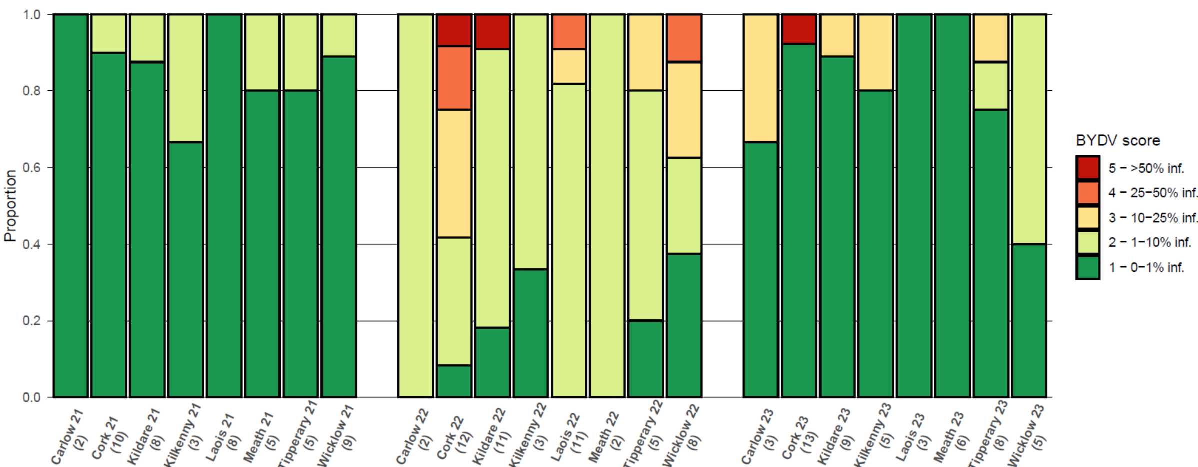

Figure 2.6: Proportion of BYDV scores of each county sampled between 2021 and 2023 across all winter barley fields.

2.3.2.6 Three year multiple factor analysis for resistance and BYDV in aphids

For all aphids collected between 2021 and 2023, a two-factor analysis was carried out to test whether aphid numbers, resistance (*kdr*), or virus presence in aphids were linked to high BYDV scores, sampling year, county, or insecticide treatments, and to test if there were interactions between the factors.

Regarding the total *S. avenae* numbers found throughout all fields in the three years, there was no significant interaction between all the factors tested with the Scheirer-Ray-Hare test, except for a significant interaction between the treatment and the sampling year (H = 7.644, p = 0.022). However, further analysis showed that the only factor that significantly influenced aphid numbers throughout all fields was the year of sampling (chi-squared = 29.201, df = 2, p-value = 4.561e-07). There was no significant difference found between the insecticide treatment (chi-squared = 2.522, df = 1, p-value = 0.1123), BYDV score (chi-squared = 7.333, df = 4, p-value = 0.119), or the county in which the aphids were sampled (chi-squared = 11.505, df = 7, p-value = 0.118).

There was also no significant interaction between the factors that had an impact on resistance levels (*kdr* positive *S. avenae*), throughout the three years of sampling (Scheirer-Ray-Hare test for interaction; all p-values > 0.1). Two significant factors for *kdr* in the fields were found to be the sampling year (chi-squared = 14.666, df =

2, p-value < 0.001) and a high BYDV-score (chi-squared = 18.595, df = 4, p-value < 0.001). However, the treatment status (whether an insecticide was applied) of the field (chi-squared = 1.0487, df = 1, p-value = 0.3058) and the sampling county (chi-squared = 5.4487, df = 7, p-value = 0.6054) had no significant impact on aphid resistance levels.

In the last comparison, there were no significant interactions found between the factors that affected the number of BYDV-infected *S. avenae* in winter barley fields throughout the three years of sampling (Scheirer-Ray-Hare test for interaction; all p-values > 0.1). However, untreated winter barley fields showed a significantly increased number of BYDV-infected *S. avenae* compared to treated fields (chi-squared = 4.4917, df = 1, p-value = 0.0341). The sampling year (chi-squared = 7.1337, df = 2, p-value = 0.0282) and a high BYDV-score per field (chi-squared = 12.825, df = 4, p-value = 0.0122) also significantly affected the number of BYDV-infected aphids. Again, there was no significant difference between the sampled counties found (chi-squared = 8.1247, df = 7, p-value = 0.3217).

2.3.2.7 Other aphid species found between 2021 and 2023

During the three years of fieldwork between 2021 and 2023, all aphids found in a crop were collected, identified, and stored. In addition to adult *S. avenae*, which have been analysed in the previous paragraphs, *M. dirhodum* and *R. padi* were also found in many fields across the years and country. Table 2.7 shows the number of all aphids caught in during three years of fieldwork.

In 2021, no aphids other than *S. avenae* and two adult *M. dirhodum* were found in all fields sampled, which is in contrast to 2022 and 2023, when adults and nymphs of all three crop-relevant aphid species were found. The sum of all aphids found was the lowest in 2021 (377), followed by slightly higher numbers in 2023 (583), and a 6-fold increase in 2022 (3026). Throughout the three years of field sampling, the numbers of *S. avenae* adults and nymphs were higher in 2021 (75 adults, 300 nymphs) and 2022 (95 adults, 378 nymphs), in comparison to 2023 (14 adults, 78 nymphs), and *R. padi* was rarely found at all. However, the number of *M. dirhodum* was found to vary greatly between years, with only two adults found in 2021,

followed by an increase to 259 adults and 2253 nymphs in 2022 and a decrease again to 87 adults and 396 nymphs in 2023.

Table 2.7
Overview of all aphid species found during fieldwork in between 2021 and 2023, including adult aphids and nymphs. T: Treated fields, UT: Untreated fields.

	2021			2022			2023		
Treatment	T	UT	Total	T	UT	Total	T	UT	Total
Fields (n)	25	25	50	27	27	54	18	33	51
S. avenae (adults)	35	40	75	12	83	95	2	12	14
S. avenae (nymphs)	58	242	300	84	294	378	11	67	78
M. dirhodum (adults)	0	2	2	88	171	259	11	76	87
M. dirhodum (nymphs)	0	0	0	677	1576	2253	33	363	396
R. padi (adults)	0	0	0	11	2	13	3	4	7
R. padi (nymphs)	0	0	0	14	14	28	0	1	1
Sum all Aphids	93	282	377	886	2140	3026	60	523	583

Insecticide spray application also had a significant impact on the overall number of aphids found across the country (chi-squared = 91.7725, df = 3, $p < 0.0001$). In 2021, 24.6% of aphids were found in treated fields and 75.4% were found in untreated fields, with a similar proportion in 2022 (29.3% in treated fields and 70.7% in untreated fields). In 2023, the gap between sprayed and unsprayed fields widened to 11.5% of the aphids found in treated fields, in comparison to 88.5% of aphids in untreated fields.

2.4 Discussion

The development of new resistances to insecticides in agricultural aphid pests is considered to be one of the biggest threats to cereal production worldwide, and the emergence of resistance to pyrethroids in *S. avenae* is another concerning addition to the list of insecticide resistances already found in aphids (Foster et al., 2017). With the loss of neonicotinoids in 2018, only pyrethroid applications remain, which raised concerns that the incidence of pyrethroid resistant *S. avenae* (and with it yield losses by BYDV damages) will drastically increase in the years after 2018 (Mc Namara et al., 2020). This thesis chapter set out to first identify, whether decreased insecticide sensitivity persists in partially resistant *S. avenae* maintained in the laboratory for years after initial collection, and second, whether resistance levels have increased in following the neonicotinoid ban in Ireland, almost 10 years after the first detection of resistant *S. avenae*.

In the dose-response bioassays, *S. avenae* from the *kdr* positive SA3 clone aphids were tested for their ability to survive pyrethroid exposure, three and six years after they were captured in 2017. The results from the first insecticide bioassays showed, that *S. avenae* carrying *kdr* have an around 35% chance of survival when being exposed to the recommended field rates for pyrethroids (at 5 g/ha active ingredient of λ-cyhalothrin). Additionally, the LC50 was found to be at 3.65 g/ha AI (95% conf. interv. 3.34 - 4.04), which is in line with previous results from 2014, where an LC50 of 3.05 g/ha AI and 3.24 g/ha AI were found (Foster et al., 2014). This is interesting, as no ancestor of the SA3 clone in this experiment experienced any insecticide exposure within a three/six-year period. Given the average generation time of *S. avenae* is ~14 days (at a day/night- and temperature cycle of 16h-24 °C/8h-20 °C; see fertility experiments Chapter 5.2.1), more than 100 generations (experiment 1; three years after collection), and more than 200 generations (experiment 2; six years after collection) of SA3 aphids were not exposed to any insecticides. Besides *kdr*, a second insecticide resistance mechanism in *S. avenae* is thought to be metabolic resistance (through fast degradation of the insecticide inside the aphid), which might also play an important role in surviving insecticide exposure (Foster et al., 2014). It has been previously shown that the resource-intensive metabolic resistance is reduced in aphids that are not frequently exposed to insecticides (Bass et al., 2014). Nonetheless, around 35% of SA3 from

experiment 1 survived a 5 h field-rate exposure and around 50% of SA3 from experiment 2 survived a 4 h field-rate exposure, years, and many generations after the last exposure. This suggests that the *kdr* mutation is the main mechanism of insecticide resistance in *S. avenae* and that metabolic resistance may only play a subordinate role under frequent exposure scenarios in the field (Foster et al., 2014; Martinez-Torres et al., 1999).

Results from three years of fieldwork showed that the numbers of *kdr* carrying *S. avenae* and BYDV levels in aphids and winter barley fields significantly differed between each year and that there was no significant link between aphid resistance and BYDV levels in fields. However, remarkable differences were found when comparing the resistance levels in *S. avenae* collected in Irish winter barley fields between 2016 to 2018 (with neonicotinoid seed treatments available) and between 2021 and 2023 (no neonicotinoids available). In this study, the average number of *kdr*-carrying aphids found in winter barley fields across all sampled years was between 0% and 18.42% (1.17% *kdr* in 2021, 18.42% *kdr* in 2022, and 0% *kdr* in 2023). This is with the exception of 2022, in contrast and far below levels found in previous testing (54% *kdr* in 2016, 25% *kdr* in 2017, and 20 % *kdr* in 2018), showing a decline from a three-year average of 33.0% (SD +/- 14.99%) to 6.53% (SD +/- 8.42%) resistance levels in Irish winter barley fields (Walsh, Schmidt, et al., 2020). Although the sampling year seems to have the greatest impact on resistance levels in Ireland, the decline in resistance leads to the conclusion that there is no evidence for the proliferation of *kdr*, following the ban on neonicotinoids in this study.

The results also indicate that pyrethroid spray applications did not lead to an increased incidence of resistant *S. avenae* in insecticide treated winter barley, as the same levels of resistant aphids were found in both treated and untreated fields across the whole country. Instead, this study showed that resistance levels significantly varied between the sampling years, most likely due to differences between clonal lineages in their spatio-temporal distribution (Ciss et al., 2014). However, it is also plausible that the fitness advantage of surviving insecticide applications in the partially resistant *S. avenae* clonal lineage SA3 has caused other fitness-reducing pleiotropic effects. For example, it has been shown that the SA3 clone showed a reduced fecundity, a worsened response to alarm pheromones, and an increased susceptibility to parasitation (Foster et al., 2007; Jackson et al., 2020).

These fitness deficits could possibly be another factor, which could explain the decrease in the incidence throughout the last years, but further data and experiments to investigate this phenomenon are needed. Nonetheless, the results of this chapter lead to the conclusion that there is no evidence between insecticide applications and an increased emergence of insecticide resistant aphids, due to favourable selection after spray applications. This rejects one of the thesis hypothesis. With the loss of neonicotinoids in 2018, several studies suggested that overreliance on a single insecticide could lead to a strong increase in the incidence of resistant *S. avenae*. However, at least in winter barley fields studied between 2021 and 2023 in Ireland, this is not the case (Dewar & Foster, 2017; Walsh, Schmidt, et al., 2020).

Stringent legal requirements for active ingredients slow down the development of new products, which could help slow resistance development through the possibility of rotating chemistries used. For example, sulfoxaflor (Isoclast™, Corteva, Indianapolis, US), a newly developed insecticide was available for aphid control for only one winter barley season in Ireland in 2021 before it was banned again because of EU regulations. Therefore, future aphid monitoring in winter barley is needed, to test whether the overreliance on pyrethroids in Ireland could become an issue, which further drives resistance in *S. avenae* and could possibly lead to resistance in other crop aphid species such as *M. dirhodum* and *R. padi*.

In the case of all *S. avenae* caught during fieldwork, only the heterozygous *kdr* mutation was found, suggesting that no fully resistant clone (through homozygous *kdr*) has become widespread yet in Ireland. This is in line with a previous field study conducted in Ireland between 2016 and 2018, when neonicotinoids were still available (Walsh, Schmidt, et al., 2020), suggesting that the use of pyrethroids after 2018 has not yet increased the selection pressure to the point of full resistance emerging in *S. avenae*. However, it is not unlikely that a homozygous *kdr* could appear in the near future, as resistant *S. avenae* have been shown to be capable of sexual reproduction (Walsh et al., 2019). Therefore, continuous monitoring for homozygous *kdr* is necessary in the future to detect if or when this occurs, as it will have a knock-on effect on insecticide efficacy in the field for controlling aphids and BYDV.

This study also found that, even though an insecticide application was not directly linked to a reduced BYDV score, a significantly reduced number of the three cereal aphid species was found when the winter barley field was treated with an insecticide, which ultimately reduces the disease pressure. Therefore, an insecticide application remains an important tool for managing aphids, especially in high-pressure years, even though a proportion of insecticide resistant *S. avenae* might survive exposure. Nonetheless, there was a high and varying number of *M. dirhodum* (an "efficient" spreader of BYDV-MAV (Van den Eynde et al., 2020)), caught during fieldwork, which could have also impacted BYDV spread in the high pressure year of 2022. Although *S. avenae* was previously found to be the main BYDV vector in Ireland, the impact of *M. dirhodum* on BYDV spread and yield losses in Irish winter barley remains unknown and therefore remains an interesting topic to study, to improve the understanding of virus epidemiology in crops (Kennedy & Connery, 2005).

To gain a better understanding of aphid abundance, resistance levels, and virus incidence in winter barley crops, direct monitoring of cereal aphids and the presence of BYDV in 155 fields over three years was conducted in this study. There are multiple crop sampling methods, such as *in situ* counting, destructive counting, sweeping, beating, or vacuuming aphids from plants in fields, all of which have their own advantages and disadvantages, as reviewed in Harrington and Hullé (2017). Counting and sweeping aphids from crops (the chosen method of in-field monitoring in this chapter), has the benefit of obtaining real-time information about aphid levels present at the date of sampling, while also allowing later molecular analyses in the laboratory (Harrington & Hullé, 2017). However, in-field sampling only provides information on aphid presence within the chosen sampling period. During fieldwork between May and June, high variations in the prevalence and abundance of aphids were found between the three years of study, with overall low aphid and resistance numbers in 2021 and 2023, but higher numbers in 2022. This variation in aphid abundance is expected, and is in line with previous studies investigating seasonal and climatic factors responsible for differences in aphid phenology (Vialatte et al., 2007; Winder et al., 1999). However, it is important to note that *S. avenae* are present and spread BYDV in winter barley from crop emergence in autumn, over the winter months, and into spring (Vialatte et al., 2007). A continuous in-field monitoring of aphids is considerably more time consuming though, and it is currently

unclear, which aphid species and numbers found in a field, and at what time of the year, will have an economically significant negative impact on yield (Harrington & Hullé, 2017).

An alternative yet effective approach to monitor aphid activity throughout the year is through automated suction towers, which continuously sample migrating aphids (Bell et al., 2015). The lack of a suction tower network in the Republic of Ireland, similar to the existing one in the UK, was considered a factor of uncertainty in the existing data on resistant *S. avenae*, which were collected in fields (Bell et al., 2015; Walsh, Schmidt, et al., 2020). In 2020, three 12.2 m suction towers have been installed in Ireland with the aim of filling this gap of knowledge around migrating and potentially BYDV-spreading aphids. In the following thesis chapter, the incidence of *kdr* and BYDV in migrating *S. avenae* collected through the Irish suction tower network is compared to the results from the fieldwork presented in this chapter.

In summary, this research chapter shows that there is no evidence for an increasing incidence of resistant *S. avenae* in post-neonicotinoid Ireland to date. Additionally, it was found that an insecticide treatment efficiently led to a reduction in the numbers of all cereal aphids found in the field. However, it was found that the main factor impacting aphid numbers, resistance levels, and BYDV-score is the sampling year. This leads to the conclusion that there are high-pressure and low-pressure years and that high aphid numbers and disease outbreaks might be linked to weather conditions, with warmer winters causing higher pressures. However, this hypothesis needs to be further investigated, as more long-term data are needed. Interestingly, some fields with a high BYDV-score (mostly untreated fields) also showed high levels of resistant *S. avenae.* This poses the question of whether the resistant *S. avenae* clone might be a more efficient BYDV-spreader than other susceptible *S. avenae* clones. This question is investigated further in Chapter 4 of this thesis.

3

Incidence of *kdr* and BYDV in migrating aphids in Ireland

3.1 Introduction

3.1.1 Insect monitoring

Insects are one of the most important functional drivers of ecological systems, and play essential roles in plant pollination, nutrient cycles, as herbivores or detritivores, and as a food resource for many higher animals (Yang & Gratton, 2014). Insect decline and loss of species, and therefore the potential loss of ecological services, is driven by anthropological factors such as habitat loss, intensive agriculture, chemical and light pollution, but also by invasive species or climate change (Sánchez-Bayo & Wyckhuys, 2019). A continuous insect decline during the last century has been reported by many scientists, but has gained international media attention in 2017, when a 75% decline in insect biomass within only 27 years was reported in natural reserves in Germany (Hallmann et al., 2017). This and other studies lead to the idea of an “Ecological Armageddon” happening around the globe, with scientists (and media) discussing a “global insect apocalypse” or “Insectageddon” (Cardoso & Leather, 2019; Leather, 2017; Thomas et al., 2019).

While the overall loss of insect species diversity and biomass is ongoing, it is currently unclear which species or groups are most affected, and at which rates the decline is happening. In recent years, the biodiversity loss “rate debate” has started, with scientists trying to identify the rates at which insect decline is ongoing (Bell et al., 2020). For example, long-term prediction of insect abundance is difficult when data collection is performed in only a small period of time, as insect numbers can fluctuate between years, depending on environmental conditions (e.g. temperature or rain). Additionally, most studies on insect decline focus on Europe or North America, creating a bias which leaves the rest of the world being underrepresented (Simmons et al., 2019). Although, it has also been shown that some species (e.g. pest species such as aphids) benefit from the decline, as their non-specialisation and flexibility allows them to adapt quicker to a changing environment, in comparison to a highly specialised insect (Bell et al., 2020). Therefore, accurate data and predictions of insect abundance must utilise as much long-term monitoring data as possible.

The Rothamsted Insect Survey program in the UK is currently the longest running insect monitoring program in the world, collecting data from flying (migrating) insects through a network of 12.2 m suction towers since 1964 (Bell et al., 2015). Their long-

term data showed that species and abundance decline rates are highest for native bees, moths, carabids, hoverflies, and butterflies, yet there are a few insect species that show no significant population trends, including aphids (Bell et al., 2020). Over 50 years of long-term data have shown that aphids, especially crop pests, might even benefit from anthropogenic impacts. For example, a warmer climate increases aphid reproduction rates, contributes to earlier first flights and an extended migration period, but also prevents aphids switching from asexual to sexual reproduction, resulting in faster life cycle completion over winter (Bell et al., 2015). This, in combination with the emergence of insecticide resistance in at least 14 crop-relevant aphid species that transmit crop diseases, might have a serious impact on agriculture in the future, underlining the importance of aphid monitoring worldwide (Foster et al., 2017).

3.1.2 Monitoring aphid migration

In contrast to monitoring aphids in the field (see Chapter 2), monitoring aphids with suction towers provides critical information on diseases and resistance carried by migrating aphids at the landscape level. This is beneficial, as it provides broader/more generalised data that are independent of farmers' practices or insecticide sprays, which individually differ from field to field. Aphid migration is caused by two main factors: 1) environmental factors such as climate, season, temperature, wind, and rain, or 2) biological factors such as the production of winged aphids by overcrowding, poor nutrition, presence of predators or life cycle induced (migration to another host) (Fereres et al., 2017). Understanding the factors that drive aphid migration will improve our knowledge of when there is a high risk of disease or resistance carrying aphids spreading throughout the country.

3.1.3 The Irish suction tower network

The Republic of Ireland suction tower network is set up similarly to other networks in the UK (including one tower in Northern Ireland), Europe, Africa, the US, and China (Harrington & Hullé, 2017). The network includes a 12.2 m suction tower in Oak Park, Carlow (established in 2019), followed by two more towers in Castlemartyr, Cork and Ashtown, Dublin, which sample for migrating aphids and

other insects that are flying over it (Fig. 3.1). The benefits of aphid sampling using suction towers are ample. As the towers run throughout the year, continuous daily data are generated, which allows the measurement of aphid flight/migration patterns under all weather conditions to quantify the aphid pressure as it happens. It has also been shown that, depending on the topography of a region, a single suction tower can represent aphid migration patterns with diameters between 30 km and 700 km around it, with 80 km being the accepted range for the UK (Bell et al., 2020; Cocu et al., 2005; Taylor, 1984). For Ireland, this means that even a small network of three suction towers can provide versatile landscape level information about aphid flight, which covers most of the crop-growing regions in the east, southeast, and south of the country (Fig. 3.1).

Figure 3.1: *left:* A 12.2 m suction tower in county Carlow. *right*: The suction tower network established in 2020 with three towers in county Dublin, Carlow and Cork. The red circles indicate an 80 km diameter around each tower.

3.1.4 Monitoring BYDV and resistance in *Sitobion avenae*

The loss of control options, paired with resistance to the only remaining chemical (pyrethroids), has caused great concern that resistant grain aphids (*S. avenae*), will become predominant and cause severe BYDV infections in the near future (Dewar & Foster, 2017). In Ireland, partially resistant *S. avenae* were first found in 2013, and they were still present in high numbers several years later (33% between 2016-

2018; 11% between 2021-2023) (Chapter 2 of this thesis; Walsh, Schmidt, et al. (2020)). However, these data were collected by ad-hoc sampling in fields, and continuous systematic sampling throughout the year (as with the Rothamsted suction tower network in the UK) was lacking in Ireland until 2020.

3.1.5 Objectives

This research chapter has three main objectives:

1) Identify aphid migration patterns and weather conditions that lead to higher aphid and BYDV pressure with the data generated by the Irish suction tower network.

2) Test the hypothesis that the incidence of resistant *S. avenae* from the suction tower network is increasing following the neonicotinoid ban in Ireland.

3) Determine whether suction tower data about prevalence, resistance and BYDV status of aphids are comparable to in-field monitoring data.

For this, data from the first four years of the Irish suction tower network were analysed, which allowed a further investigation of migration patterns and abundance of crop-relevant aphids between 2020 and 2023 in Ireland. These data were correlated with weather data to improve the understanding of aphid migration in Ireland. Additionally, *S. avenae* caught in suction towers were tested for resistance and virus presence, and the incidence in the suction tower network and in-field monitoring were compared. Finally, the importance of different monitoring techniques (suction towers vs. in-field monitoring) for IPM is discussed, as the prediction of aphid migration and aphid populations in fields would help inform spray decisions, which ultimately lead to a reduction in costs and improved biodiversity in winter barley fields by reducing prophylactic insecticide use.

3.2 Materials and Methods

3.2.1 Suction tower sampling

In 2019 a 12.2 m suction tower was established in Oak Park (Carlow), followed by two more suction towers in Cork and Ashtown (Dublin) to complete the Republic of Ireland suction tower network. This thesis chapter will only focus on analysing data from the Oak Park and Cork suction towers, which were sampled on a daily basis, in contrast to the Ashtown suction tower, which was emptied sporadically for the first two years.

Daily insect samples, year-round, were collected in 50 ml plastic bottles filled with 15-20 ml of 50% ethanol, which were mounted on a rotating carrousel underneath the 12.2 m long tube of the suction tower (Fig. 3.2). Within four years of the Oak Park suction tower, and three years of the Cork suction tower (Castlemartyr), approximately 2500 daily samples were analysed, giving a total of over 230,000 insects collected. All aphids (~23,000) were separated from other insects under a microscope, and relevant cereal aphids (such as *S. avenae*, *R. padi,* and *M. dirhodum*) were identified with a key (Blackman & Eastop, 2000). All collected samples were stored in 90% ethanol for subsequent molecular analyses. Weather data was taken from the Irish Meteorological Service (Met Eireann; https://www.met.ie/climate/available-data/historical-data).

Figure 3.2: Collection bottles in the 12.2 m suction tower in Oak Park, Carlow.

3.2.2 Resistance and BYDV testing of *Sitobion avenae*

Resistance and BYDV testing of grain aphids (*S. avenae*) caught in the suction tower network was performed as in Chapter 2.2.4

3.2.3 Data analysis

To measure correlations between aphid numbers and weather data, the Pearson correlation coefficient was calculated with R-values greater than 0.5 or less than -0.5 showing strong correlations; R-values between 0.5 to 0.3 and -0.5 to -0.3 showing moderate correlations, and R-values between 0.3 to 0 and -0.3 to 0 showing weak correlations. All total numbers of aphids and numbers of resistant and BYDV positive aphids were tested with a chi-square test for significant differences between the sampling years and all p-values < 0.05 were considered significant.

A Generalized Linear Model (GLM) with a negative binomial distribution was employed to analyse the relationship between the daily counts of *S. avenae* and various predictors, including daily weather conditions (maximum temperature, wind

speed, and rain), year, month, calendar week, and location. This model was chosen because of the presence of overdispersion in the count data, where the variance exceeds the mean, which is a common characteristic of ecological counting data. The negative binomial distribution provides a way to model this overdispersion by introducing an additional parameter (theta) to account for it. The model also explored interactions, specifically between maximum temperature and location, to assess whether the effect of temperature on aphid counts varied between the two study locations (Oak Park and Cork). The analysis was conducted using the glm.nb function of the MASS package. A heatmap was created using the vivid package following the approach from Inglis et al. (2022). All analyses were performed using R (version 4.2.1) and using the packages ggplot2 reshape2, scales, plyr, viridis, tidyverse, lubridate, dplyr, Rcpp, rcompanion, FSA, MASS, ggpubr, ggmagnify, and ggnewscale (Ihaka & Gentleman, 1996; Wickham, 2011).

3.3 Results

3.3.1 Aphid migration data from 2020 to 2023

Table 3.1 shows the number of aphids and other insects caught in both Oak Park and Cork suction towers from 2020 to September 2023. The most predominant aphid species found in both suction towers was *R. padi* with an average of 1049.7 (SD +/- 652.7) aphids per year in Oak Park and an average of 1885.5 (SD +/- 632.5) aphids per year in Cork. In comparison to *R. padi*, the averages for *S. avenae* were lower in both suction towers with an average of 229.7 (SD +/- 172.9) aphids per year in Oak Park and an average of 93.5 (SD +/- 7.5) aphids per year in Cork. There were no averages calculated for *M. dirhodum*, as counting only began in 2022. However, the average numbers of all three relevant cereal aphids combined, in comparison to the average of all other aphids caught, showed that 41.07% of all aphids caught in Oak Park and 43.84% of aphids caught in Cork were the major crop damaging and BYDV transmitting cereal aphids (*S. avenae*, *R. padi,* and *M. dirhodum*).

Table 3.1
Numbers and averages of *S. avenae* (Sa), *R. padi* (Rp), *M. dirhodum* (Md), other aphids, and other insects caught in the Oak Park and Cork suction towers in between 2020 and 2023. SD: Standard deviation.

Location	Year	Sa	Rp	Md**	Sum cereal aphids	Other aphids	All aphids	Other insects
Oak Park	2020	75	1194	-	1269	805	2074	25392
	2021	471	1767	-	2238	3088	5326	45559
	2022	142	188	139	470	1814	2284	47715
	2023*	34	217	22	273	1379	1652	13396
	all	722	3366	161	4249	7086	11335	132062
	Avrg/ Year*	229.3 (SD +/- 172.9)	1049.7 (SD +/- 652.7)	-	1325.7 (SD +/- 722.9)	1902.3 (SD +/- 934.1)	3228.0 (SD +/- 1486.0)	39555.3 (SD +/- 10053.6)
Cork	2020	-	-	-	-	-	-	-
	2021	101	2509	-	2610	2035	4645	30104
	2022	86	1262	161	1509	3240	4749	33928
	2023*	22	250	82	354	1968	2322	14083
	all	209	4021	243	4473	7243	11716	78115
	Avrg/ Year*	93.5 (SD +/- 7.5)	1885.5 (SD +/- 632.5)	-	2059.5 (SD +/- 550.5)	2637.5 (SD +/- 602.5)	4697.0 (SD +/- 52.0)	32016.0 (SD +/- 1912.0)

*2023 data shown only until August and is not included in the calculation of averages; ***M. dirhodum* numbers were only counted in 2022 and 2023.

3.3.2 Overview of *Sitobion avenae* flight in relation to weather conditions

A total of 931 *S. avenae* were caught in suction towers in Oak Park and Cork between January 2020 and August 2023. As each suction tower was sampled daily, the number of aphids caught was linked to weather data on the sampling day, to identify the weather conditions, in which *S. avenae* migrate. Figure 3.3 shows an

overview of the number of *S. avenae* caught per day in both Oak Park and Cork, in relation to the maximum temperature and sum of rain on the day of capture. The lowest maximum temperature, at which *S. avenae* flight was recorded was 13.0 °C (one aphid caught on 28/05/2021 in Oak Park), and the highest maximum temperature was 31.6 °C (six aphids caught on 18/07/2022 in Oak Park). Looking at the precipitation, out of the 931 *S. avenae* caught in total, 707 (75.94%) were caught on days were there was no, or almost no rain (between 0 mm and 0.1 mm) at all. The highest number of aphids caught on a single day was 45 (on 15/07/2021 in Oak Park; max temp. 24.8 °C and 0 mm rain).

Figure 3.4 shows the daily number of aphids caught in relation to the maximum temperature and sum of rain over a four-year period, split into the month of capture. No migrating *S. avenae* were caught between the five winter months from November to March, and only one *S. avenae* each was caught in October and April. The results show that *S. avenae* migration in Ireland starts in May, peaks in July, and declines through August until it finishes in September. Of the 931 *S. avenae* caught in between 2020 and 2023, 692 (74.33%) were caught in July, making it the most abundant month for *S. avenae* migration in Ireland.

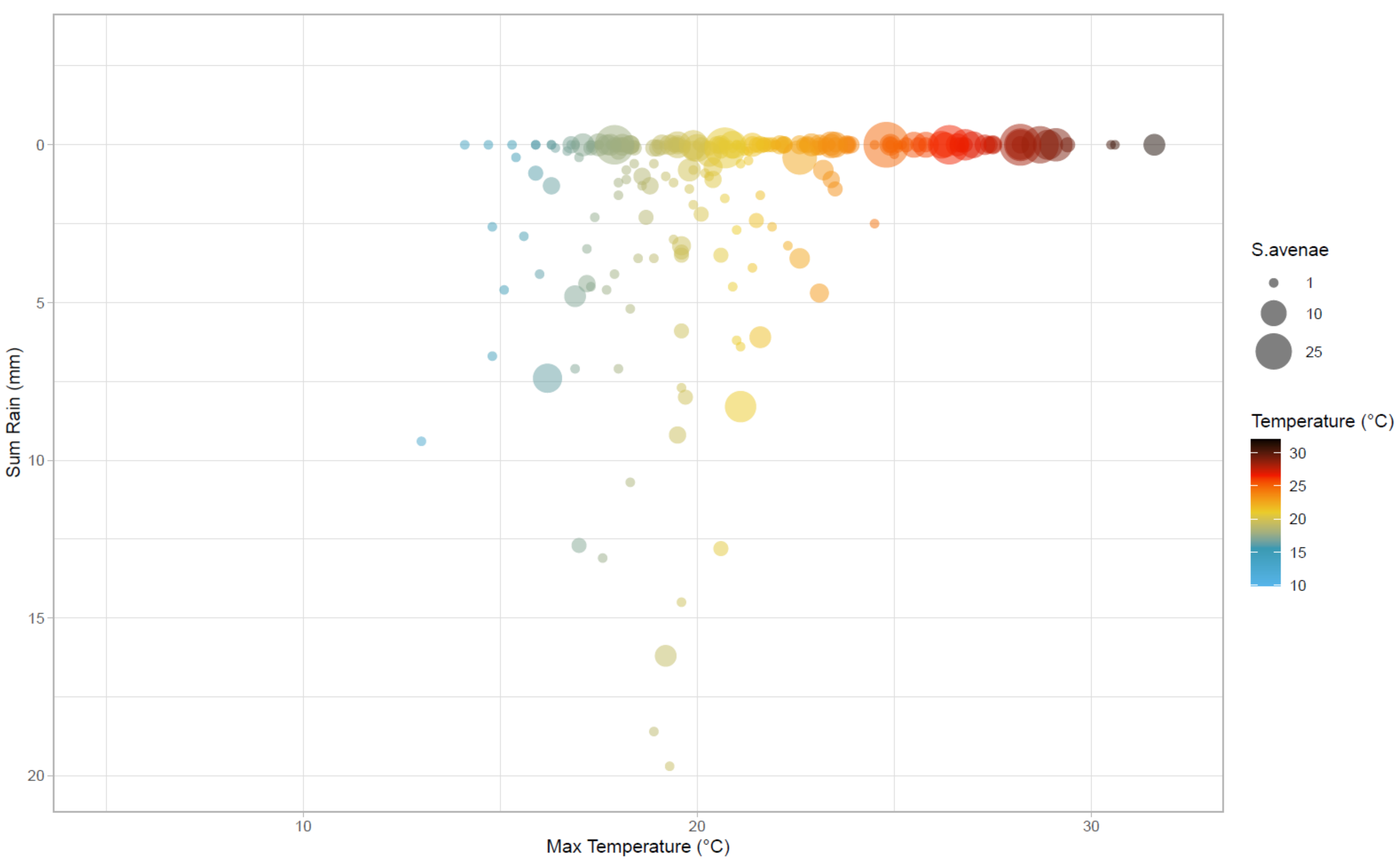

Figure 3.3: Daily number of migrating *S. avenae* depending on two weather conditions (Temperature and Rain) caught by two Irish Suction Towers (Cork and Carlow) in between 2020 and 2023.

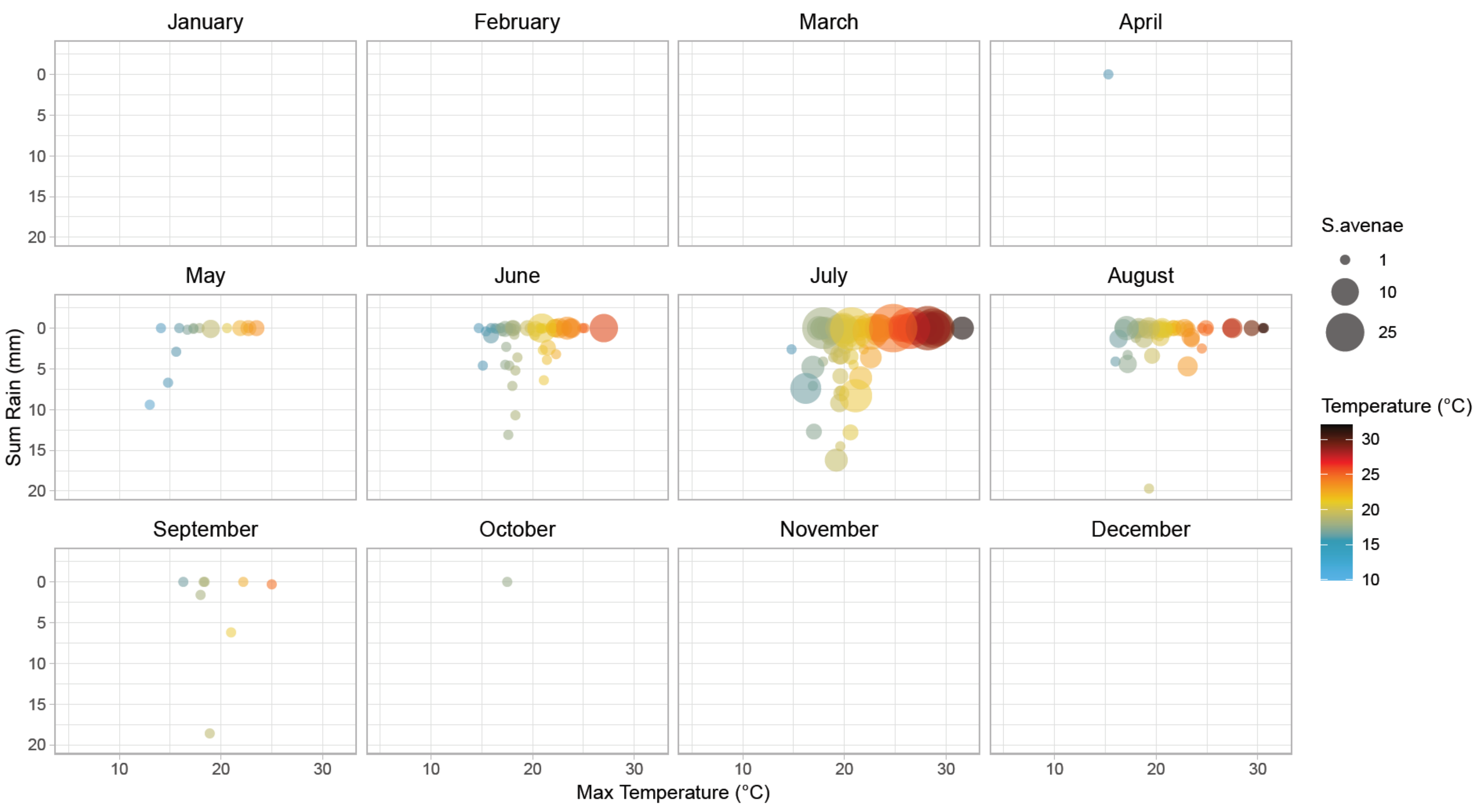

Figure 3.4: Daily number of migrating *S. avenae* depending on two weather conditions (Temperature and Rain) caught by two Irish Suction Towers (Cork and Oak Park) in between 2020 and 2023, separated by month.

3.3.3 Correlation of *Sitobion avenae* migration and weather parameters

3.3.3.1 Daily temperature and *Sitobion avenae* migration

The daily number of *S. avenae* caught in two suction towers in Cork and Oak Park between 2020 and 2023 was correlated with the maximum temperature on the day of capture. The results show a moderate and significant correlation between an increased temperature and the number of aphids caught per day (Fig. 3.5) (R = 0.32, $p < 0.001$).

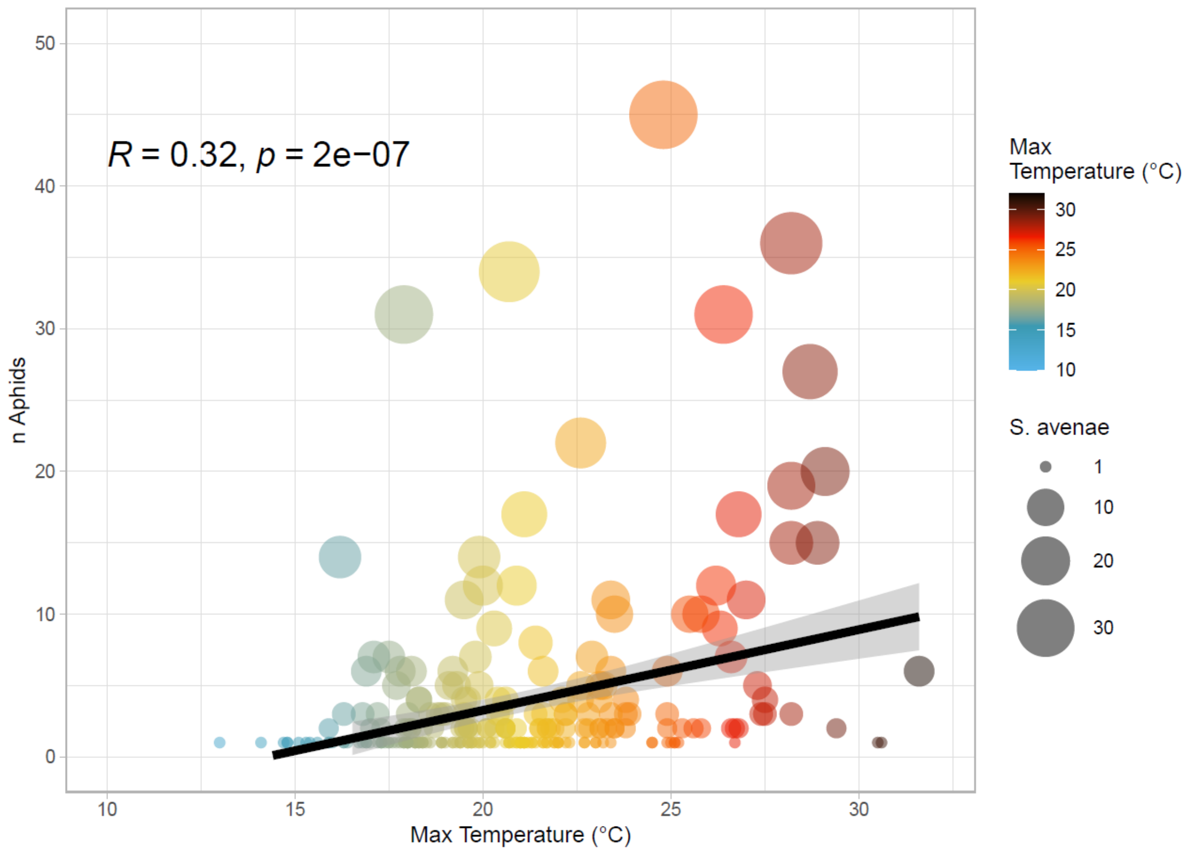

Figure 3.5: Daily numbers of migrating *S. avenae* caught in the Cork and Oak Park suction towers between 2020 and 2023, depending on the daily maximum temperature.

3.3.3.2 Daily sum of rain and *Sitobion avenae* migration

The daily number of *S. avenae* caught in the suction towers in Carlow and Cork between 2020 and 2023 was also correlated with the sum of rain per day. The results show a significant but very weak negative correlation with fewer aphids caught in increasing amounts of rain per day (Fig. 3.6) (R = -0.071, p = 0.049). However, most *S. avenae* (68.24%) were caught on days without rain.

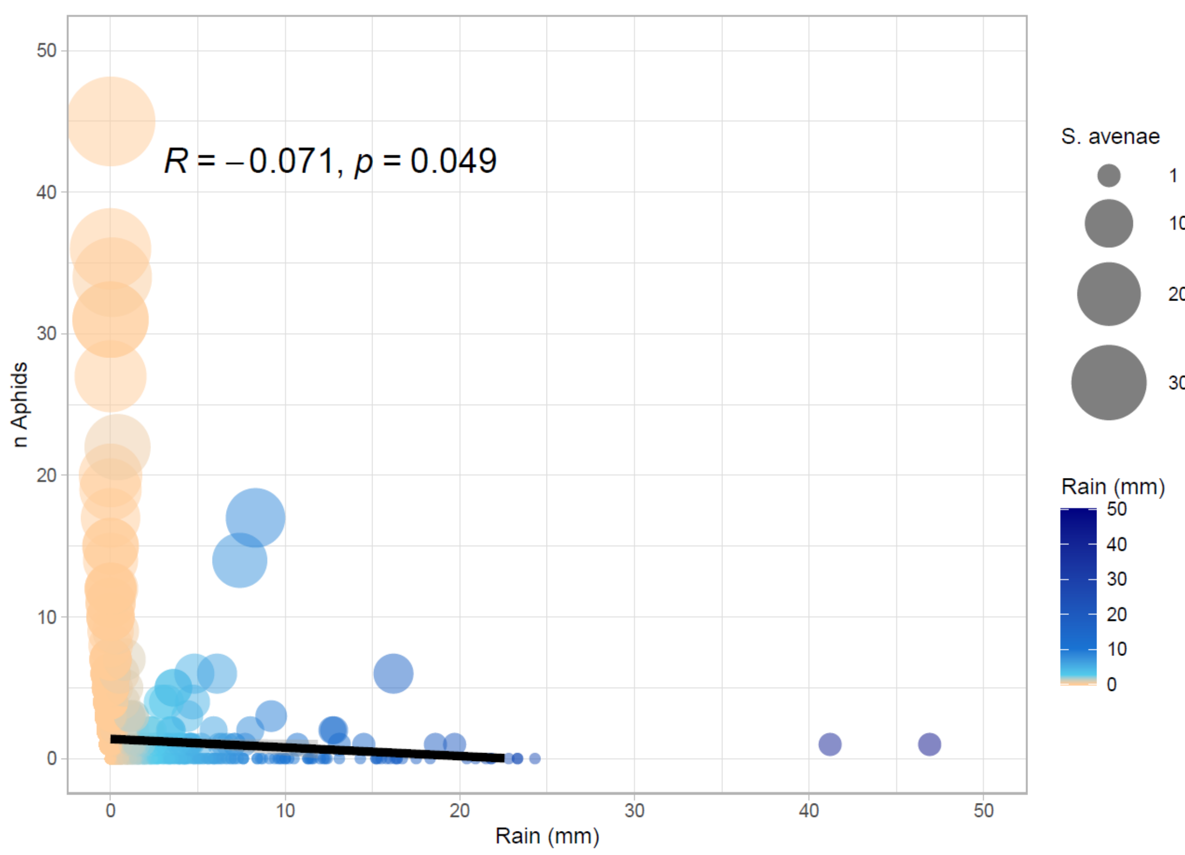

Fig. 3.6: Daily numbers of migrating *S. avenae* caught in the Cork and Oak Park suction towers between 2020 and 2023, depending on the sum of rain on the day of capture.

3.3.3.3 Wind speed and *Sitobion avenae* migration

The daily number of *S. avenae* caught in the Cork and Oak Park suction towers between 2020 and 2023 was correlated with the respective average wind speed on the day of capture. The results show a significant weak negative correlation between increased wind speed and a reduced number of aphids caught per day (Fig. 3.7) ($R = -0.22$, $p < 0.001$).

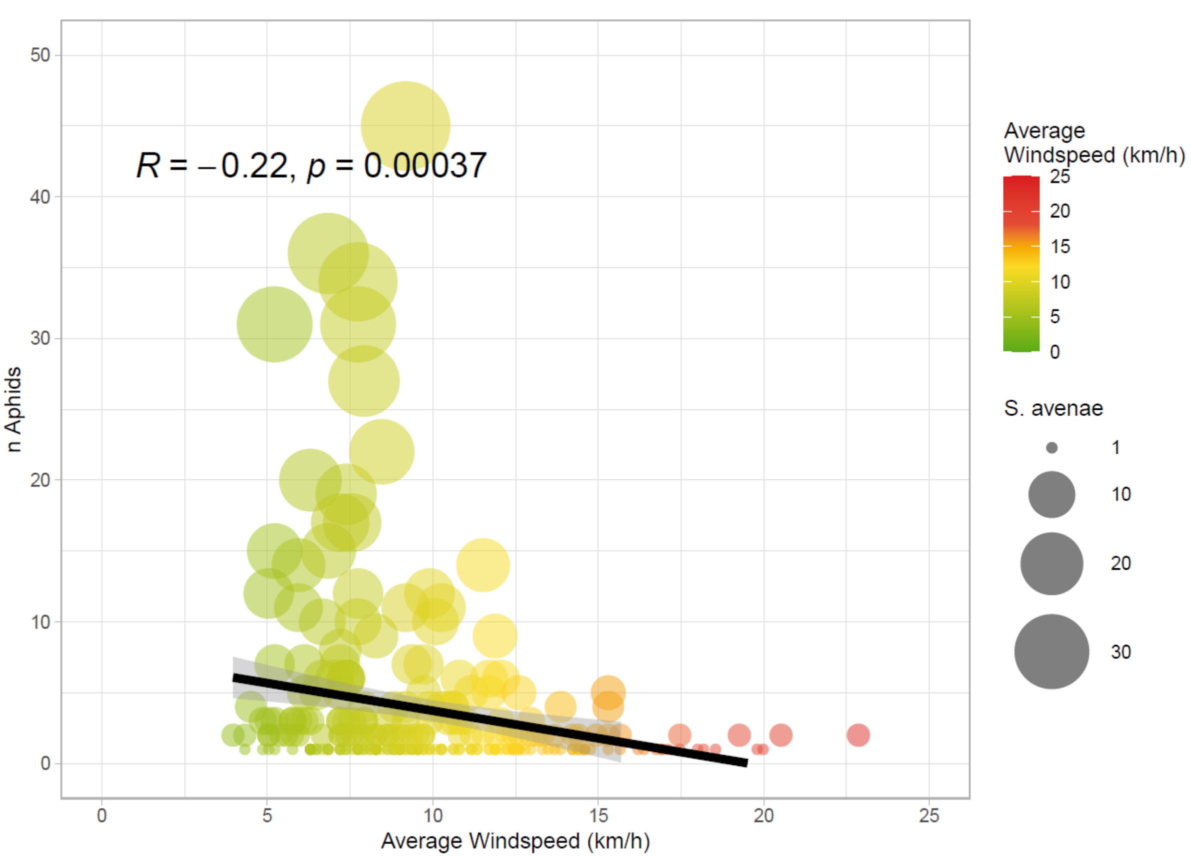

Figure 3.7: Daily numbers of migrating *S. avenae* caught in the Cork and Oak Park suction towers between 2020 and 2023, depending on the average wind speed on the day of capture.

3.3.3.4. General linear model of *Sitobion avenae* numbers

In order to analyse the relationship between environmental factors which influence *S. avenae* flight, a generalized linear model with multiple predictors including daily weather conditions (max temperature, wind, and rain), year, month, calendar week and suction tower location was employed, to find the best predictor for *S. avenae* migration, and to test whether there is any interaction between the factors.

Table 3.2
Analysis of deviance between the predictors.

predictors	LR Chisq	Df	p-value
Temperature (max)	14.81	1	0.0001186
Rain	0.2779	1	0.5981
Wind	19.4	1	1.061e-05
Year	36.17	3	6.909e-08
Month	2.007	5	0.8481
Location	42.54	1	6.909e-11
Calendar Week	22.29	19	0.2702

The analysis of deviance (Table 3.2), for the model with no interaction between the main effects showed how each predictor contributed to explaining the variability in the daily numbers of *S. avenae*. The results showed that the maximum temperature, wind, year, and location were significant for the model (at 5%). This shows that temperature and wind are significant environmental predictors of *S. avenae* abundance, with temperature being positively associated and wind being negatively associated with aphid numbers. Rainfall, calendar week, and month did not significantly affect daily *S. avenae* numbers. However, there are significant yearly variations in *S. avenae* numbers, with 2021 showing a notable increase compared to the other years. Finally, the main effects model indicated that the Oak Park suction tower had significantly higher *S. avenae* counts than the Cork suction tower.

A second model, including the interaction between the predictors, showed that there is a significant interaction between maximum temperature and the Oak Park location

(p = 0.00116) with a coefficient of 0.10020, indicating that the relationship between temperature and *S. avenae* numbers differs between the two suction tower locations.

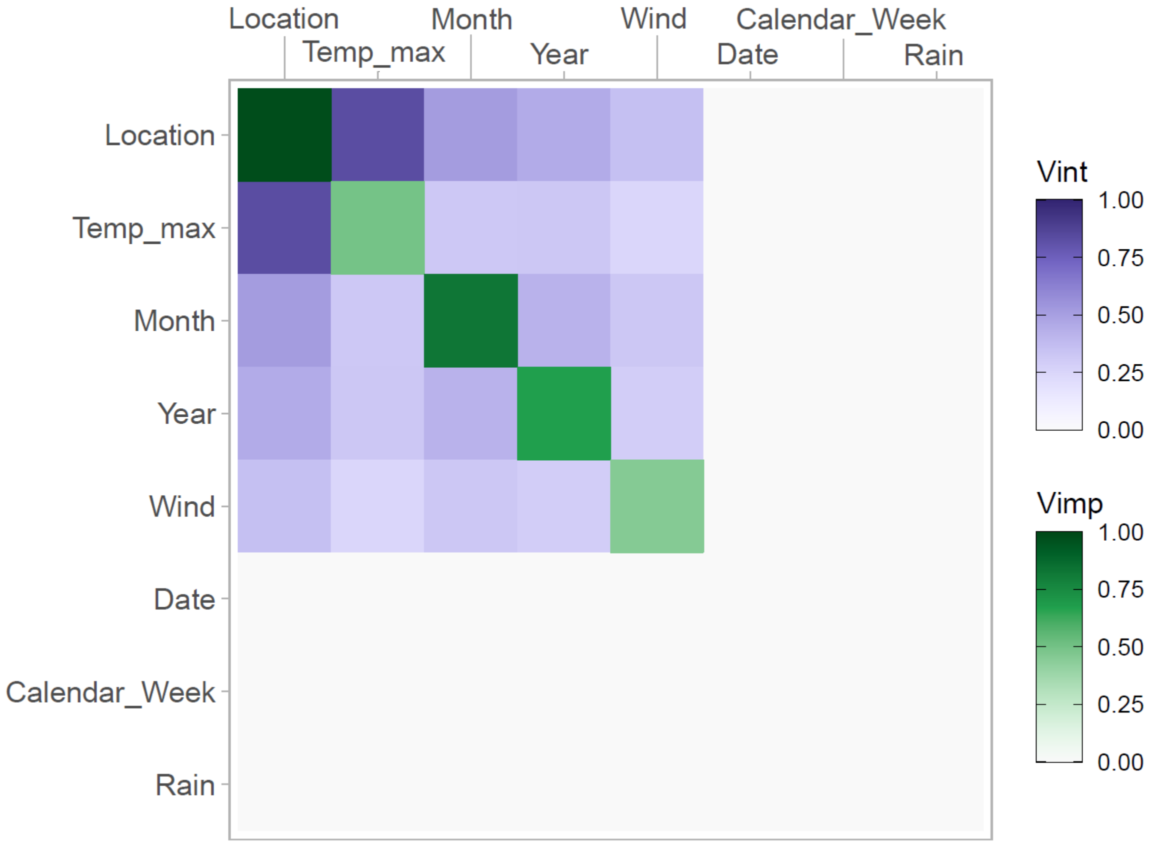

Figure 3.8: Heatmap showing the most important variables (Vimp) and interactions (Vint) within the model that explain daily *S. avenae* numbers caught in the suction tower network.

A heatmap was created to show the most important factors influencing *S. avenae* migration (Vimp), as well as their interaction (Vint) (Fig. 3.8). The results show that the suction tower location is the most important variable, followed by temperature, month, year, and wind, whereas collection date, calendar week, and rain are not relevant. Furthermore, the primary interaction effect identified is between suction tower location and temperature.

3.3.4. Incidence of resistance and BYDV in migrating *Sitobion avenae*

S. avenae caught in the Oak Park suction tower in 2020, 2021 and 2022, were tested for both resistance (*kdr*) and BYDV status. The weekly numbers of *S. avenae*

(including their resistance and BYDV status) caught per week in both suction towers are shown in Figure 3.9 (combined three-year period of sampling) and in Figure 3.10 (split between 2020, 2021 and 2023).

Between 2020 and 2022, a total of 559 migrating *S. avenae* (75 in 2020, 460 in 2021, and 127 in 2022) were caught in the Oak Park suction tower, of which 11.8% tested positive for resistance (*kdr*) and 14.7% tested positive for BYDV. There was no significant correlation between aphid resistance and BYDV status found (chi-squared = 0.0108, df = 1, p-value = 0.9172). However, resistance levels significantly varied between the sampling years with only 7.4% resistance (although very high aphid numbers) in 2021, 21.4% in 2020 and 28.4% of resistance in 2022 (chi-squared = 43.8713, df = 1, p-value < 0.001) (Tab. 3.3). The three-year average percentage of resistance in migrating *S. avenae* was 19.06% (SD +/- 8.73%). The BYDV status of migrating *S. avenae* also significantly varied between the three years of sampling (chi-squared = 25.957, df = 1, p-value < 0.001), with the highest percentage of BYDV positive *S. avenae* in 2020 (32.0%), followed by 2021 (12.2%), and 2022 (7.8%) (Tab. 3.3). The three-year average percentage of BYDV-carrying migrating *S. avenae* was 17.37% (SD +/- 10.50%).

Table 3.3
Percentages of BYDV and *kdr* carrying *S. avenae* caught in the Oak Park suction tower between 2020 and 2022. SD: Standard deviation

Year (n aphids per year)	BYDV positive		BYDV negative	
	kdr pos.	*kdr* neg.	*kdr* pos.	*kdr* neg.
2020 (n=75)	6.7%	25.3%	14.7%	53.3%
2021 (n=460)	0.9%	11.3%	6.5%	81.3%
2022 (n=127)	2.4%	5.5%	26.0%	66.1%
All (n=559)	1.8%	12.9%	10.0%	75.3%
Three year average	3.33% (SD +/- 2.46%)	14.03% (SD +/- 8.31%)	15.73% (SD +/- 7.99%)	66.9% (SD +/- 11.44%)

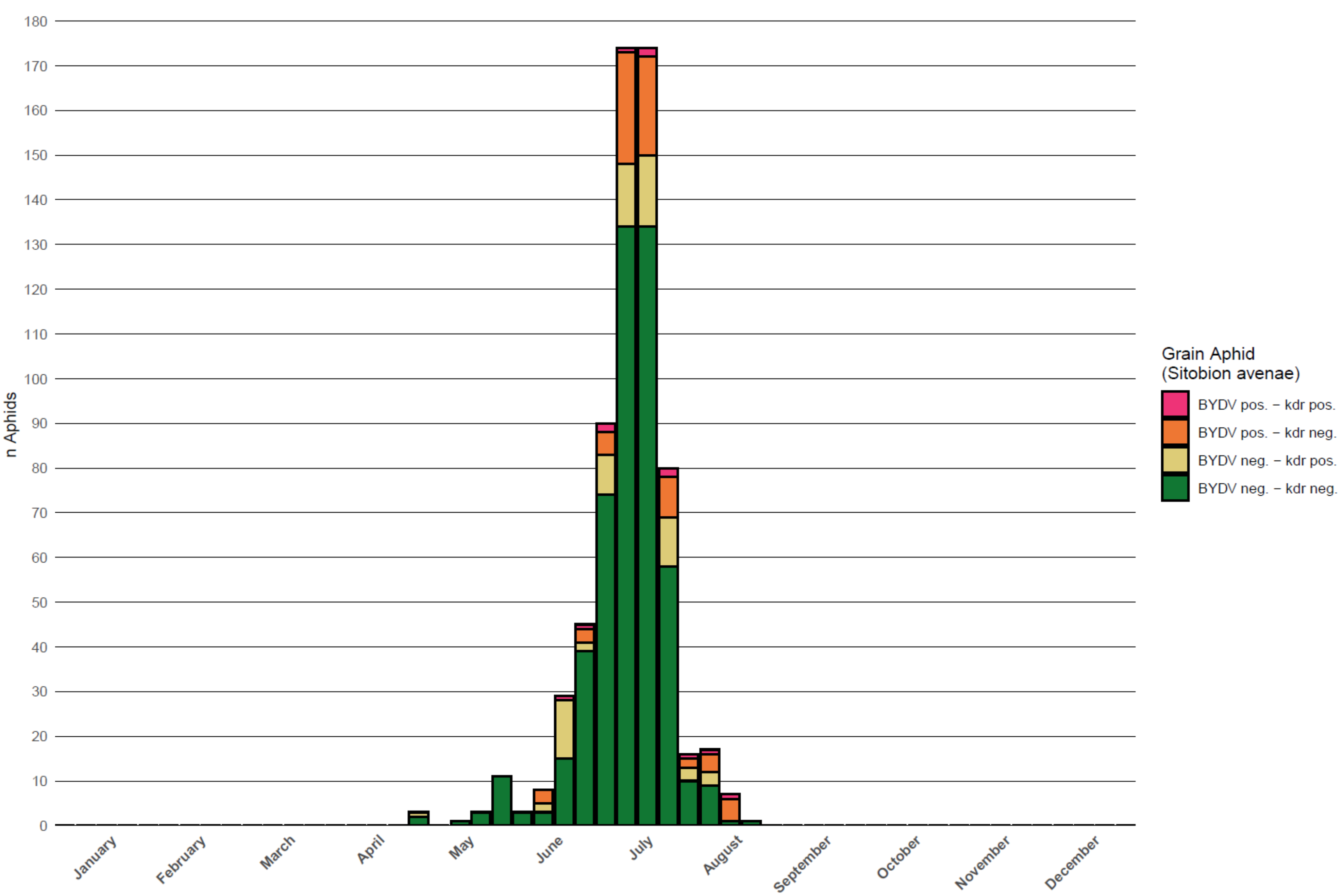

Figure. 3.9: Migration timing, resistance and BYDV status of *S. avenae* caught in between January and December in the Oak Park suction tower between 2020 and 2023.

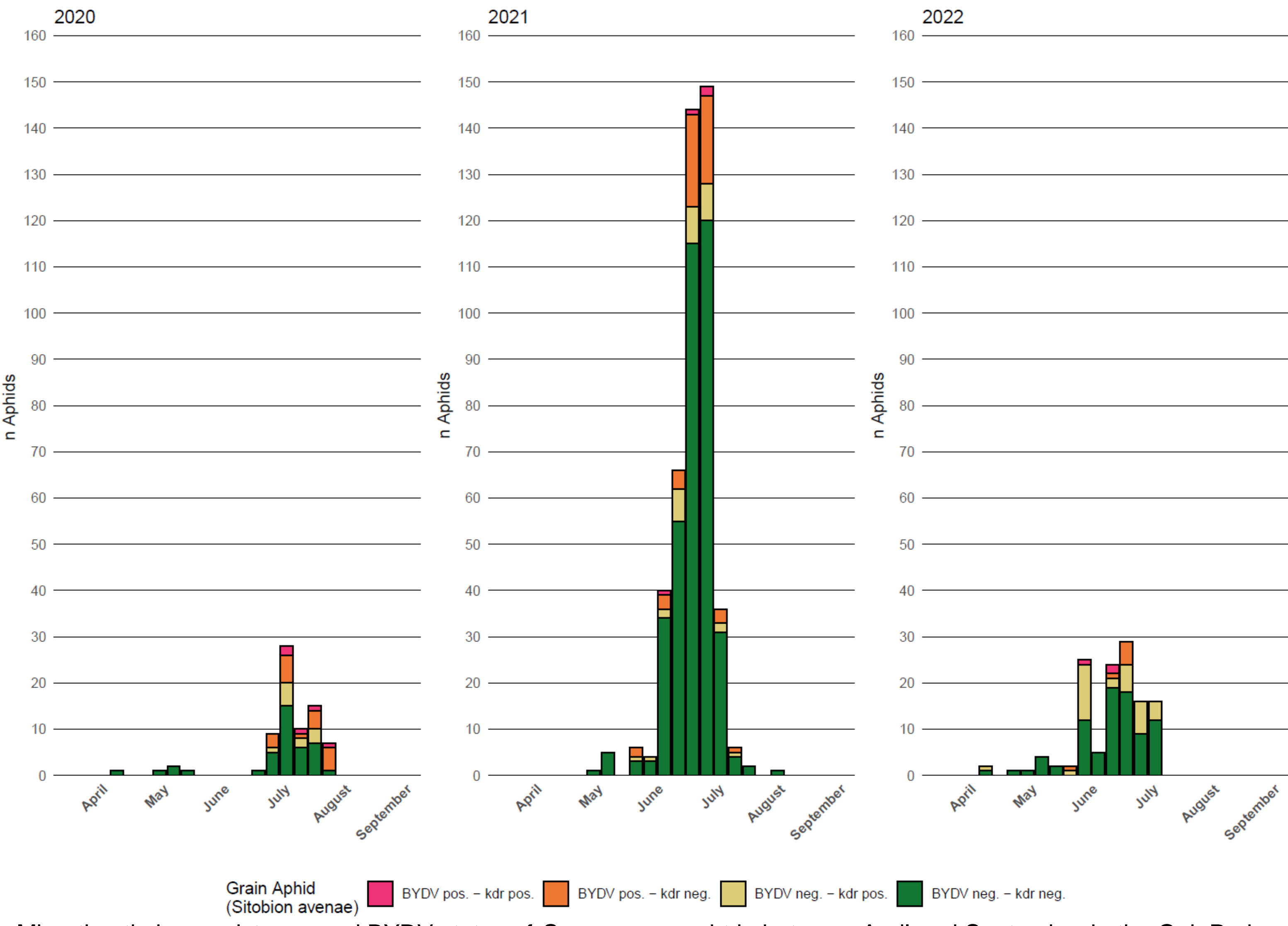

Figure. 3.10: Migration timing, resistance and BYDV status of *S. avenae* caught in between April and September in the Oak Park suction tower, split by sampling year (2020, 2021 and 2022)

3.3.5 Comparison of fieldwork and suction tower data

Table 3.4 shows a direct comparison between BYDV and resistance (kdr) levels in *S. avenae* caught during fieldwork (in between 2021 and 2023) and *S. avenae* caught in the Oak Park suction tower (in between 2020 and 2022). The results showed that the three-year average levels of resistance in migrating *S. avenae* caught between 2020 and 2022 were at 19.06% (SD +/- 8.73%) and that 17.37% (SD +/- 10.50%) of *S. avenae* carried BYDV. In comparison, the three-year averages of *S. avenae* that were collected in winter barley fields in between 2021 and 2023 differed, with slightly lower resistance levels at 11.34% (SD +/- 14.71), but 2.7 x higher BYDV levels at 47.57% (SD +/- 24.9%).

Table 3.4
Comparison between BYDV and resistance (*kdr*) carrying *S. avenae* collected from Fieldwork (Chapter 2) and through the Oak Park suction tower (this Chapter). SD: Standard deviation.

	Fieldwork						Oak Park Suction Tower					
	BYDV positive		BYDV negative		BYDV positive total	*kdr* positive total	BYDV positive		BYDV negative		BYDV positive total	*kdr* positive total
Year	*kdr* positive	*kdr* negative	*kdr* positive	*kdr* negative			*kdr* positive	*kdr* negative	*kdr* positive	*kdr* negative		
2020	-	-	-	-	-	-	6.7%	25.3%	14.7%	53.3%	32.0%	21.4%
2021	0%	13.2%	1.9%	84.9%	13.2%	1.9%	0.9%	11.3%	6.5%	81.3%	12.2%	7.4%
2022	19.8%	38.3%	12.3%	29.6%	58.1%	32.1%	2.4%	5.5%	26.0%	66.1%	7.7%	28.4%
2023	0%	71.4%	0%	28.6%	71.4%	0%	-	-	-	-	-	-
Three year average	6.60% (SD +/- 9.33%)	40.96% (SD +/- 23.83%)	4.74% (SD +/- 5.41%)	47.70% (SD +/- 26.31%)	47.57% (SD +/- 24.9%)	11.34% (SD +/- 14.71%)	3.33% (SD +/- 2.46%)	14.03% (SD +/- 8.31%)	15.73% (SD +/- 7.99%)	66.9% (SD +/- 11.44%)	17.37% (SD +/- 10.50%)	19.06% (SD +/- 8.73%)

3.4. Discussion

Beginning in 2019, a 12.2 m suction tower network, consisting of three towers in Oak Park (Carlow), Ashtown (Dublin), and Castlemartyr (Cork), was established in the Republic of Ireland, to sample migrating cereal aphids (such as *S. avenae*, *M. dirhodum,* and *R. padi*). A suction tower network has the benefit of providing a better understanding of aphid flight dynamics as well as landscape-level information about resistance and disease pressure throughout the year (Bell et al., 2015).

This research chapter set out to identify the length of the migration season and migration patterns, as well as the resistance and disease data of *S. avenae* caught in the first four consecutive years of sampling by a suction tower network. This creates the first dataset of its kind in the Republic of Ireland. The results showed that *S. avenae* migration in Ireland starts between April and May, peaks in July, and diminishes quickly throughout August and September, which is in line with previous data from the UK and Western Europe (Llewellyn et al., 2003; Taylor et al., 1981). In addition, *S. avenae* flight positively correlated with increasing temperatures, and negatively correlated with increasing rain and wind. However, even though these correlations were significant, they were weak, and analyses from the generalised linear model showed, that only temperature and wind significantly affected daily *S. avenae* flight numbers.

Data from four years of suction tower sampling also showed that the number of migrating *S. avenae* significantly varied between the years and the suction tower locations, which is expected due to differences in the spatial and temporal distribution of *S. avenae* from season to season (Winder et al., 1999). Aphid phenology and migration patterns depend on both climate drivers and agricultural practices (e.g. an unexpectedly warm winter or the ban of an insecticide), which also vary annually (Bell et al., 2015; Benton et al., 2002). The results from the generalised linear model showed that the number of *S. avenae* in the respective suction tower location also interacted with temperature, possibly explaining the higher *S. avenae* numbers in Oak Park than in Cork. These results indicate that complex models that also take seasonal, climate, and location data into account are required, to predict *S. avenae* migration accurately.

Aphid monitoring and modelling are important, as long-term data from the UK showed that aphid pest species have expanded their flight season and spatial synchrony, although a higher total number of aphids, due to a warmer climate, is not expected (Bell et al., 2015; Bell et al., 2020). However, any potential long-term trends in Ireland are still unknown, as aphid monitoring by the suction tower network has only been fully operational since 2020. Therefore, more long-term data will help to accurately study, predict, and model *S. avenae* migration, and therefore, resistance and disease pressure in Ireland in the future.

This study was the first to test both resistance and BYDV levels in migrating *S. avenae* collected in a 12.2 m suction tower over a three-year consecutive period in Ireland. The results indicated that the three-year average levels of resistance in migrating *S. avenae* caught between 2020 and 2022 were at 19.06% (SD +/- 8.73%) and 17.37% (SD +/- 10.50%) of *S. avenae* carried BYDV.

The suction tower data also showed that there was no evidence that insecticide resistant *S. avenae* are more likely to carry BYDV, as only 3.33% of *S. avenae* tested in a three-year period were carrying both *kdr* and BYDV. However, similar to the total number of *S. avenae* caught, resistance levels and BYDV levels also varied significantly in between the sampling years, which again, could be because *S. avenae* migration is mostly linked to seasonal patterns (Winder et al., 1999). Interestingly, the year with the highest number of *S. avenae* caught (460 aphids in 2021) was also the year with the lowest levels of resistance (7.4% *kdr*), compared to 2020 (75 aphids, 21.4% *kdr*) and 2022 (127 aphids, 28.4% *kdr*). A possible explanation for this phenomenon could be a fitness disadvantage that was shown to affect resistant aphids, which could benefit non-resistant *S. avenae* clones in years when the selection pressure by insecticides is low and the selection pressure by natural predators is high (Jackson et al., 2020). However, the analyses in this study for resistance and BYDV levels in migrating *S. avenae* only included one out of three suction towers in Ireland and was also limited to a period of three years. More long-term monitoring data are needed to determine if there is evidence that resistant *S. avenae* show a different migration pattern than other non-resistant *S. avenae* (Winder et al., 1999).

Investigating under which environmental conditions and at what time of the year resistant or BYDV carrying *S. avenae* migrate is of special importance, as it will improve our understanding of when crops are potentially at risk. Furthermore, including other cereal aphids in the monitoring and testing them for the presence of resistance genes or viruses could be a game changer in designing a DST. For example, a plausible scenario could be that the number of migrating aphids is high, but testing these aphids showed that resistance and BYDV levels are low, which would decrease the overall risk of infection. Although data from this thesis are limited to three years of fieldwork (between 2021 and 2023) and three years of suction tower sampling (between 2020 and 2022), the comparison of in-field monitoring and aerial monitoring showed that numbers of all cereal aphids differ, depending on the sampling method and the sampling year (Table 3.3). For example, *R. padi* was the most abundant cereal aphid in the suction tower network, but rarely found in any winter barley fields during fieldwork, while *M. dirhodum* was only present in the field in some years, but seems to have a stable number in the suction towers.

Comparing *S. avenae* abundance, resistance and virus level data from the suction tower network to data from in-field monitoring showed that *S. avenae* resistance and virus levels in Ireland also differ between the sampling methods. First, the three-year average *S. avenae* resistance levels were 1.6 times higher in the suction tower (19.06%, SD +/- 8.73%), in comparison to aphids collected in winter barley fields (11.34%, SD +/- 14.71; Chapter 2), although the variation in between the sampling years was high for both sampling methods. Second, *S. avenae* collected in winter barley fields showed 2.7 times higher levels of BYDV than *S. avenae* from the suction tower. This observed variation in resistance and BYDV levels between the field and aerial sampling could possibly be explained by the different sampling timings and setups. In-field monitoring of *S. avenae* was carried out exclusively in winter barley fields between May and June, just before aphid migration started. The suction tower, however, continuously samples throughout the year, showing a peak in *S. avenae* migration in July. From spring to summer, *S. avenae* change from unwinged to winged phenotypes due to their life cycle or nutritional changes in their host plant, and start to migrate to another host plant (Fereres et al., 2017). *S. avenae* have an extensive range of host plants, reaching from most cereals (e.g. Barley, Wheat, Maize, Rice etc.) to many wild Poaceae (Blackman & Eastop, 2008). As

suction tower sampling provides landscape-level information, it is impossible to determine from which of the many hosts a migrating aphid caught originated. This is in contrast to the aphid sampling from winter barley fields and could explain the 2.7 times higher levels of BYDV found, as aphids were collected directly from the main host of the virus. On the other hand, in two out of three years of field sampling, almost no resistant aphids were found in winter barley fields, while resistant *S. avenae* were always present in the suction tower. It is plausible that a background population of resistant *S. avenae* exists on the non-winter barley hosts. These resistant aphids are then detected in the suction towers during the migration season, even though they might not necessarily be present in winter barley crops (Winder et al., 1999). Nonetheless, in the future, aphid abundance data from both in-field and aerial sampling methods will be used to create aphid forecasting and DST to predict aphid and virus levels to allow efficient insecticide application, if necessary.

The survival of insecticide resistant aphids after spray application can affect BYDV epidemiology and crop production in Ireland. Through in-field and aerial monitoring, the first two research chapters of this thesis aimed to determine whether the incidence of pyrethroid resistant aphids increased after the 2018 ban on neonicotinoids, leaving pyrethroids as the only active ingredient available. In contrast to the hypothesis, the overall resistance levels in *S. avenae* have declined compared to the initial study by Walsh, Schmidt, et al. (2020). However, resistant *S. avenae* are thought to be better BYDV spreaders, as they were found to be associated with high BYDV levels in Ireland and as reported by Dewar and Foster (2017) in the UK. The next chapter therefore investigates the ability of the resistant *S. avenae* clone to successfully transmit the two predominant BYDV species in Ireland.

4

Investigating BYDV transmission efficiencies of insecticide resistant *Sitobion avenae* for two BYDV strains found in Ireland

4.1 Introduction

Crop infections by barley yellow dwarf viruses (BYDVs) can cause significant yield losses from 5% to 80%, and therefore present a high risk for cereal production worldwide (Perry et al., 2000; Poehling et al., 2017; Riedell et al., 2003). Symptoms of BYDV infection include yellowing of leaves, stunted growth, and reduced grain size, leading to an overall loss of grain quality and yield (Edwards et al., 2001). Seven species of the single-stranded RNA+ BYDVs are generally accepted (BYDV-PAV, BYDV-MAV, BYDV-PAS, BYDV-GAV, BYDV-SGV, BYDV-kerII, and BYDV-kerIII), all located within the genus *Luteovirus* in the taxonomic family Tombusviridae (Miller & Lozier, 2022). With a host range of over 100 Poaceae, including perennial grasses and crops such as barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), BYDVs are highly versatile in the range of plants they can infect (Irwin & Thresh, 1990). However, host range and severity of symptoms vary greatly between BYDV species (Baltenberger et al., 1987; Griesbach et al., 1990).

To spread within crops, BYDVs depend on aphid vectors (e.g. the English grain aphid *Sitobion avenae*; the rose-grain aphid *Metopolophium dirhodum,* and the bird cherry-oat aphid *Rhopalosiphum padi*). Aphids acquire the phloem-limited viruses by feeding on the sap of the infected plants. Virions in the sap then move through the vectors hindgut, haemocoel and saliva glands, to be transmitted via the aphid saliva onto a new host plant (known as circulative transmission) (Brault et al., 2010; Dáder et al., 2017). The initial viral load acquired by the aphid during the feeding process is important, as BYDV does not replicate within the vector (non-propagative virus) (Gray et al., 1991). Additionally, BYDV is transmitted in a persistent (long-time inoculation) manner, which can range from a few hours to multiple days of feeding time, meaning that transmission success will increase with a longer feeding time (Stevens & Lacomme, 2017). However, not all aphids can transmit different BYDV species with the same efficiency. For example, previous research has shown that *S. avenae* is a “very efficient” transmitter of BYDV-MAV (75-100% transmission rate), an “efficient” transmitter of BYDV-PAV (25-75% transmission rate), and that it can transmit BYDV-PAS, but the transmission rates are unknown (Van den Eynde et al., 2020).

To measure transmission efficiency, virus transmission experiments investigate the inoculation access period (IAP) and acquisition access period (AAP) (Dedryver et

al., 2005; Gray et al., 1991; Power et al., 1991). The inoculation access period describes the feeding time required by an infected aphid to successfully transmit the plant virus to a healthy plant (Gray et al., 1991). The acquisition access period describes the feeding time needed to acquire the virus from an infected plant and to successfully transmit the virus afterwards (Power et al., 1991). Both IAP and AAP can change under different environmental conditions, such as temperature or humidity. For example, the transmission efficiency can drop by 50% in a 10 °C colder environment and, almost no transmission is possible at temperatures below 12 °C (Choudhury et al., 2017; Van der Broek & Gill, 1980). However, variations in transmission efficiency can also be linked to the aphid species, aphid clonal lineage or different species/isolates of the virus itself (Dedryver et al., 2005).

To understand the complex relationships between each virus and their vector, it is essential to identify the viruses and aphids present in the respective crop. Worldwide, BYDV-PAV and its main vector *R. padi* are considered as the most widespread in winter barley (Aradottir & Crespo-Herrera, 2021). In Ireland though, previous studies found that the most prevalent BYDV species in winter and spring barley is BYDV-MAV (Kennedy & Connery, 2001, 2005). It is important to note that serological testing in these early studies was performed with ELISA (which is known to be prone to cross-reactions) only for these specific BYDV species; however more BYDV species exist in crops (Miller & Lozier, 2022). Additionally, new winter barley cultivars, which claim to be tolerant to specific BYDV species (e.g. the cultivar “Joyau“, tolerant to BYDV-PAV infections), have become available on the market. However, it is currently unknown whether the BYDVs species, against which the new barley varieties are tolerant, are widespread within Irish crops. A better understanding of all BYDV species present in crops and the impact of BYDV-tolerant plants is needed to determine whether BYDV resistance/tolerance genes can lead to a reduction in yield losses. In addition, Kennedy and Connery (2005) identified that in Ireland, the most important aphid for BYDV transmission in winter barley is *S. avenae,* which is in contrast to continental Europe, where *R. padi* is predominant.

S. avenae is a major agricultural pest worldwide including Ireland (Dixon, 1987; Kennedy & Connery, 2005; Kolbe & Linke, 1974). It feeds on the phloem sap of the leaves and ears of crops, causing damage directly by sap reduction or stomata blockage (by secreted honeydew) and indirectly through the transmission of BYDV.

Farmers often rely on insecticides to manage *S. avenae* in crops and reduce the risk of BYDV damage. However, repeated insecticide applications create a selection pressure that has led to the development of insecticide resistance to multiple compounds in at least 14 important agricultural aphid pest species such as *Myzus persicae*, *Aphis gossyphii, Schizaphis graminum,* and most recently *S. avenae* (Foster et al., 2017). In *S. avenae,* resistance (known as knock-down resistance, *kdr*) is caused by a heterozygous mutation in the voltage-gated sodium channel gene, causing up to 40-fold higher resistance to pyrethroids, compared to the wild type (Foster et al., 2014). In Europe, *kdr* in *S. avenae* was first detected in a clonal lineage (SA3) after spray failures in the UK in 2012 (Foster et al., 2014). Soon afterwards, more discoveries of partially resistant SA3 have followed in Ireland in 2016 (Walsh et al., 2019), in Germany and Belgium in 2017 (Poehling et al., 2017), in China in 2021 (Gong et al., 2021) and most recently in France in 2022 (Fontaine et al., 2023). Given the recent European Union regulations banning outdoor use of neonicotinoids due to their risk to pollinators, pyrethroids are the only insecticide compounds available to control aphid pests in crops (European Commission, 2018). This, in combination with the emergence of partial resistance in *S. avenae,* could challenge future crop production in Europe, as aphids that survive spray applications can continue to transmit and spread BYDVs (Blake, 2018; Jactel et al., 2019).

Little is known about whether the development of insecticide resistance affects the transmission efficiency of BYDV in *S. avenae*. Previous studies have shown that the fitness advantage of insecticide resistance is associated with pleiotropic fitness- and behavioural trade-offs, such as reduced fecundity, worsened response to alarm pheromones, and increased susceptibility to parasitation (Foster et al., 2007; Jackson et al., 2020). Given reports that widespread insecticide spray failures are linked to high BYDV outbreaks, it was hypothesised that the resistant *S. avenae* clone might be a more efficient BYDV transmitter than other clones of the population (Dewar & Foster, 2017).

4.1.1 Objectives

Because of its highly destructive potential as a crop pest, it is critical to study the BYDV transmission efficiencies of the resistant *S. avenae* clone to understand their role in BYDV epidemiology. This research chapter has three main objectives:

1) Identify the most predominant BYDV species in Irish barley.

2) Conduct BYDV transmission experiments to test the hypothesis, that the insecticide resistant *S. avenae* clonal lineage is a better BYDV transmitter than other susceptible *S. avenae* clones.

3) Conduct virus transmission experiments between different winter barley varieties, to test whether varietal BYDV tolerance affects virus transmission.

For objective 1), the most predominant BYDV species in Ireland were first identified through high-throughput sequencing of barley samples, which were collected during fieldwork in 2021 and 2022 (Chapter 2, Byrne et al. (2024)). For objective 2) and 3), *S. avenae* from eight different Irish counties were collected and genotyped, and AAP and IAP experiments with two BYDV species commonly found in Ireland (BYDV-MAV and BYDV-PAS) were conducted to identify potential differences in the BYDV transmission rates between multiple *S. avenae* clonal lineages. The transmission efficiency of partially resistant *S. avenae* (SA3 clone) was then compared to that of other clonal lineages without the *kdr* mutation. Additionally, in a third experiment, the transmission efficiencies of *S. avenae* and *R. padi* for transmitting BYDV-MAV and BYDV-PAS from a BYDV-susceptible winter barley variety (cultivar Etincel) onto a BYDV-tolerant variety (cultivar Joyau) were assessed, to investigate possible changes in transmission efficiency between different winter barley varieties.

4.2 Material and Methods

4.2.1 High-throughput sequencing of BYDV in Irish barley

4.2.1.1 Leaf sampling and RNA isolations

Leaf samples from eight Irish counties were collected from spring and winter barley crops in Ireland in 2021 and 2022. Within a transect of 20-50 m (depending on the field size), three to four leaves displaying symptoms of BYDV infection (yellowing of the leaves) were randomly sampled. Three non-overlapping transects were sampled per field, to obtain a total sample size of nine to twelve leaves per field. The samples were stored at -80 °C until further processing. To extract the leaf juice, the samples were placed in extraction bags (Bioreba, Reinach, Switzerland) with 2 ml of phosphate-buffered saline (PBS, pH 7) and ground with the aid of a tissue homogeniser attached to a motorised drill. Aliquots of the homogenised solution were used for ELISA and nucleic acid extraction. A double antibody sandwich enzyme-linked immunosorbent assay (ELISA) was performed to ensure the presence of BYDV in each sample before sequencing (see Chapter 4.2.6), and 48 samples were selected for RNA isolations and sequencing (3 aphid colony samples, 29 winter barley samples, and 16 spring barley samples (see Appendix Table A1) (Clark & Adams, 1977).

A Spectrum Total Plant RNA extraction kit (Sigma Aldrich, St. Louis, MO, USA) was used to isolate total RNA from the homogenised solution of samples confirmed to have BYDV infection, following a slightly modified protocol from the manufacturer. For this, 100 µl of homogenised leaf extract was transferred to a 1.5 ml micro-centrifuge tube and 400 µl of lysis buffer was added and vortexed. The lysate was transferred onto a (blue) filter column, centrifuged at 14,000 x g and an equal volume of 70% ethanol was added and vortexed. The lysate was then transferred onto a (red) binding column and spun down to allow binding of RNA onto the column. After a washing step and DNAse digestion with 80 µl of DNAse1/Digest buffer for 15 min at RT, the column was washed again with two washing buffers. Total RNA was eluted in 50 µl elution buffer and the concentration was measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The extracted RNA was shipped on dry ice to GeneWiz (Berlin, Germany) for library

preparation with rRNA depletion and sequencing on an Illumina platform (PE 150 bp).

4.2.1.2 Assembly and mapping of RNA sequencing data & phylogenetic analyses

Assembly, mapping, and phylogenetic analyses of RNA sequencing data was carried out as part of another study (Byrne et al., 2024). In the assembly, raw sequencing data, which usually consists of short RNA fragments, gets merged together to reconstruct the full RNA sequence (Bushmanova et al., 2019). These full sequences are then mapped against a database of BYDV reference genomes (Miller & Lozier, 2022; Sõmera et al., 2021), in order to identify each BYDV species and calculate their by phylogenetic relationships through maximum-likelihood methods (Guindon et al., 2010). The BYDV sequencing data were deposited in the Sequence Read Archive (SRA) at NCBI under the BioProject PRJNA918968. The final consensus sequences are available in GenBank under accession numbers OQ686645, OR771726-OR771729, and OQ686648-OQ686695.

4.2.2 *S. avenae* sampling, genotyping and virus rearing

Grain aphids (*S. avenae*) from eight Irish counties were collected in winter barley fields and reared in pint-sized bug dorms or 30x30x30 cm nylon cages (NHBS GmbH, Bonn, Germany) on winter barley (*Hordeum vulgare*; cultivar Etincel) at a day/night and temperature cycle of 16 h-24 °C / 8 h-20 °C. The aphids were genotyped by the James Hutton Institute, Invergowrie, Scotland, following a published protocol and five microsatellite loci (Sm10, SM12, Sm17, SaΣ4 and S16b) (Malloch et al., 2016; Simon et al., 1999). Eight *S. avenae* clones from seven different Irish counties were selected for the transmission experiments, including the insecticide resistant *S. avenae* clonal lineage SA3 and a previously unidentified clone “New_Irl” (Table 4.1). *R. padi* (RpIA clone) was collected in the Department of Yonne, France in 2012 and maintained on wheat (T*riticum aestivum*, cultivar Rubisko) at the identical day/night and temperature conditions of 16 h-24 °C / 8 h-20 °C (Pichon et al., 2022).

BYDV-MAV was reared on winter barley colony originating from a single virus positive *S. avenae* collected in county Laois, Ireland in 2021. After the development of symptoms in the plants, further serological tests and virus sequencing confirmed the virus species. BYDV-PAS (GenBank accession number OQ686687) was acquired from a single R. padi collected in France in 1989 by Chain et al. (2007) and also reared on winter barley in a colony of *R. padi* at a day/night and temperature cycle of 16 h-24 °C / 8 h-20 °C. Three months prior to all experiments, a BYDV-MAV infected *S. avenae* colony (SA27) and a BYDV-PAS infected *R. padi* (RpIA) colony were set up, which served as a BYDV source for the experiments described in Chapter 4.2.5, Chapter 4.2.6 and Chapter 4.2.7.

Table 4.1
S. avenae and *R. padi* clones used in the virus transmission experiments

Species	Clone	*kdr*	County / Country	Collection date	Crop
S. avenae	New_Irl	-	Wexford / Ireland	May 2021	Winter Barley
S. avenae	SA27	-	Carlow / Ireland	May 2021	Winter Barley
S. avenae	SA3	+	Carlow / Ireland	2017	Winter Wheat
S. avenae	SA38	-	Meath / Ireland	May 2021	Winter Barley
S. avenae	SA38_2	-	Kildare / Ireland	May 2021	Winter Barley
S. avenae	SA44	-	Tipperary / Ireland	May 2021	Winter Barley
S. avenae	SA44_2	-	Cork / Ireland	May 2021	Winter Barley
S. avenae	Sct_2016	-	Wicklow / Ireland	May 2021	Winter Barley
R. padi	RpIA	x	Yonne / France	2012	Winter Wheat

4.2.3 Resistance detection in *S. avenae*

All aphids used in the experiments were tested for the presence of *kdr,* as described in Chapter 2.2.4.

4.2.4 Achieving consistency in virus transmission experiments

Virus transmission experiments such as investigating the acquisition access period (AAP) or inoculation access period (IAP) are large-scale experiments that require accurate planning over a timeframe of multiple months. The experiments are therefore limited, as investigating multiple aphid clonal lineages or multiple plant cultivars at multiple AAP and IAP timings all at once would create an unmanageable experimental size. Therefore, it is important that all aphid clones/colonies and plants during the preparation and the experiments are receiving the exact same conditions. The experiments described below (in 4.2.5, 4.2.6 and 4.2.7) were therefore split up and each aphid clone was tested separately, with one clone tested per week. This ensures the same colony age for each clone, reared in consistent climatic conditions, and therefore guarantees comparable data throughout the experiments.

4.2.5 Acquisition access period

4.2.5.1 Acquisition access period experiment preparation

To produce BYDV-MAV or BYDV-PAS infected plants for the acquisition access period experiments, 10 day old winter barley plants (cultivar Etincel) were inoculated with three aphids from viruliferous colonies for 24 h. Aphids were removed and the plants were allowed to grow for three additional weeks in order to provide enough time for systemic virus infection of the plant. Each plant was tested for virus presence using ELISA (described in 4.2.8), prior to the experiments.

4.2.5.2 Acquisition access period experiments

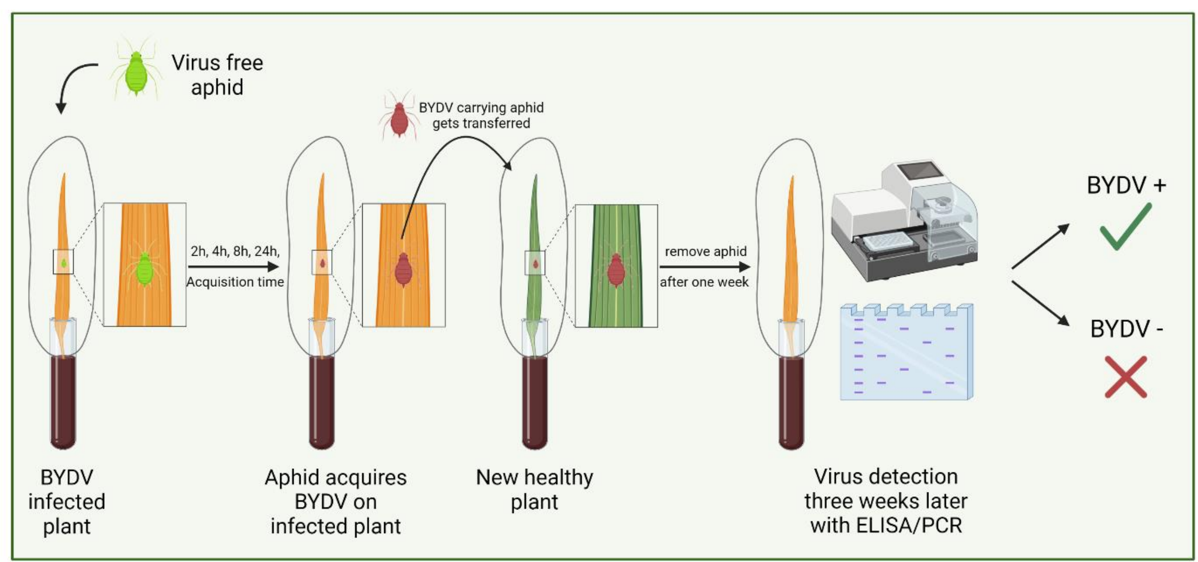

Figure 4.1: Experimental design of an acquisition access period experiment.

The virus acquisition access period (AAP) was tested in two separate experiments. Virus-free aphids were transferred onto infected barley plants for 2 h, 4 h, 8 h 24 h or 48 h for BYDV-PAS (experiment 1) and 24 h for BYDV-MAV and BYDV-PAS (experiment 2) (Fig. 4.1). After each respective AAP, single aphids (2nd to 4th instar nymphs) were transferred onto single plants and kept alive for a week to guarantee enough time for successful virus transmission. The aphids were reared on the barley plants for one week at a day/night and temperature cycle of 16 h-20°C / 8 h-20°C before insecticide treatment with Pirimor G® 0.1% vol/vol (Syngenta, Basel, Switzerland), followed by another two week growth period in the greenhouse to allow virus replication in the plants. The plants were tested for virus using either ELISA (BYDV-PAS; experiment 1) or by PCR (BYDV-PAS and BYDV-MAV; experiment 2). For each timing and clone, 20 plants were tested in three replicates, leading to a total of 1920 plants tested in AAP experiment 1 and 480 plants tested in AAP experiment 2.

4.2.6 Inoculation access period

4.2.6.1 Inoculation access period experiment preparation

Viruliferous aphid colonies are required to perform IAP experiments. These were set up by preparing virus-infected plants (as described in "AAP Preparation" section)

first, on which virus-free aphids of each respective clone were put onto for 24 h AAP. After this, the infected aphids and plants were transferred to a new 30x30x30 cm cage with uninfected winter barley plants. The colonies were given three weeks in order to allow the virus to spread inside the colony, prior to the IAP experiments.

4.2.6.2 Inoculation access period experiments

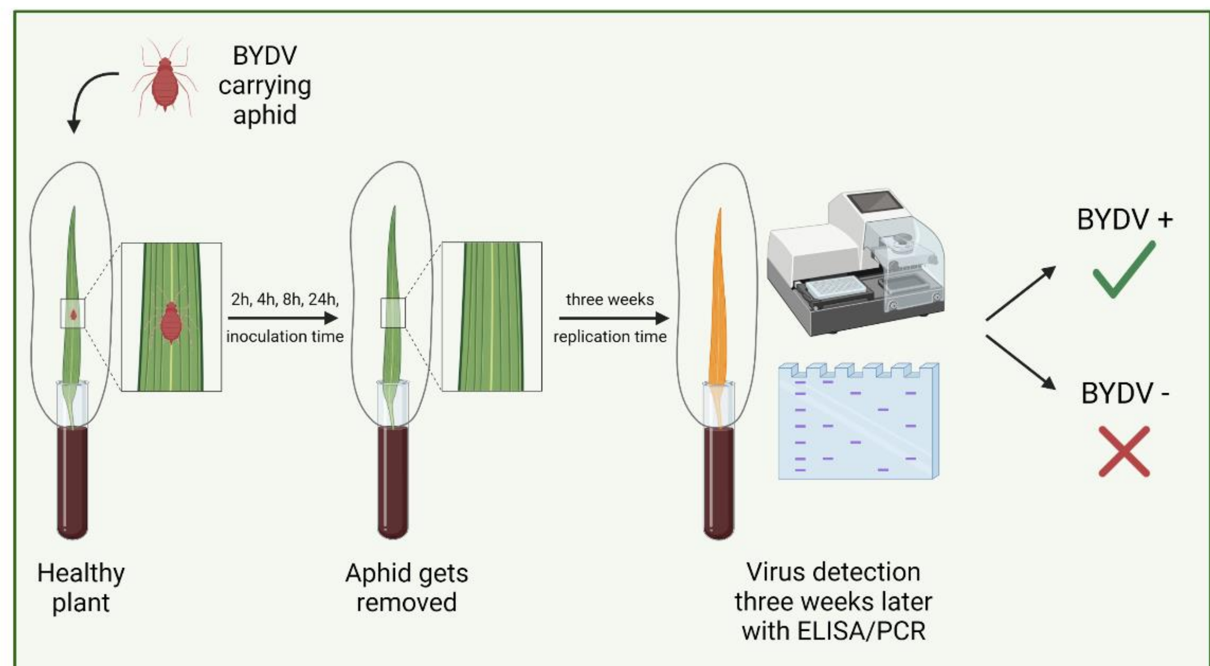

Figure 4.2: Experimental design of an inoculation access period experiment

Two separate Inoculation Access Period experiments were carried out with IAP's of 2 h, 4 h, 8 h, 24 h or 48 h for BYDV-PAS (experiment 1) and 24 h for BYDV-MAV and BYDV-PAS (experiment 2) (Fig. 4.2). For this, single 2nd to 4th instar nymphs from viruliferous colonies were placed on single healthy barley plants. At the end of each respective IAP, the aphids were removed and the plants were treated with the insecticide Pirimor G® 0.1% vol/vol (Syngenta, Basel, Switzerland), followed by a three-week growth period in the greenhouse at a day/night- and temperature cycle of 16 h-20 °C / 8 h-20 °C. Afterwards, the plants were tested for BYDV either by ELISA (experiment 1) or by PCR (experiment 2). For each IAP and aphid clone used in the experiments, 20 plants were tested in three replicates, leading to a total of 1920 plants tested in IAP experiment 1 and 480 plants tested in IAP experiment 2.

4.2.7 Etincel to Joyau transmission experiments

In a third experiment, the transmission ability of BYDV-PAS and BYDV-MAV from a BYDV-susceptible winter barley variety (cultivar Etincel) onto a BYDV-tolerant variety (cultivar Joyau) was assessed. For this, the AAP and IAP after 8 h and 24 h of aphids that were either carrying BYDV-PAS (*R. padi)* or BYDV-MAV (*S. avenae,* SA3 clone) were transferred from Etincel plants onto Joyau plants as described above (4.2.5 and 4.2.6). For each timing, virus and AAP/IAP, four replicates of 20 plants were tested, leading to a total of 640 plants tested.

4.2.8 Virus detection in aphids and plants

Virus detection in plants (for virus sequencing and experiment 1) was performed using a double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977), using commercially available kits for BYDV (Bioreba, Reinach, Switzerland) or per Pichon et al. (2022). Virus detection in experiments 2 and 3 was performed using cDNA synthesis, PCR and gel electrophoresis.

For the pre-screening ELISA for the BYDV sequencing, commercially available kits for BYDV (BYDV-B complete kit 960, BYDV-F complete kit 960 and BYDV-RPV complete kit 960; Bioreba, Reinach, Switzerland) were used, following a slightly modified protocol provided by Bioreba ("Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA): test specifications"; Version 4 – 18/10/2017). For this, 100 µl of 1:1000 diluted antigen/coating buffer was pipetted into each well of a Nunc MaxiSorop F96 microtiter plate. After a 3.5 hr incubation at 37 °C, the wells were washed three times with 150 µl washing buffer. 100 µl of the plant extract and 100 µl of positive and negative controls (provided with the kits) were added to each well and overnight incubated at 4 °C. After another washing step, 100 µl of 1:1000 diluted antibody/conjugate buffer was added and incubated at 37 °C for 4 h, followed by a final washing step. Next, 100 µl of pNPP/substrate buffer (1mg/ml concentration), was pipetted into each well, followed by incubation for 30 min, 1 h and 2 h to allow the coloration of the wells. After each of the respective times, the plates were scanned using an ELISA Reader at a wavelength of 405 nm. Where necessary, threshold levels were manually calculated after the provided protocol by Bioreba. For this, a cut-off ("step") value was determined and the mean of all values

below this step value was calculated. Three times the standard deviation was added to this value and the value was multiplied by 1.1. All values above the cut-off were considered as BYDV positive, however, in most cases, the sample value was already 5-10 times higher than that of the control, making the cut-off calculation obsolete. From over 200 winter- and spring barley fields tested with ELISA, a variety of the highest scoring samples from each county were further selected for sequencing (see Appendix Table A1).

For the ELISA in experiment 1 (BYDV-PAS, AAP, and IAP), Nunc™ MaxiSorp™ flat-bottom 96 well plates were prepared with 100 µl of 1:1000 diluted antigen/coating buffer (polyclonal anti-BYDV antibodies PAV52, H. Lapierre, INRAE; Coating buffer: 15 mM Na2CO3, 35 mM NaHCO3, pH 9.6). After a 3.5 h incubation at 37 °C, the plates were washed three times with 150 µl phosphate-based saline washing buffer (PBS: 137 mM NaCl, 8 mM Na2HPO4, 12H2O, 2.7 mM KCl, 1.5 mM KH2PO4). Plant material was extracted using either a leaf juice press (MEKU) or by hand in leaf juice extraction bags provided by Bioreba. 100 µl of the plant extract was added to each well and incubated overnight at 4 °C. After another washing step, 100 µl of 1:1000 diluted antibody/conjugate buffer (alkaline phosphatase-coupled antibody PAV52, H. Lapierre, INRAE; Conjugate buffer: Phosphate-buffered saline with Tween 20 (PBST) with 2% wt/vol ovalbumin; 137 mM NaCl, 8 mM Na_2HPO_4, $12H_2O$, 2.7 mM KCl, 1.5 mM KH_2PO_4, 0.05% vol/vol Tween 20) was added and incubated at 37 °C for 3 h. After the final washing step, 100 µl of pNPP/substrate buffer (p-nitrophenylphosphate (1 mg/ml); substrate buffer: diethanolamine (1 M, pH 9.8), was pipetted into each well following an incubation time of 30 min, 1 h, and 2 h to allow coloration of the wells. After each of the respective times, the plates were scanned using an ELISA spectrophotometer (Multiskan™ FC; Thermo Scientific™) at a wavelength of 405 nm. The positive threshold of the test was three times the OD_{405} value of the healthy plant controls, with a minimum value of OD_{405}=0.1.

BYDV cDNA synthesis in experiments 2 and 3 was carried out by extracting aphids and plants using same sucrose DNA extraction method as described in Chapter 2.2.4. Leaf material from plants was acquired by standardised cutting and discarding 4 cm of the tip of the main shoot, followed by another 0.5 cm cut, which was taken as the sample for extraction. For cDNA synthesis, 10.5 µl master mix (consisting of

1 µl random hexamers (0.2 µl/µg) and 9.5 µl ddH_2O) were combined with 2 µl plant extraction material in a 96-well PCR plate, following an incubation at 65 °C for 5 min to allow the random primers to bind to the RNA. The cDNA synthesis was performed by adding another 7.5 µl master mix (consisting of 4 µl of 5X Reaction Buffer RevertAid (200 U/µl), 0.5 µl of Ribolock RNase (40 U/µl), 2 µl of dNTP Mix (10mM) and 1 µl of RevertAid Reverse Transcriptase (5X)) to each reaction well of the plate. The final volume of 20 µl was incubated at 25 °C for 10 min, 42 °C for 60 min and 70 °C for 10 min. The cDNA was stored at -80 °C. The PCR was conducted with 1 µl of cDNA and a 24 µl of master mix consisting of 1 µl BYDV forward primer (10 µM; BYDV-PAS: GAAGAGGGCCAAATTCTATACC; BYDV-MAV: GTTACAAGATCACAAACGTCAAG), 1 µl of Reverse primer (10 µM; YanR: TGTTGAGGAGTCTACCTATTTG), 12.5 µl of Classic++ Taq MM (2X) and 9.5 µl of ddH_2O per well (Sõmera et al., 2021). The viral cDNA was amplified using a three stage amplification program starting with a pre-step for 1 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 15 s at 62 °and 15 s at 72 °C, and finally 3 min at 72 °C. Amplification of cDNA was confirmed using gel electrophoresis. For this, 5 µl of the PCR product was mixed with 1 µl of 10% loading buffer and 4 µl of H_20 and transferred on a 1.5% agarose gel (3.75 g agarose, 250 ml 0.5% TBE buffer) alongside a molecular ladder, run for 60 min at 90 V and analysed on an ENDURO™ GDS Gel Documentation System (Labnet, Cary, USA) .

4.2.9 Data analysis

The transmission percentages for each replicate were arcsin square root transformed and tested using a Shapiro test for normality. Normally distributed data were analysed by two-way ANOVA; non-normally distributed data were analysed by Kruskal-Wallis test, followed by Dunn test for multiple comparisons. Results were considered statistically significant at $p<0.05$. All statistical analyses were carried out using R studio (Ihaka & Gentleman, 1996) and the packages reshape2, scales, dplyr, emmeans, FSA, drc, ggpubr, and superb. Data graphs were created using ggplot2 (Wickham, 2011) and experimental design figures were created using Biorender (www.BioRender.com).

4.3 Results

4.3.1 BYDV-PAS transmission

The first experiment was conducted to compare the BYDV-PAS transmission rates of the insecticide resistant SA3 clone versus seven other susceptible *S. avenae* clones. Overall, BYDV-PAS transmission was low in both acquisition access period (AAP) and inoculation access period (IAP) experiments, with most clones showing average transmission rates below 10% after 24h. For AAP (Fig. 4.3), no significant differences between the clonal lineages were found (chi-squared = 11.391, df = 7, p-value = 0.1224). The newly detected Irish clonal lineage "New_Irl" showed the highest average transmission rate of 12%, while "SA44" and the insecticide resistant clonal lineage "SA3" showed 0% BYDV-PAS transmission rates after 24h AAP. As the differences between the clones were not significant, the mean AAP of BYDV-PAS value of all Irish *S. avenae* combined lies at 2.94% (SD +-3.86%).

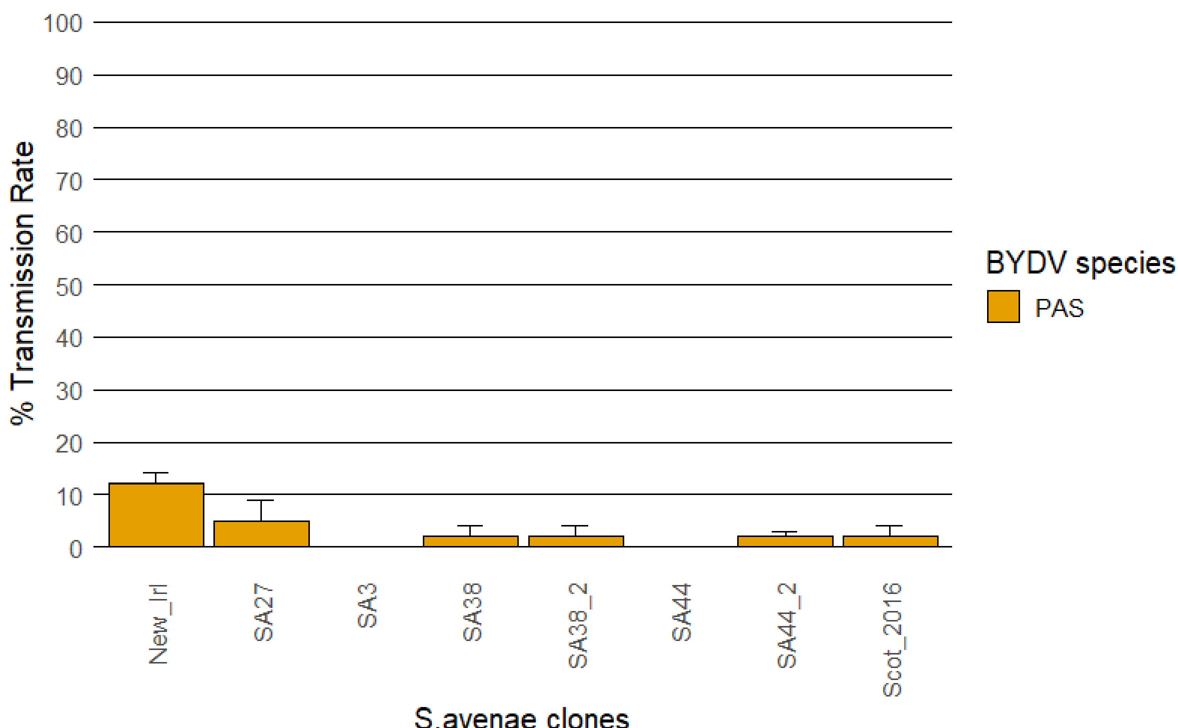

Figure 4.3: Average BYDV-PAS transmission rates onto healthy winter barley plants (Horteum vulgare, cultivar Etincel) after a 24 h acquisition access period (AAP) on infected winter barley plants by eight *S. avenae* clones. For each clone, three replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with ELISA for virus presence. Average values and the standard deviation for each clonal lineage (New_Irl. SA27, SA3, SA38, SA38_2, SA44, SA44_2 and Scot_2016) are displayed.

Transmission rates obtained for the four different AAPs of BYDV-PAS tested are listed in Table 4.2. The results show that the overall transmission rates were low and that there was no increase in transmission with an increase in the AAP. As the experiment continued for multiple months and the first results indicated unexpectedly low BYDV-PAS transmission rates after 24 h, the last two clones (SA44 and SA44_2) were tested for an AAP of 48 h in order to check whether the AAP needs to be extended. However, an AAP of 48 h also showed no increase in transmission (Table 4.2). As a control for the transmission experiments, a single rep of 80 plants was conducted with *R. padi* instead of S. avenae as a vector. This procedure showed a BYDV-PAS transmission rate ranging from 30% at 2 h AAP to 60% at 24 h AAP (Table 4.2).

Table 4.2
Average BYDV-PAS transmission rates of eight *S. avenae* clones after five acquisition access periods. *A single rep of 80 plants using *R. padi* was tested, in order to confirm that the experimental setup was working. SD: Standard deviation.

		AAP 2h	AAP 4h	AAP 8h	AAP 24h	AAP 48h
Clone	County	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)
New_Irl	Wexford	0 (0)	1.7 (2.9)	1.7 (2.9)	11.7 (2.9)	-
SA27	Carlow	0 (0)	1.7 (2.9)	0 (0)	5.0 (5.0)	-
SA3	Carlow	0 (0)	0 (0)	0 (0)	0 (0)	-
SA38	Meath	3.3 (2.9)	1.7 (2.9)	0 (0)	1.7 (2.9)	-
SA38_2	Kildare	1.7 (2.9)	0 (0)	0 (0)	1.7 (2.9)	-
SA44	Tipperary	-	0 (0)	3.3 (5.8)	0 (0)	3.3 (2.9)
SA44_2	Cork	-	1.7 (2.9)	0 (0)	1.7 (2.9)	5.0 (8.7)
Sct_2016	Wicklow	0 (0)	0 (0)	0 (0)	1.7 (2.9)	-
*R. padi**	-	*30 (-)*	*30 (-)*	*50 (-)*	*60 (-)*	-

Transmission rates of BYDV-PAS after 24 h IAP were also found to be low, with most average transmission rates under 10% (Fig. 4.4). Again, no significant differences between the clonal lineages were found (chi-squared = 9.9667, df = 7, p-value = 0.1905). The mean IAP transmission rate of all *S. avenae* lineages combined after 24h was found to be at 2.13% (SD +-2.4%).

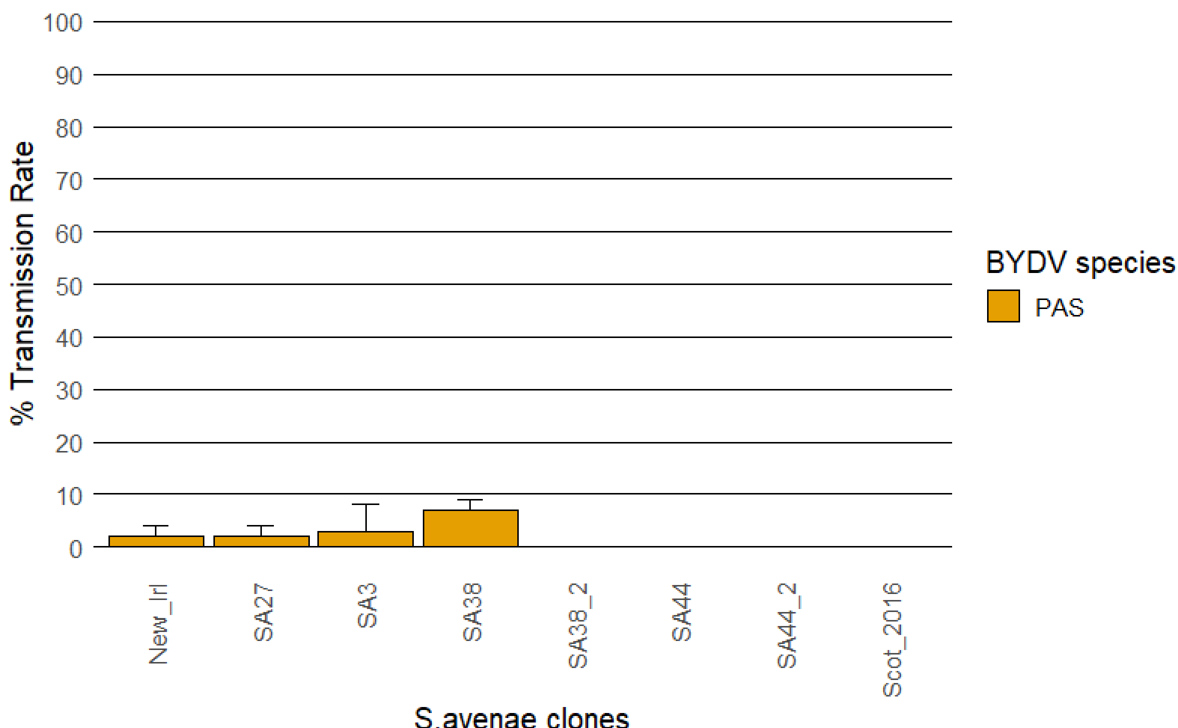

Figure 4.4: Average BYDV-PAS transmission rates of viruliferous aphids onto healthy winter barley plants (Horteum vulgare, cultivar Etincel) after a 24 h inoculation access period (IAP) by eight S. avenae clones. For each clone, three replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with ELISA for virus presence. Average values and the standard deviation for each clonal lineage (New_Irl. SA27, SA3, SA38, SA38_2, SA44, SA44_2 and Scot_2016) are displayed.

The IAP transmission rates of BYDV-PAS over time are displayed in Table 4.3 with results showing that there are no significant differences in inoculation times towards transmission rates and that there is no increase in transmission over time. Three clones (SA38_2, SA44 and SA44_2) were tested for an IAP of 48 h, but the longer inoculation time did not increase the BYDV-PAS transmission rates.

Table 4.3
Average BYDV-PAS transmission rates of eight *S. avenae* clones after five inoculation access periods. SD: Standard deviation.

		IAP 2h	IAP 4h	IAP 8h	IAP 24h	IAP 48h
Clone	County	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)	Mean % (+/- SD)
New_Irl	Wexford	0 (0)	0 (0)	0 (0)	1.7 (2.9)	-
SA27	Carlow	0 (0)	0 (0)	0 (0)	1.7 (2.9)	-
SA3	Carlow	0 (0)	0 (0)	0 (0)	3.3 (5.8)	-
SA38	Meath	0 (0)	0 (0)	1.7 (2.9)	6.7 (2.9)	-
SA38_2	Kildare		1.7 (2.9)	0 (0)	0 (0)	5.0 (8.7)
SA44	Tipperary	-	1.7 (2.9)	0 (0)	3.3 (5.8)	0 (0)
SA44_2	Cork	-	1.7 (2.9)	0 (0)	0 (0)	0 (0)
Sct_2016	Wicklow	1.7 (2.9)	0 (0)	0 (0)	0 (0)	-

4.3.2 BYDV-MAV transmission

In the second experiment, three *S. avenae* clones were tested for their ability to transmit BYDV-MAV compared to BYDV-PAS. After 24 h AAP (Fig. 4.5), the insecticide resistant clone "SA3" showed the highest average transmission efficiency (40%, SD +-5%), followed by "SA44" (34%, SD +-9%) and "SA27" (26%, SD +-5%). However, transformed data indicate no significant differences between the insecticide resistant clone "SA3" and the two other susceptible clones tested ($F=3.4362$, $p=0.1013$). The mean BYDV-MAV AAP transmission rate of all three clones combined after 24 h was at 33.07% (SD +-7.21%). Additionally, BYDV-PAS transmission rates from experiment 1 were replicated with *R. padi* showing 49% (SD +-10.1%) transmission, and again, all *S. avenae* clones showed low BYDV-PAS transmission rates.

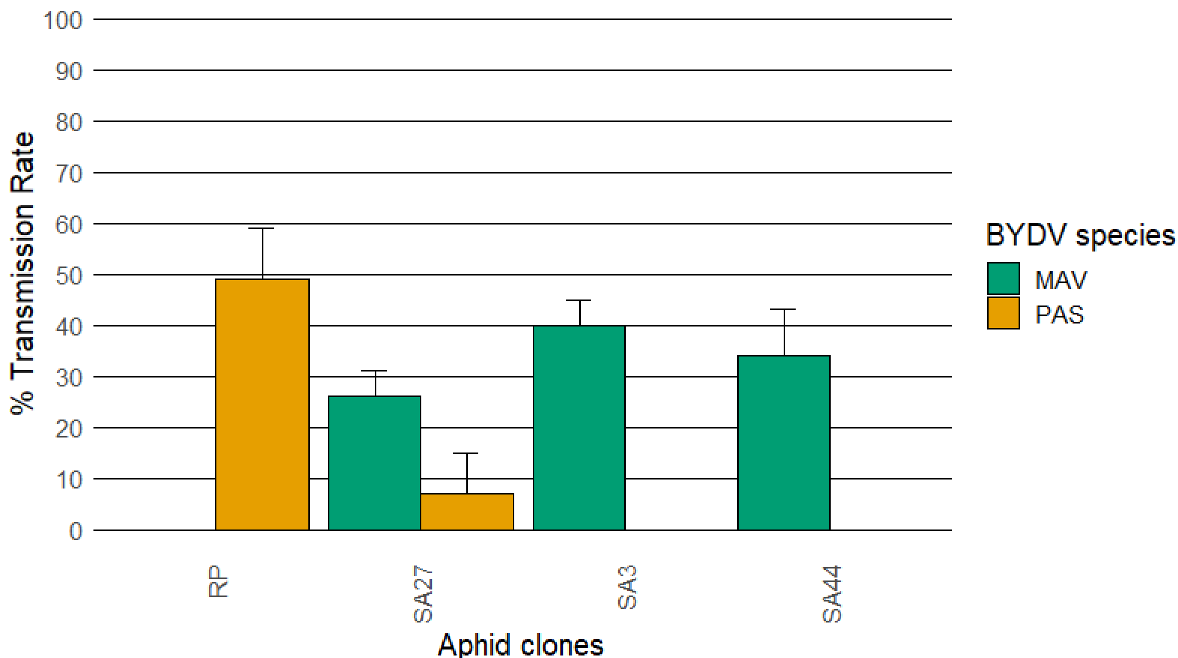

Figure 4.5: Average BYDV-MAV and BYDV-PAS transmission rates onto healthy winter barley plants (Horteum vulgare, cultivar Etincel) after a 24 h acquisition access period (AAP) on infected winter barley plants by one *R.padi* and three *S.avenae* clones. For each clone, three replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with PCR for virus presence. Average values and the standard deviation for each clonal lineage (R.padi, SA27, SA3 and SA44) are displayed.

The results of the 24 h IAP transmission rates of BYDV-MAV are shown in Figure 4.6. In this experiment, the insecticide resistant clonal lineage showed the lowest average transmission rate (26%, SD +-4%). However, "SA44" (28%, SD +-13%) and

"SA27" (30%, SD +-10%) showed only slightly higher transmission rates. Again, no significant differences between the clonal lineages were found (F=0.0788, p=0.9252) and the mean transmission rate of all *S. avenae* clonal lineages combined was found to be at 27.83% (SD +-2.04%).

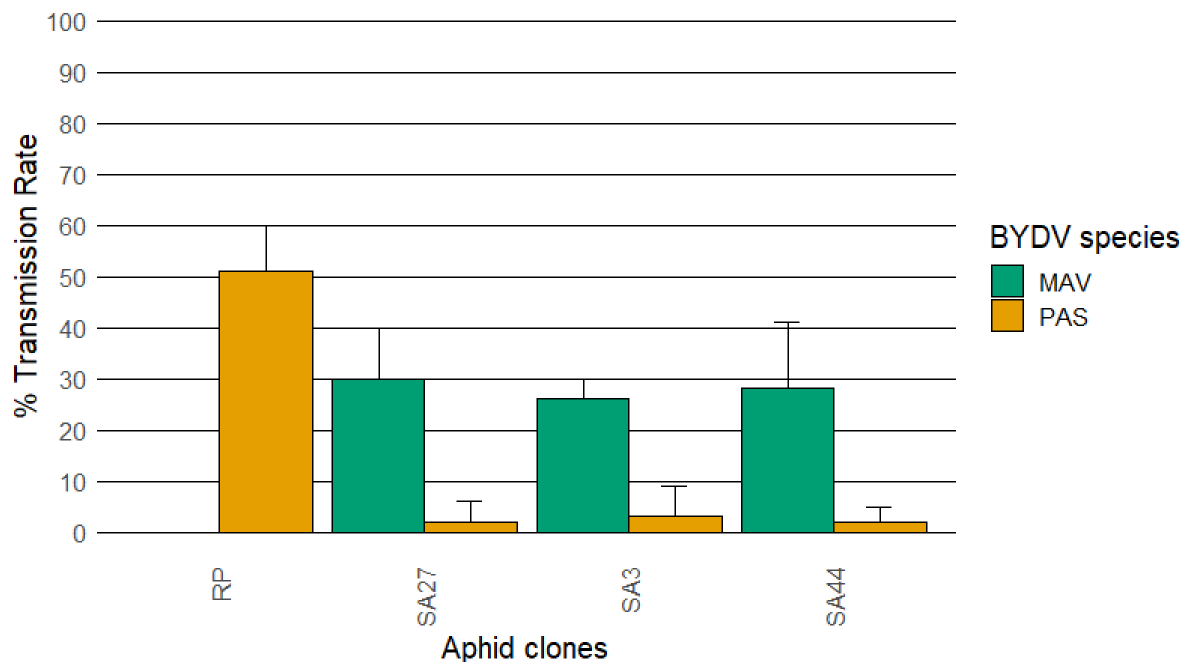

Figure 4.6: Average BYDV-MAV and BYDV-PAS transmission rates of viruliferous aphids onto healthy winter barley plants (Horteum vulgare, cultivar Etincel) after a 24 h inoculation access period (IAP) by one *R. padi* and three *S. avenae* clones. For each clone, three replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with P for virus presence. Average values and the standard deviation for each clonal lineage (R.padi, SA27, SA3 and SA44) are displayed.

4.3.3 Virus transmission rates with a BYDV-tolerant variety

In the third experiment, the virus transmission rates of BYDV-MAV (*S. avenae*, SA3 clone) and BYDV-PAS (*R.padi*) from a BYDV-susceptible winter barley (cultivar Etincel) onto a BYDV-tolerant variety (cultivar Joyau) were tested for an AAP and IAP of 8 h and 24 h, respectively (Fig. 4.7). For BYDV-MAV, which was transmitted by *S. avenae*, the average transmission rate after an 8 h AAP was at 12% (SD +- 5.5%), but increased to 29.1% (SD +-15.7) after 24 h. For BYDV-PAS and *R. padi*, the average transmission rates were 6.5% (SD +- 2.3%) after 8 h AAP, and 6.8% (SD +-4.8%) after 24 h AAP, showing no increase in transmission rates after a longer AAP.

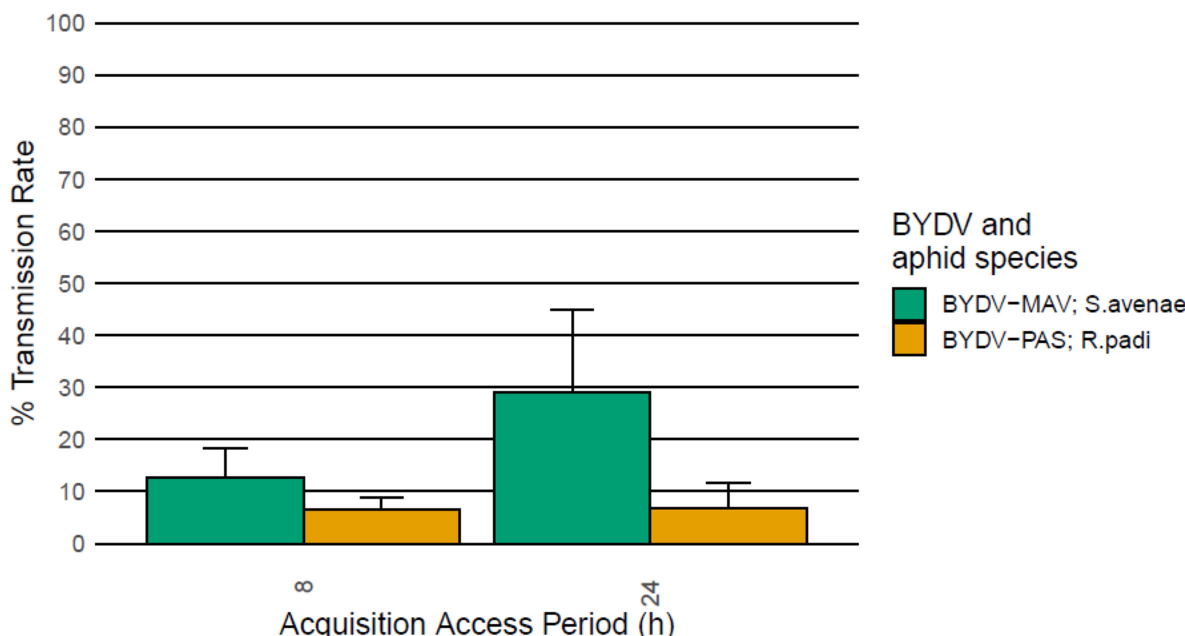

Figure 4.7: Average BYDV-MAV and BYDV-PAS transmission rates of viruliferous aphids from infected winter barley plants (cultivar Etincel) onto healthy winter barley (cultivar Joyau) after 8 h and 24 h acquisition access period (AAP) by *R. padi* and *S. avenae*. For each species, four replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with PCR for virus presence. Average values and the standard deviation for the two acquisition access periods, BYDVs and aphid species are displayed.

The results of the average transmission rates of BYDV-PAS onto Joyau after an IAP of 8 h and 24 h respectively are displayed in Figure 4.8. (Note: By the time this thesis was written, the IAP experiments for BYDV-MAV and *S. avenae* were not yet completed). The average BYDV-PAS transmission rates from Etincel to Joyau were

found to be at 26% (SD +-1.3%) after an IAP of 8 h, and at 30.7% (SD +-3.1%) after 24 h.

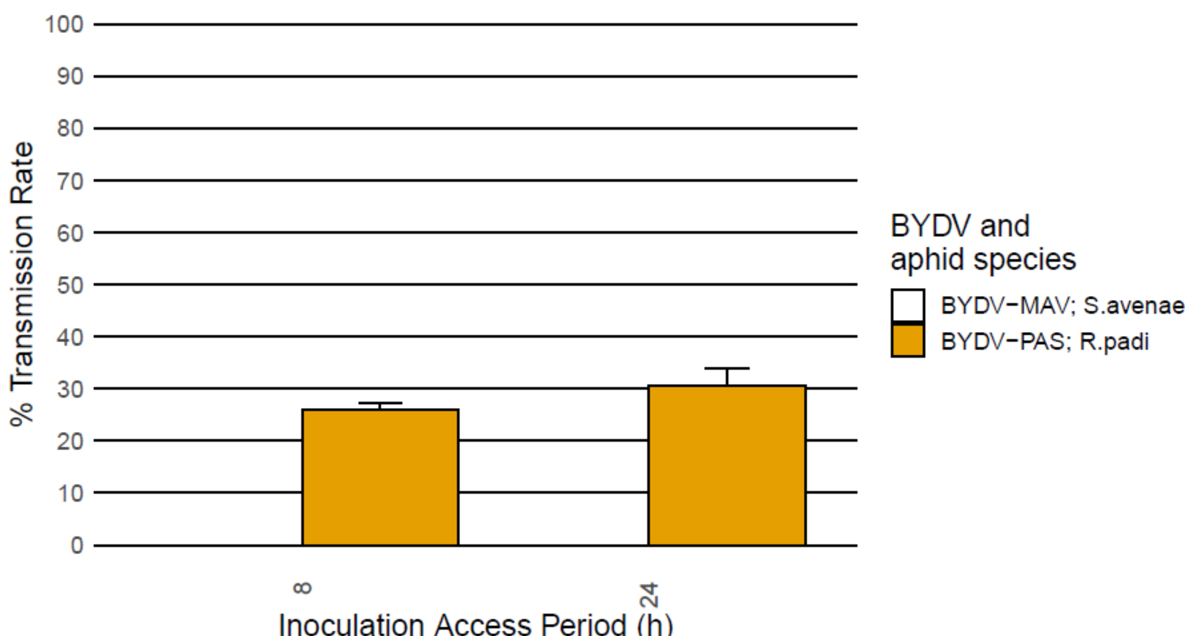

Figure 4.8: Average BYDV-MAV and BYDV-PAS transmission rates of viruliferous aphids from infected winter barley plants (cultivar Etincel) onto healthy winter barley (cultivar Joyau) after 8 h and 24 h inoculation access period (IAP) by *R. padi* and *S. avenae*. For each species, four replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with PCR for virus presence. Average values and the standard deviation for the two inoculation access periods, BYDVs and aphid species are displayed. NOTE: S. avenae data not yet available.

4.3.4 Comparison of BYDV transmission rates between Etincel and Joyau

A comparison of the average transmission rates between the varieties Etincel (from experiments 1 and 2) and Joyau (experiment 3) after an AAP of 24 h showed a significant decrease from 49.0% to 6.8% in average BYDV-PAS transmission by *R. padi*, when the virus was transmitted to the BYDV-tolerant variety Joyau (F=26.573, p=0.0036) (Fig. 4.9). However, there was no significant decrease in BYDV-MAV transmission by *S. avenae*, when the target plant variety changed from Etincel to Joyau (F=1.3532, p=0.2972).

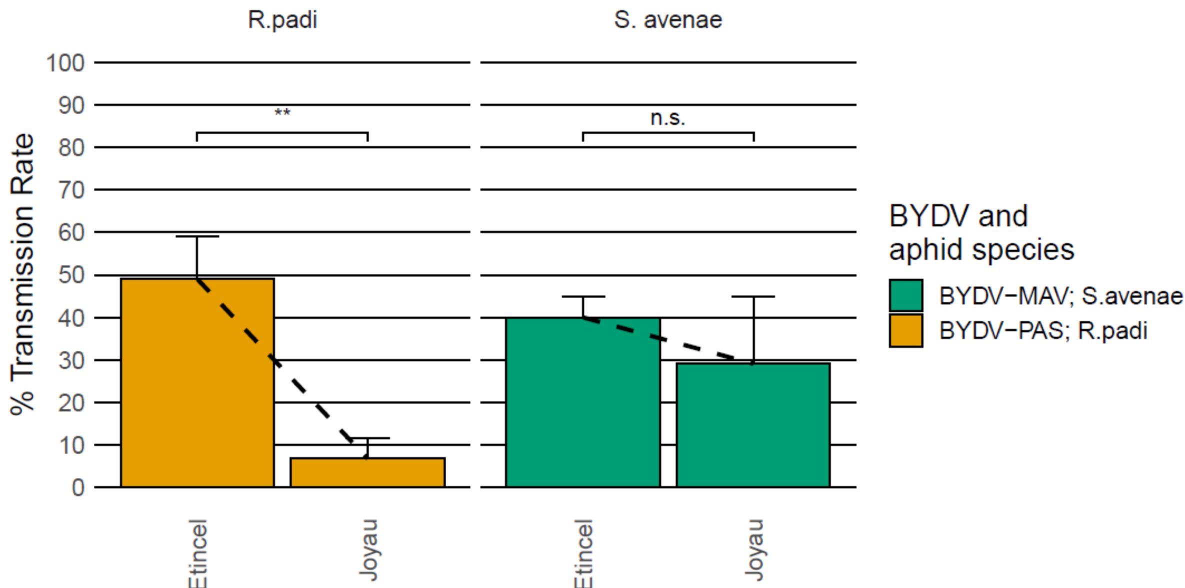

Figure 4.9: Comparison of average BYDV-MAV and BYDV-PAS transmission rates of viruliferous aphids from infected winter barley plants (cultivar Etincel) onto healthy winter barley cultivars (either Etincel or Joyau) after 24 h acquisition access period (AAP) by *R. padi* and *S. avenae*. For each species and cultivar, four replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with PCR for virus presence. Average values and the standard deviation for the acquisition access periods, BYDVs and aphid species are displayed.

There was also a significant decrease from 51% to 30.7% between Etincel and Joyau in the average BYDV-PAS transmission by *R. padi* after an IAP of 24 h, showing another reduction in transmission efficiency when switching varieties to the BYDV-tolerant Joyau (F=20.999, p=0.0059) (Figure 4.10). The transmission rates of BYDV-MAV and *S. avenae* after an IAP of 24 h remain to be determined.

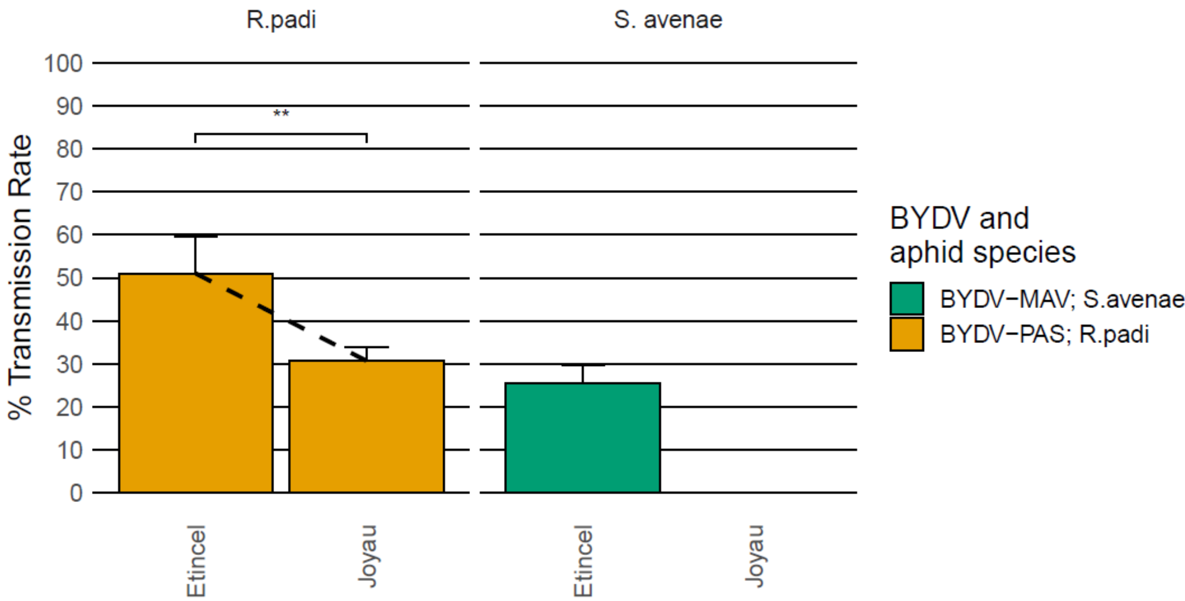

Figure 4.10: Comparison of average BYDV-MAV and BYDV-PAS transmission rates of viruliferous aphids from infected winter barley plants (cultivar Etincel) onto healthy winter barley cultivars (either Etincel or Joyau) after 24 h inoculation access period (IAP) by *R. padi* and *S. avenae*. For each species and cultivar, four replicates of 20 plants were tested independently. Three weeks after the experiments, the plants were tested with PCR for virus presence. Average values and the standard deviation for the inoculation access periods, BYDVs and aphid species are displayed.

4.4 Discussion

This thesis chapter investigated the transmission efficiencies of two BYDV species (BYDV-MAV and BYDV-PAS) commonly found in Ireland by insecticide resistant and susceptible *S. avenae* and *R. padi*. Additionally, sequencing of BYDV from Irish fields filled a knowledge gap about which BYDV species are present in spring and winter barley in Ireland, as previous work was solely based on serological assays (ELISA), which are prone to cross-reactions between viruses and do not cover all known BYDV species (Byrne et al., 2024; Kennedy & Connery, 2001, 2005).

Control of BYDVs in cereals in Europe has been considered a "low-level problem" in recent decades, primarily because a the availability of chemical control options for aphids (Mc Namara et al., 2020). However, with the loss of neonicotinoid seed treatments in 2018 and the emergence of resistance to the only available insecticide (pyrethroids) in *S. avenae*, crops were thought to be under strong BYDV pressure in the future due to insecticide resistant aphids surviving spray applications (Mc Namara et al., 2020). Since the identification of insecticide resistant *S. avenae* in 2013, this thesis chapter reports the first virus transmission efficiencies of two common Irish BYDV species (BYDV-MAV and BYDV-PAS). The results indicated that the insecticide resistant clonal lineage SA3 cannot transmit BYDV more efficiently than other *S. avenae* clones, which is in contrast to the hypothesis and other studies reporting a larger range of transmission efficiencies between different clonal lineages of the same species (Dedryver et al., 2005; Papura et al., 2002). Previous findings of resistant *S. avenae* that were linked to strong BYDV outbreaks are therefore, according to the results of this chapter, not caused by a higher transmission efficiency in the insecticide resistant lineage (Dewar & Foster, 2017). This is important, as these findings, in combination with already discovered fitness deficits, potentially reduce the overall risk of insecticide resistant *S. avenae* to crops (Jackson et al., 2020).

The BYDV transmission efficiencies for multiple clonal lineages of Irish *S. avenae* were identified in the first experiment, including the first study of the insecticide resistant clonal lineage SA3 and both BYDV-PAS and BYDV-MAV species. With an overall BYDV-PAS transmission rate of around 3% after 24 h AAP and around 2% after 24 h IAP, all *S. avenae* clones tested fall into the category between "not efficient" and "rare" transmitters; which excludes the *S. avenae* as a potential main

vector species (Van den Eynde et al., 2020). In comparison, previous research on *S. avenae* found "efficient" transmission rates of the closely related BYDV-PAV, of which *R. padi* is the main vector species (Papura et al., 2002). It is therefore plausible that, instead of *S. avenae*, *R. padi* could qualify as the main vector species of BYDV-PAS, however, *Metopolophium dirhodum* and *Rhopalosiphum maidis* are BYDV-PAS transmitters too, but their respective transmission efficiencies are yet to be investigated (Van den Eynde et al., 2020).

For BYDV-MAV, "efficient" rates between 25% and 40% were found for *S. avenae*. These rates are lower than the "very efficient" transmission reported in previous studies (Creamer & Falk, 1989; Gray et al., 1991; Power et al., 1991). This could be due to the slightly lower temperatures and shorter timings for AAP and/or IAP used in this experiment, but it is also plausible that geographical differences are a factor, as Irish climate, BYDV species and aphid populations differ from continental Europe or North America where previous studies were conducted (Dedryver et al., 2005). However, it has also been shown different cereal aphid clonal lineages can show differences in transmission efficiencies, and higher BYDV transmission rates were caused by the presence of "vector alleles" in the respective clone (Leybourne et al., 2024). Therefore, even though molecular marker analyses are beyond the scope of this research chapter, different genetic traits in the *S. avenae* clones tested might be another explanation for differences in virus transmission observed, but this needs to be further investigated. Additionally, the rose-grain aphid *Metopolophium dirhodum*, which is frequently found in Irish crops (see Chapter 2), is also a "very efficient" BYDV-MAV vector, potentially compensating for the less efficient transmission by Irish *S. avenae* (Creamer & Falk, 1989).

Preliminary experiments have investigated the transmission efficiencies of aphids transferring BYDV onto a BYDV-tolerant winter barley variety (Joyau), which is commercially available in Ireland since 2022. BYDV transmission in tolerant varieties has previously been described only with BYDV-PAV and *R. padi*; hence, the experiments conducted in this study were the first to describe BYDV-MAV and BYDV-PAS transmission on a BYDV-tolerant variety (Alquicer et al., 2023). While there were no differences in BYDV-MAV transmission by *S. avenae*, BYDV-PAS transmission by *R. padi* was found to be significantly reduced when the aphids transmitted BYDV onto Joyau in comparison to BYDV transmission rates on the

susceptible cultivar Etincel. This is interesting, as BYDV tolerance genes in Joyau do not reduce virus replication and symptom expression (Hu et al., 2019). Therefore, it was expected that virus transmission rates would be the same, yet these preliminary results suggest that there is a reduction in transmission when BYDV-PAS is transferred by *R.padi* onto the tolerant variety. As the presence of resistance genes conferring tolerance to BYDVs cannot explain the reduction in transmission of only BYDV-PAS by *R. padi*, another possible explanation could be potential physiological differences between the two barley varieties tested (e.g. a thicker stem epidermis), which could have an impact on the feeding behaviour of *R. padi*. However, further experiments are required to investigate this hypothesis. Overall, the experiments confirmed that Joyau plants can still be infected with both BYDV-MAV and BYDV-PAS; but further experiments (e.g. large-scale field trials) are needed to test if the tolerance genes also confer a reduction in yield losses when the plants are infected with BYDV-MAV. This is of great interest, as BYDV-tolerant and/or BYDV-resistant varieties are another promising option to reduce yield losses caused by BYDVs, yet there is a lack of data under field and laboratory conditions.

It is important to note that the experiments in this study were conducted under controlled conditions and tested on only two winter barley varieties (Etincel and Joyau). Virus transmission in-field depends on many factors such as host genetics, host age, temperature, co-existence of other viruses/aphids, and plant nutrition status (Choudhury et al., 2017). Thus, the results would need to be confirmed under field conditions, especially as BYDVs and both aphid species have a wide range of hosts (Miller & Lozier, 2022).

Based on sampling symptomatic plants and serological confirmation (Appendix table A1), BYDVs were found in all 45 field samples (Chapter 2) and in three aphid colonies; with BYDV-MAV being present in all field samples (Appendix table A2; Byrne et al. (2024)). This is in agreement with previous studies carried out in Ireland using ELISA on leaf samples of winter and spring barley from counties Carlow and Cork (Kennedy & Connery, 2001, 2005). These were tested by D/TAS-ELISA using F- and B-type antibodies, which can detect BYDV-MAV and BYDV-PAV/RPV, respectively (Clark & Adams, 1977; Plumb, 1974). In these studies between 1990 to 1993, and 1996 to 2001, 52.8% of collected samples tested positive for F-type (BYDV-MAV) virus; however only 1.5% tested positive for B-type (BYDV-PAV/RPV)

virus. In the sequencing study of barley in Ireland, 100% of samples tested positive for BYDV-MAV, 44% of samples (bulk samples of 9-12 leaves per field) were co-infected with BYDV-PAV, and 29% of samples were co-infected with BYDV-PAS, showing an increase in the abundance of both BYDV-PAV and BYDV-PAS species (see Appendix table A2, Byrne et al. (2024)). This first finding of the more severe BYDV-PAS species in Ireland, in combination with the novelty of studying BYDV-PAS transmission in *S. avenae*, led to the decision to investigate BYDV-PAS transmission efficiencies in this research chapter, even though BYDV-PAV is slightly more common in Ireland. Nonetheless, the presence of both BYDV-PAS and BYDV-PAV suggests either an increase in BYDV-PAV and BYDV-PAS spread within the last two to three decades in Ireland, or a higher detection rate due to an increased sensitivity of the high-throughput sequencing, in comparison to ELISA. Additionally, this chapter covered a wider range of locations, than previous work in Ireland (Kennedy & Connery, 2001, 2005), which may have increased the chances of capturing greater viral diversity.

Another interesting aspect of virus transmission in-field is that plants can be co-infected with multiple BYDV species simultaneously, and that both viruses can benefit from each other, causing greater damage (Marchetto & Power, 2018; Seabloom et al., 2009). Although, previous studies have also found that there is competition between different BYDV species, when they are transmitted simultaneously. For example, BYDV-PAV was shown to be the predominant virus species in a single plant 45 days after being co-inoculated with BYDV-PAS (Hall & Little, 2013). Through sequencing of 45 field samples, BYDV-MAV and BYDV-PAS co-infected crops were found commonly in Ireland, although the BYDV-PAS transmission rates were found to be poor in the virus transmission experiments. A plausible mechanism explaining the common spread of BYDV-PAS could therefore be the co-infection of plants by co-transmission of the virus, and by BYDV-PAS outcompeting BYDV-MAV when being co-transmitted to a new host. However, this hypothesis needs to be tested in future co-infection experiments. The results of these co-infection experiments, in addition to testing aphids from the field and suction tower network (Chapters 2 and 3) for the presence of multiple viruses, could fundamentally improve our understanding of BYDV.

Although there were no significant differences in the BYDV transmission efficiencies in between resistant and susceptible *S. avenae* clones, the insecticide resistant SA3 clone remains a problem, as control options are limited to only one active ingredient (pyrethroids). So far, only partial resistance to pyrethroids has been discovered in *S. avenae*, but due to the continuous selection pressure and the overuse of pyrethroids, full resistance in *S. avenae* or in other crop aphids might arise, conferring new risks and virus transmission efficiencies to be studied again (Foster et al., 2014).

In conclusion, virus transmission was not improved in the insecticide resistant *S. avenae* clonal lineage SA3. However, further research is needed that includes different barley varieties, BYDV co-infection, and elevated CO_2- and temperature levels in line with predicted future climate change scenarios. This will help improve our understanding of BYDV epidemiology, to adapt IPM methods and BYDV control options in the future.

5

Virus-vector manipulation in *Sitobion avenae* by BYDV

5.1 Introduction

Host manipulation by a pathogen or parasitoid to enhance their own chances of spread is a common phenomenon that can be found throughout the animal and plant world (Heil, 2016; Hernandez-Caballero et al., 2022; Holmes et al., 1972; Lafferty & Shaw, 2013; Poulin, 2010). In insects, a famous example is the host manipulation of ants by *Cordyceps sp.* fungi, which promote their own spread by changing the behaviour of infected ants to climb up and cling to a branch, where the fungal spores have a better location to spread than on the ground (Shang et al., 2015). Another example of host manipulation is the hairworm *Spinochordodes tellinii*, which induces suicidal behaviour in the cricket *Nemobius sylvestris*, by making it drown itself, as the worm is dependent on water to reproduce (Libersat et al., 2009).

Many pathogens also rely on vectors for transmission to a new host (e.g. *Plasmodium falciparum*, which causes malaria, uses mosquitos to be transferred between human hosts). In arthropods, especially insects, over a thousand species of pathogens, including fungi, bacteria, phytoplasmas, trypanosomes, *Plasmodia,* or viruses have been described to be reliant on insect vectors (Hurd, 2003; Ingwell et al., 2012). This also led to the discovery of an active behavioural manipulation by the pathogens as, for example, mosquitoes infected with *Plasmodium falciparum* showed an increased biting and feeding frequency, but preferred infected humans to feed on, when they were not carrying the pathogen (Koella et al., 1998).

Pathogen-induced changes in the host physiology and behavioural manipulation of vectors also exist in plant pathosystems (Figure 5.1, reviewed in Mauck et al. (2019)). However, as plants are sessile, pathogens mostly spread from plant to plant through vectors, which are therefore the only targets to actively induce behavioural changes (Ingwell et al., 2012). Plant viruses such as BYDV are known to cause changes in both the host plant and the vector, which is described as a multi-layered interaction (Dáder et al., 2017). Insect manipulation by plant viruses can be either indirect, through virus-induced changes in plant volatiles, colour or morphology (for example to enhance the attractiveness of the infected host), or direct, by enhancing vector fertility, movement abilities, or volatile detection abilities (Figure 5.1; Mauck et al. (2019)).

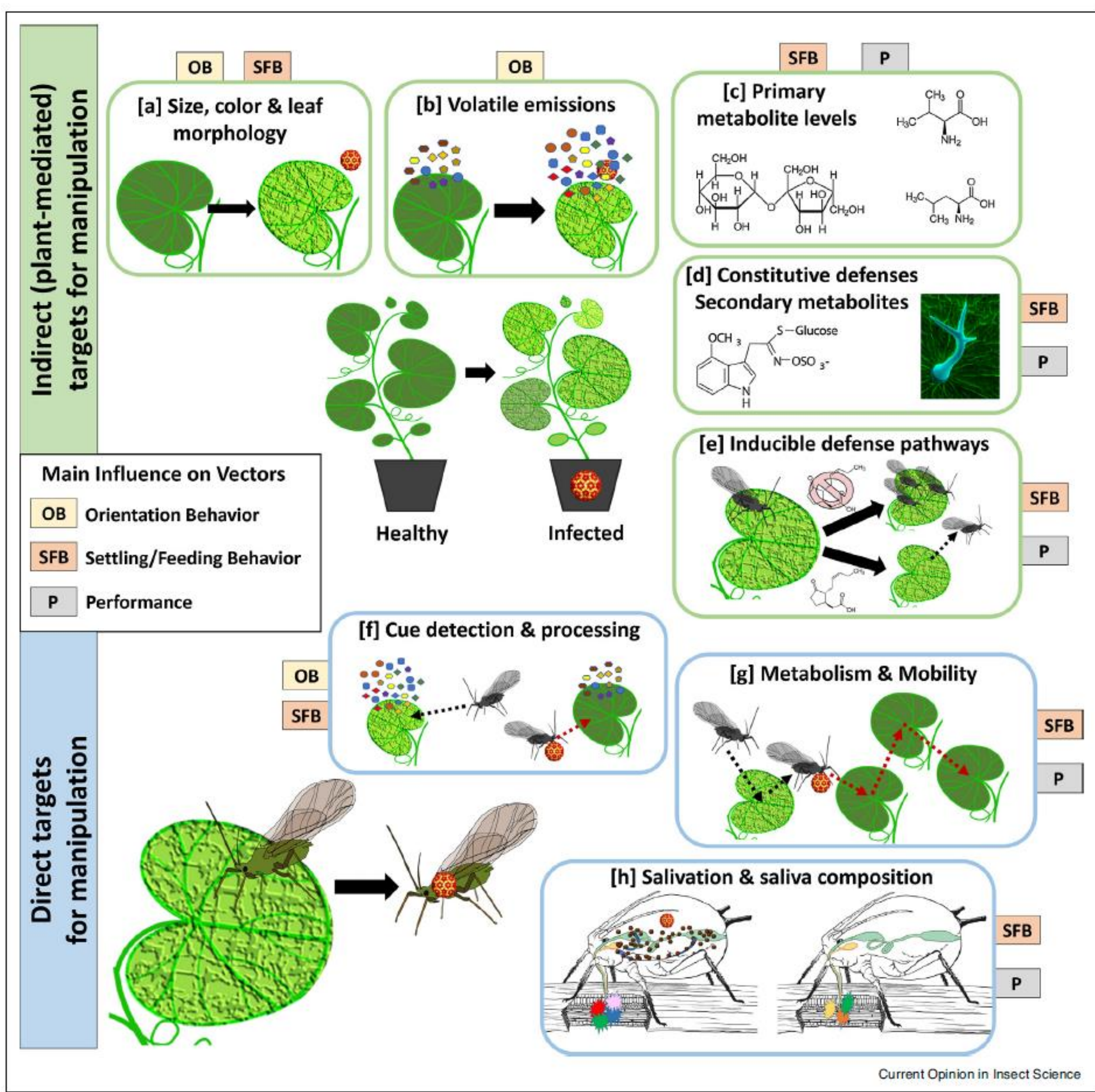

Figure 5.1: Aspects of the host phenotype (top - indirect effects in green boxes [a-e]) and components of vector behaviour and physiology (bottom - direct effects in blue boxes [f-h]) that are frequently altered following virus infection or acquisition. Tags on the side of the boxes indicate whether virus manipulation is likely to alter vector orientation behaviour (OB), settling/feeding behaviour (SFB), or performance (P). Image taken from Mauck et al. (2019).

Vector manipulation, to enhance plant virus spread, also plays an important role in agricultural crops where the high plant density in monocultures eases virus infections, as distances between host plants are minimal (Mauck & Chesnais, 2020). In Irish winter and spring barley (*Hordeum vulgare)*, the most common viruses are BYDVs and their vectors, the English grain aphid *Sitobion avenae*, the bird cherry-oat aphid *Rhopalosiphum padi* and the rose-grain aphid *Metopolophium dirhodum* (Kennedy & Connery, 2005).

Crops become primarily infected by either winged aphids that fly/migrate into the field (long-distance migration) or by aphids from the side/hedges that walk into the field (short-distance migration). Direct vector manipulation by the virus is known to play an important role in this process, as it has been shown that BYDV-PAS carrying (viruliferous) *R. padi* prefer uninfected plants to settle and feed, whereas BYDV-free aphids prefer infected plants (Medina-Ortega et al., 2009; Minato et al., 2022).

Secondary infection (BYDV spread within the field) is caused by offspring of the first generation that feed on the primarily infected plants. In case of BYDV, it has been shown that physiological changes (causing indirect manipulations in the vector) in BYDV infected plants encouraged aphid feeding and also enhanced development and population growth rate, ultimately benefiting virus spread (Jiménez-Martínez et al., 2004; Montllor & Gildow, 1986).

There is also increasing evidence that other Luteoviridae directly manipulate aphid locomotive activity *post-acquisition,* with viruliferous aphids showing a 3-4 times faster movement speed in a behavioural experiment where aphids are placed in a clue-free arena (see Fig. 5.3, Chesnais et al. (2020)). The locomotive activity of an aphid can be measured in four factors: 1) dispersion describes the furthest distance of an aphid reached from the starting point within 300 seconds; 2) velocity measures the time of an aphid spent in each zone; 3) reaction time is defined by the time it takes before the aphid leaves the first zone; and 4) movement describes the total number of zones crossed.

However, *post-acquisition* locomotive vector manipulation has not yet been shown with BYDV, and it is also unknown when the manipulation occurs after acquisition, as no virus-encoded genes were found that could explain direct vector manipulation (Mauck et al., 2019). As BYDV is a circulative virus, it is thought that vector manipulation occurs in between the encapsulation of virions in the mid-gut and binding to the salivary glands (Ingwell et al., 2012).

As described in Chapter 4 of this thesis, through recent sequencing, BYDV-MAV and BYDV-PAS have been found in winter and spring barley in Ireland (see Appendix Table A2, (Byrne et al., 2024)). Additionally, *S. avenae* was found to be an "efficient" BYDV-MAV transmitter and a non-efficient or rare BYDV-PAS transmitter, while *R. padi* showed efficient BYDV-PAS transmission rates, even

though it cannot transmit BYDV-MAV (Chapter 4). This poses the question of whether virus transmission efficiency and vector manipulation are related to each other (e.g. does a poorly transmitted virus compensate through high manipulation) or if they are unrelated (e.g. viruses manipulate independently of the vector species)?

5.1.1 Objectives

This chapter contains of two main research questions:

1) Can BYDV indirectly manipulate aphid fecundity?

2) Can BYDV actively manipulate aphid behaviour?

For this, a large-scale vector manipulation experiment with two BYDV species (BYDV-MAV and BYDV-PAS) and two aphid species (*R. padi* (1 clone) and *S. avenae* (3 clones)) was conducted to 1) test the indirect effects of virus-vector manipulation on aphid fecundity and 2) quantify direct virus-vector manipulation on aphid dispersion, velocity, movement, and reaction time. These experiments are the first to investigate the direct vector manipulation hypothesis in the pathosystem of BYDV.

5.2 Materials and Methods

5.2.1 Fertility experiment

To create virus-infected plants, 10-day-old plants were inoculated with either three BYDV-PAS or BYDV-MAV infected aphids or left untouched (Fig. 5.2). After 24 h virus inoculation period, all aphids were removed from the plants and the plants were kept growing for two weeks, in order to allow the virus to replicate. Next, winged adult aphids from three different clonal lineages (SA3, SA27 and SA38) were placed on a new set of healthy plants to produce nymphs overnight. The next day, 60 single first instar nymphs of each clone were transferred onto either 20 BYDV-PAS, BYDV-MAV, or healthy plants each. The number of offspring produced by each aphid was counted after 14 days of development and asexual reproduction. The experiment was replicated three times giving a total of 445 aphids studied (Tab. 5.1).

Table 5.1
Overview of aphids and BYDV species tested in the fertility experiments

Aphid	Virus-free (n)	BYDV-MAV (n)	BYDV-PAS (n)	total
S. avenae –SA3	35	42	39	116
S. avenae – SA27	35	41	37	113
S. avenae – SA38	32	39	35	106
Sum *S. avenae*	102	122	111	335
R. padi	37	37	36	110
all	139	159	147	445

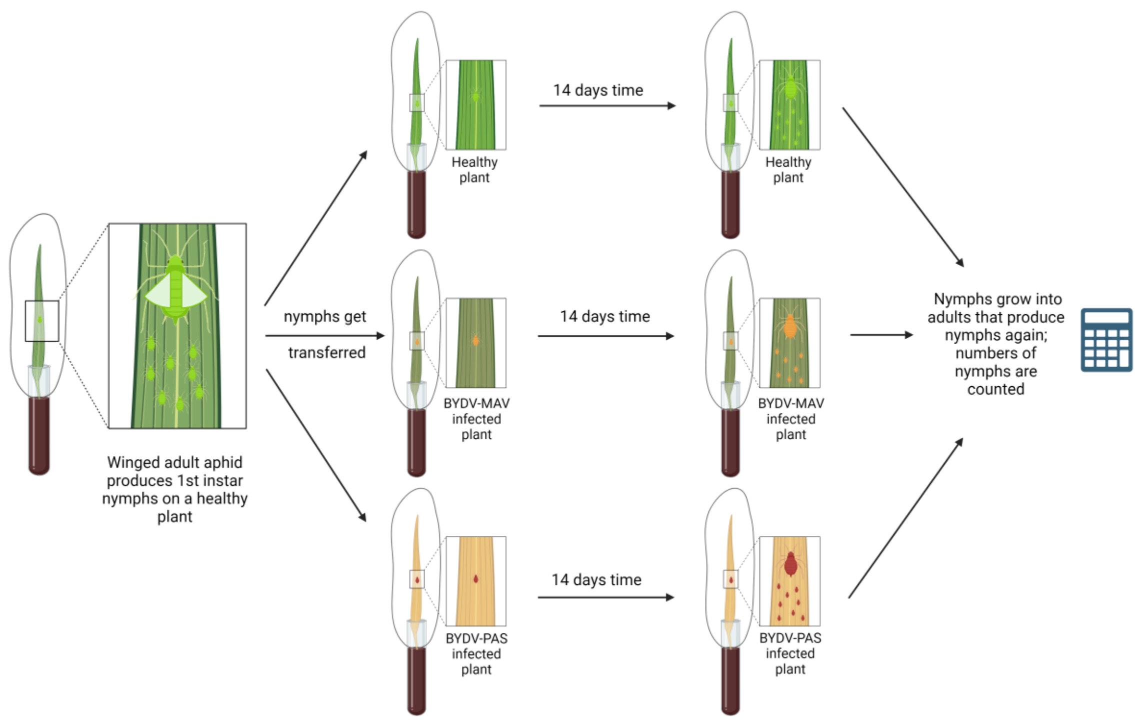

Figure 5.2: Setup of the fertility experiment.

5.2.2 Movement experiment

Apterous adult aphids from the second generation of offspring from the fertility experiment were used in the movement experiment. For this, a single aphid was carefully transferred into an arena of 10 circles with a 1.5 cm radius between each circle, which was placed in a white walled box of paper under a constant light source (Fig 5.3). Aphid movement behaviour was recorded for 300 s and four events were noted: 1) dispersion: the furthest (from the centre) circle reached after 300 s; 2) movement: the sum of lines crossed; 3) velocity: the average time the aphid spent within one circle, and 4) initial movement: the time until the aphid left the starting circle. Each experiment was replicated three times and a total of 467 aphids were studied (Tab. 5.2).

Table 5.2
Overview of aphids and BYDV species tested in the movement experiments

Aphid	Virus-free (n)	BYDV-MAV (n)	BYDV-PAS (n)	total
S. avenae – SA3	36	37	40	113
S. avenae – SA27	36	38	30	104
S. avenae – SA38	35	42	40	117
Sum *S. avenae*	107	117	110	334
R. padi	55	47	31	133
all	162	164	141	467

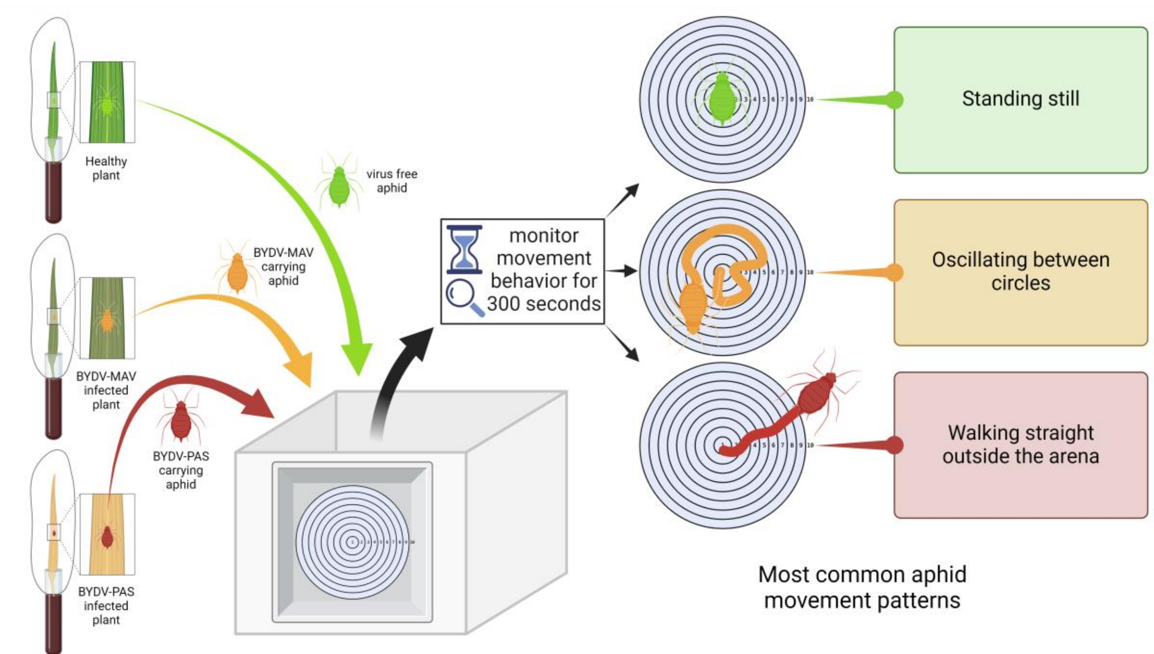

Figure 5.3: Setup of the behavioural manipulation experiment.

5.2.3 Virus testing

Virus testing of leaves and aphids was conducted as described in Chapter 4.2.7. In addition to testing the plants, a random selection of 88 aphids from all three experimental replicates were tested for the presence of BYDV, with all aphid samples confirming the presence of virus as described in Chapter 4.2.7.

5.2.4 Statistical analyses

Dispersion data were tested using the Shapiro-Wilk test for normality and with Levene's test for homogeneity of variance. As the data were not normality distributed and homogeneity of variance was given, they were evaluated using a non-parametric ranked ANOVA (R package: "Rfit"; p-value adjustment with "fdr" method). When a factor or an interaction between factors was significant, the data was further tested for multiple-comparisons between the factors (R-package "nparcomp", p-value adjustment "Tukey" method). Results were considered significant at a p-value <0.05.

Log-transformed data from the velocity experiment (excluding all aphids that did not move for 300 s) were tested with a Shapiro-Wilk test for normality and for interaction between the variables (clones and virus-free/viruliferous) using Scheirer-Ray-Hare-test. Both species (*S. avenae* and *R. padi*) were analysed separately, as significant differences between them were found. As normality was confirmed, parametric ANOVA was used to test the data. If the results showed a significant p-value <0.05, the Tukey-HSD-test was used to test for differences between the treatments.

The cumulative total number of zones crossed per aphid was tested using the Shapiro-Wilk test for normality and for interaction between the variables (clones and virus-free/viruliferous) using the Scheirer-Ray-Hare-test. The dataset was found to be non-normally distributed; therefore, a non-parametric Kruskal-Wallis test was used to test the effects of the treatments. If results showed a significant p-value <0.05, testing was followed with Dunn's test to test for differences between the treatments.

Initial movement time and fertility data were tested using the Shapiro-Wilk test to check for normality and evaluated with a non-parametric ranked ANOVA (R package: "Rfit"; p-value adjustment with "fdr" method). *R. padi* and *S. avenae* data were significantly different, resulting in both species being separated in the further analyses. Both datasets were tested for the impact of the virus with ANOVA, followed by a Tukey-HSD-test to test for differences between the treatments. Statistical significance was set at p-values < 0.05.

All statistical analyses were performed using R (version 4.2.1) and using the packages ggplot2 reshape2, scales, plyr, viridis, tidyverse, lubridate, dplyr, Rcpp,

rcompanion, FSA, MASS, ggpubr, ggmagnify, and ggnewscale (Ihaka & Gentleman, 1996; Wickham, 2011).

5.3 Results

5.3.1 Dispersion manipulation

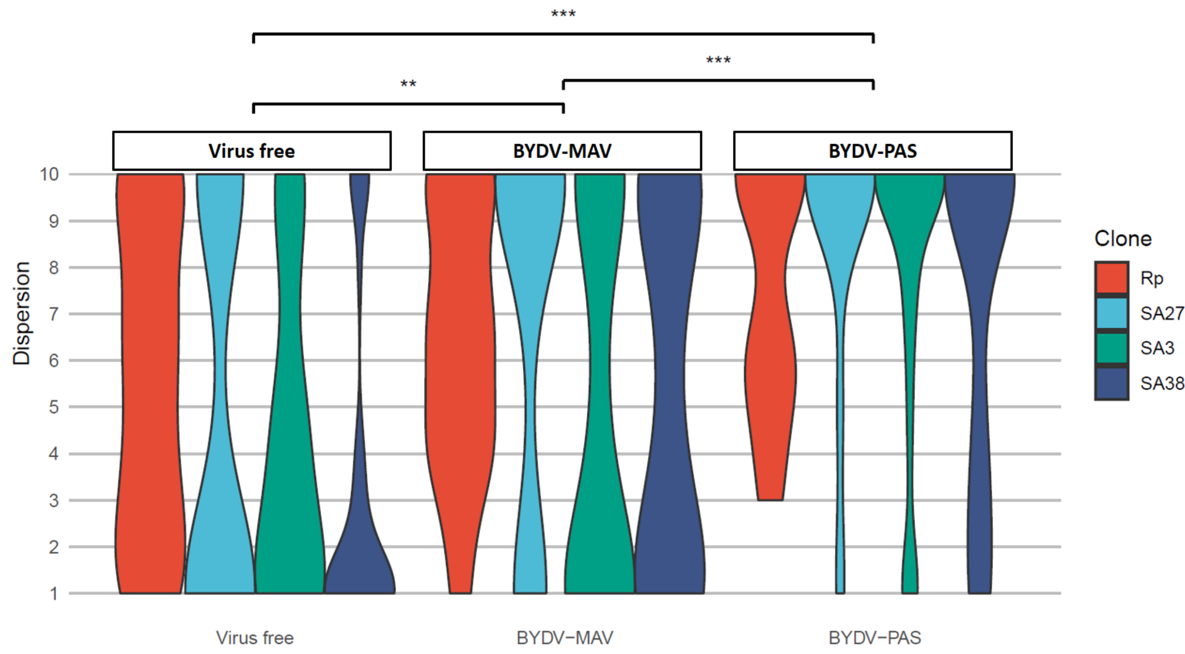

Figure 5.4: Maximum Dispersion of Virus free, BYDV-MAV and BYDV-PAS infected *S. avenae* (3 clones) and *R. padi*. The width of each column indicates the relative distribution of aphids showing a maximum dispersion after 300 seconds and the asterisk indicate significant differences between the tested groups ("**"=p<0.01; "***"=p<0.001).

The maximum dispersion distance was found to be insignificant between all aphid species and clones after 300 s (Fig. 5.4) (ANOVA, p=0.257); thus all the data were analysed together. Independent of aphid species or clone, the maximum dispersion after 300 s significantly increased when aphids were carrying BYDV-MAV, in comparison to virus-free aphids (ANOVA p<0.006). The same phenomenon was found in BYDV-PAS carrying aphids compared to virus-free aphids (ANOVA, p<0.001). Additionally, there was also a significant increase of the maximum dispersion when comparing BYDV-MAV carrying aphids to BYDV-PAS carrying aphids (ANOVA, p<0.001), showing a significantly greater effect of locomotive vector manipulation by BYDV-PAS.

5.3.2 Velocity manipulation

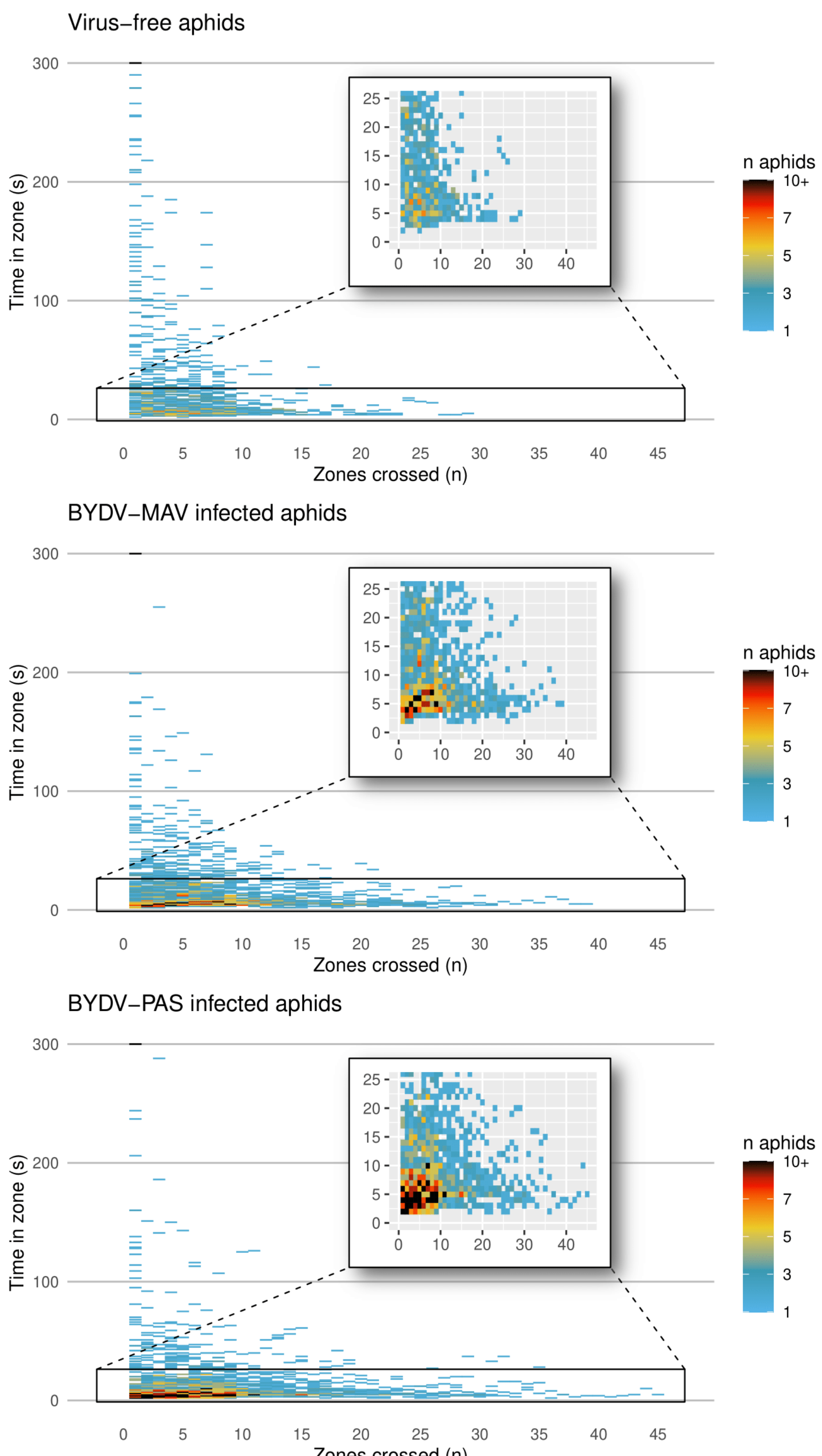

Figure 5.5: Heatmap showing the number of zones crossed and the time spent within the respective zone per aphid. Coloration of each dot is linked to the number of aphids showing identical zone/time numbers.

Data on aphid velocity (time spent per zone in seconds) in comparison to the number of zones crossed are displayed in a heatmap (Fig. 5.5). Data from 163 virus-free aphids, 164 BYDV-MAV infected aphids, and 141 BYDV-PAS infected aphids showed an increase in aphids spending less time in each zone and crossing more zones when they were viruliferous.

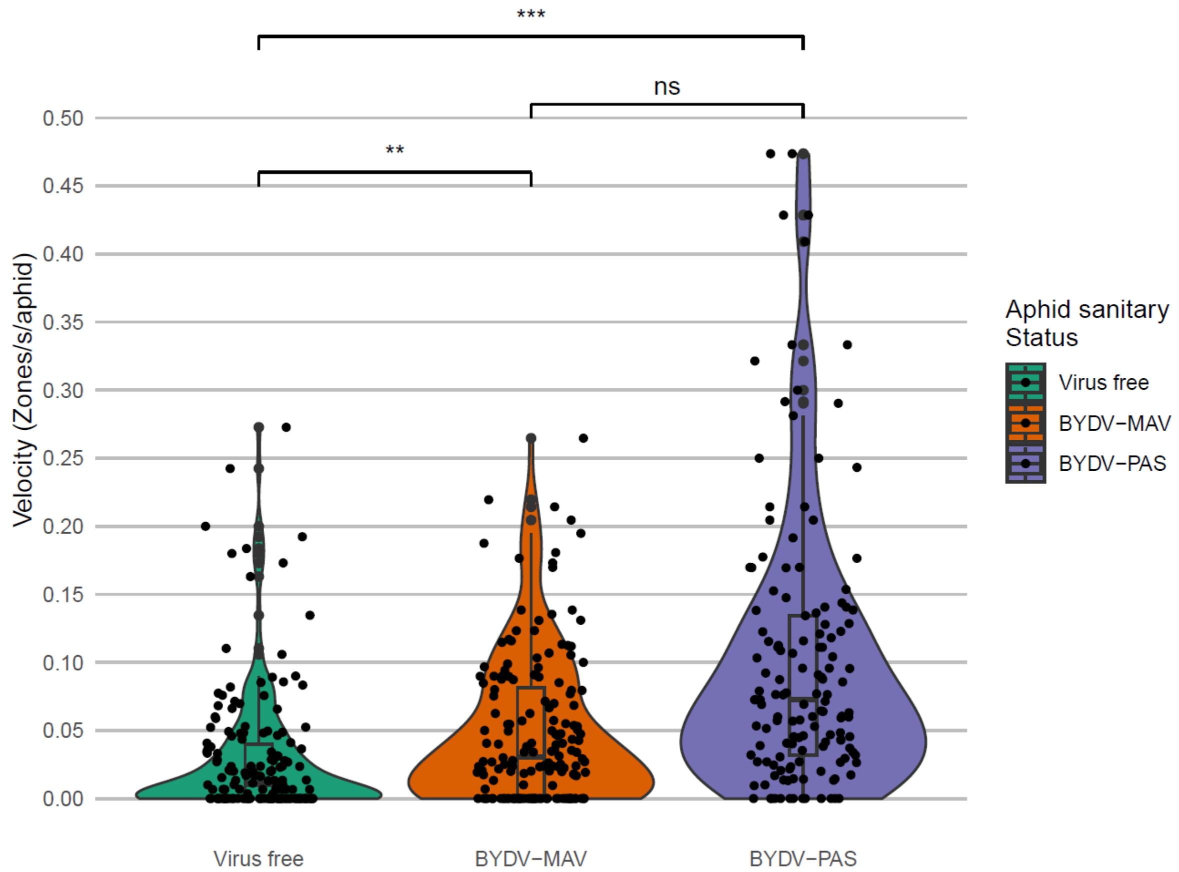

Figure 5.6. Average velocity for aphids of all *S. avenae* clones (in zones/second), which were either virus-free, or viruliferous with BYDV-MAV or BYDV-PAS. The width of each column indicates the relative distribution of aphids at a certain velocity and the asterisk indicate significant differences between the tested groups (“ns”=p>0.05; “**”=p<0.01; “***”=p<0.001).

Velocity data of all *S. avenae* clones are displayed in Figure 5.6, showing that BYDV-PAS carrying aphids spent the least time in each zone (showing an increased velocity). For statistical analyses, all aphids that did not move at all (time in zone = 300 s) were removed from the dataset and preliminary tests showed significant differences between the aphid species *S. avenae* and *R. padi* (ANOVA, p<001); thus both aphid species were analysed separately. The results showed a significantly increased velocity (seconds/zone crossed) when the aphids were viruliferous (*S. avenae*: ANOVA, F=13.45, p<0.001; *R. padi*: ANOVA, F=7.1661,

p<0.001). In *S. avenae*, both BYDV-MAV- and BYDV-PAS viruliferous aphids showed a significantly increased velocity compared to virus-free aphids (Virus-free vs BYDV-MAV: Tukey-HSD, p<0.007; Virus-free vs BYDV-PAS: Tukey-HSD, p<0.001). However, there was no significant increase in velocity between BYDV-MAV and BYDV-PAS (Tukey HSD, p=0.075). In *R. padi*, both BYDV-MAV and BYDV-PAS carrying aphids showed a significant increase in velocity (Virus-free vs BYDV-MAV: Tukey-HSD, p<0.0233; Virus-free vs BYDV-PAS: Tukey-HSD, p<0.001). Again, there was no significant difference between BYDV-MAV and BYDV-PAS (Tukey HSD, p=0.482).

5.3.3 Movement manipulation

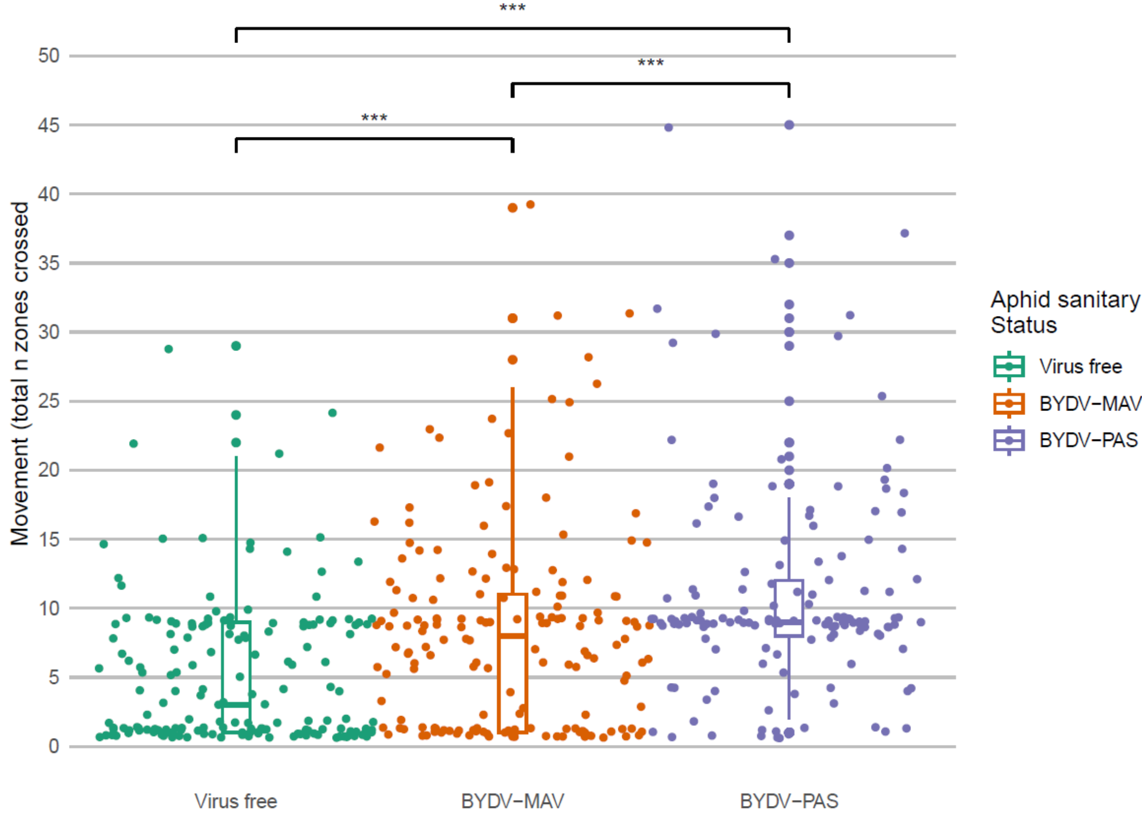

Figure 5.8: Total number of zones crossed per aphid. The asterisks indicate significant differences between the tested groups of either virus free, BYDV-MAV viruliferous and BYDV-PAS viruliferous aphids ("***"=p<0.001).

The total number of zones crossed per aphid, depending on whether it was virus-free or viruliferous with BYDV-MAV or BYDV-PAS are displayed in Figure 5.8. Preliminary testing found no significant difference between the aphid species or

clones; thus all were analysed together. The total number of zones crossed significantly increased when the aphids were viruliferous (Kruskal-Wallis chi-squared = 60.542, df = 2, p<0.001). Post-hock testing using Dunns' test showed that the increase was significant between virus-free and BYDV-MAV carrying aphids (p<0.001), virus-free and BYDV-PAS carrying aphids (p<0.001), and significantly higher in BYDV-PAS carrying aphids compared to BYDV-MAV carrying aphids (p<0.001).

5.3.4 Initial movement manipulation

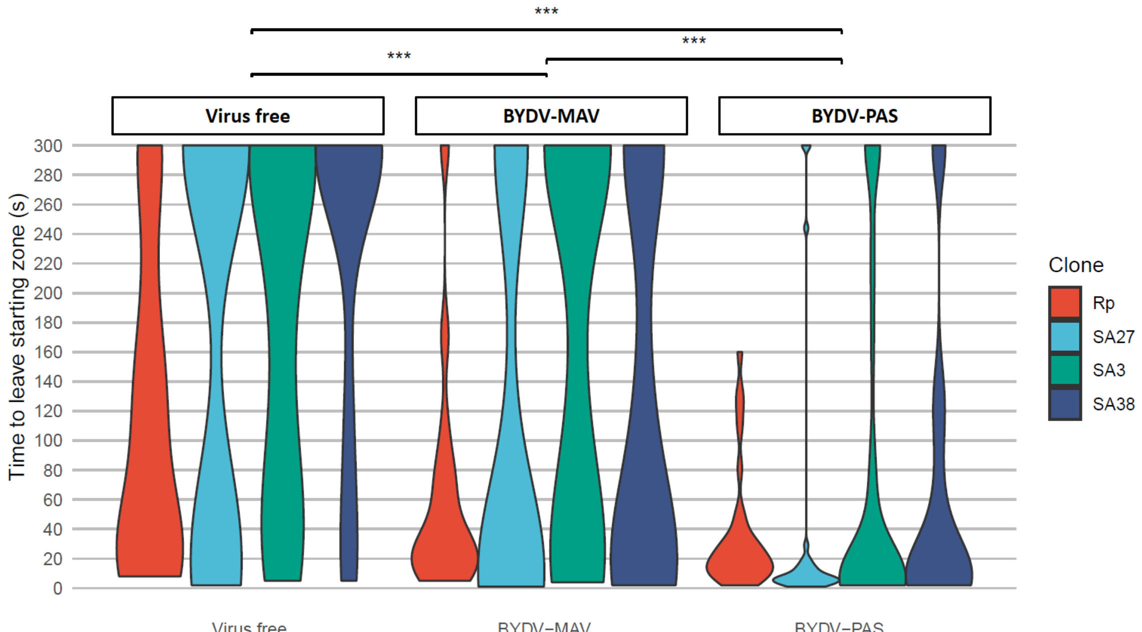

Figure 5.7: Initial movement time in seconds until leaving starting circle (centre). The width of each column indicates the relative distribution of aphids showing a certain initial movement time and the asterisks indicate significant differences between the tested groups of either virus free, BYDV-MAV viruliferous and BYDV-PAS viruliferous aphids ("**"=p<0.01; "***"=p<0.001).

The initial movement time to leave the starting zone for all aphids and viruses is displayed in Figure 5.7. Preliminary testing found no significant differences between aphid species or clones; thus they were analysed together. Both viruses significantly decreased the initial movement time of aphids (Virus-free vs BYDV-MAV: ANOVA, p<0.001; Virus-free vs BYDV-PAS: ANOVA, p<0.001). There was also a significant difference between BYDV-MAV and BYDV-PAS (ANOVA, p<0.001), showing even stronger manipulation by BYDV-PAS.

5.3.5 Fertility

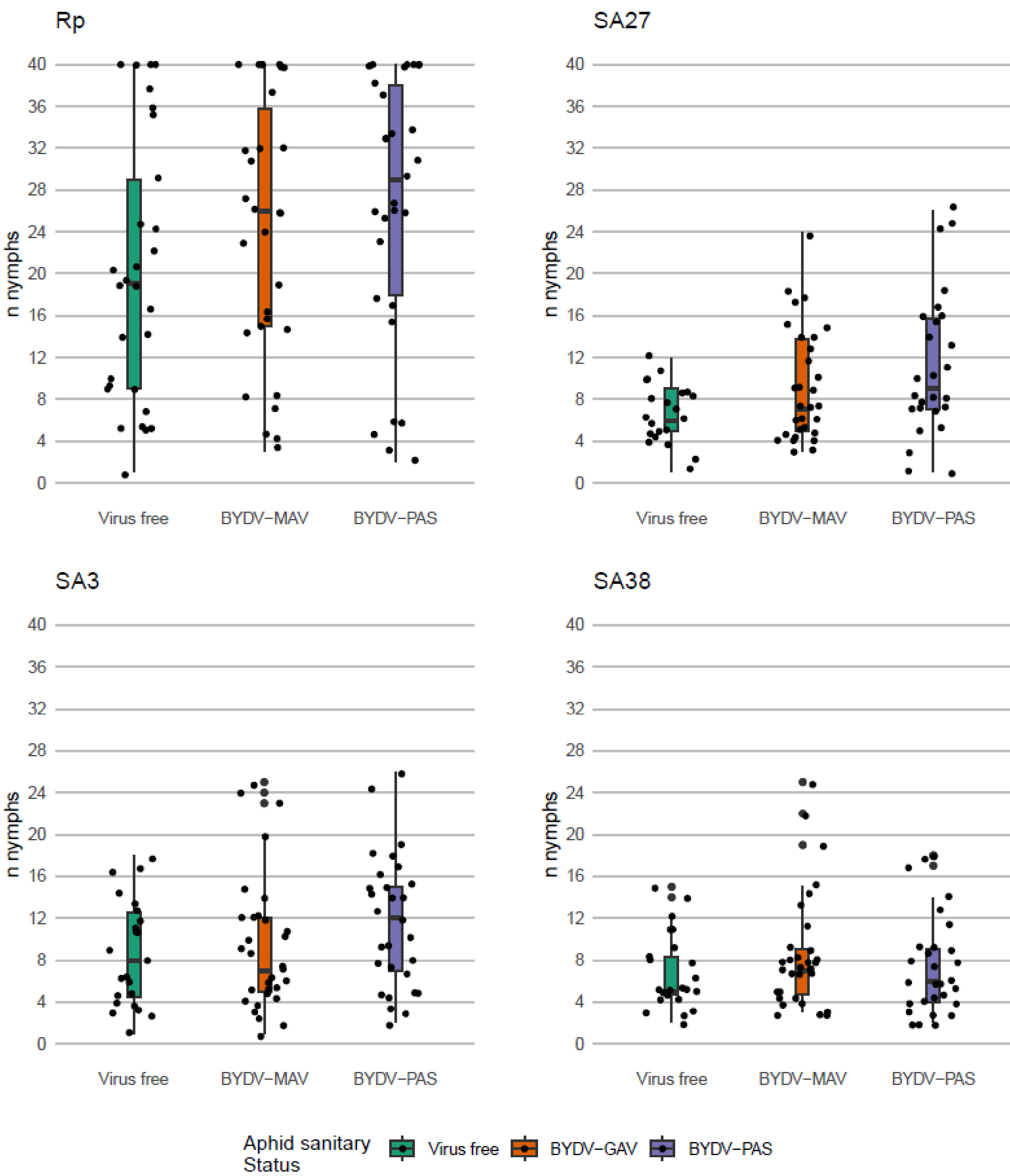

Figure 5.9: Fertility of *R. padi* and three *S. avenae* clones after 14 days of growth starting from a single 1st instar nymph.

The results of the fertility experiment are shown in Figure 5.9. Preliminary testing between aphid species showed that the number of nymphs produced by *R. padi* after 14 days significantly differed between *S. avenae,* but there were no differences between the *S. avenae* clones. An increase in fertility (number of offspring produced by a single aphid after 14 days) was found in all *S. avenae* clones when the aphids

grew and started to produce nymphs on a BYDV infected plant (ANOVA: $F=4.366$; $p=0.0137$). However, post-hock testing showed that only BYDV-PAS significantly increased number of nymphs in *S. avenae* (Tukey-HSD: $p=0.010$). There were no significant differences in fertility between aphids on virus-free and BYDV-MAV infected plants (Tukey-HSD: $p=0.139$) or between aphids on BYDV-MAV and BYDV-PAS infected plants (Tukey-HSD: $p=0475$). There was also no significant increase in fertility in *R. padi* on either virus free, BYDV-MAV or BYDV-PAS infected plants (ANOVA $F=1.6495$ $p=0.198$).

5.4 Discussion

Post-acquisition locomotive activity and dispersal behaviour in aphids carrying persistent and circulative Luteoviruses have been described only twice, yet there are over 100 peer-reviewed studies investigating plant virus-vector manipulation (Mauck et al., 2019). In the first study, BYDV carrying winged *R. padi* were found to have a shorter flight duration than virus-free aphids in a wind-tunnel experiment, suggesting a shorter flight window for long-distance migration in viruliferous aphids (Levin & Irwin, 1995). The second study, which is similar to this thesis chapter, investigated ground locomotive behaviour (short-distance migration) of unwinged aphids, showing that Turnip yellow virus (TuYV) carrying *Myzus persicae* had a 3-4 times increased velocity (time per zone) in comparison to virus-free aphids (Chesnais et al., 2020). In this research chapter, virus manipulation experiments were performed to investigate potential interclonal differences in viruliferous *S. avenae* and to compare them with *R. padi* using two different BYDV species (Fig 5.10).

Throughout all locomotive experiments, significantly increased maximum dispersion, velocity, movement, and initial movement times were found in both BYDV-MAV and BYDV-PAS carrying aphids. These results are congruent with the findings of increased locomotive activity in other circulative virus pathosystems, further supporting the virus-vector-manipulation hypothesis (Ingwell et al., 2012). When placed into a new environment, aphids usually show three behavioural types when trying to locate a new host (sitting still, circulating, or walking straight in a line) (Gottlieb et al., 2017). The results from the dispersion experiment showed a clear shift from a large proportion of virus-free aphids showing sitting behaviour, towards a large proportion of viruliferous aphids walking in a straight line and leaving the arena. Additionally, a significantly increased circulating movement pattern was found along with an overall increased velocity when the aphids were viruliferous, further supporting direct vector manipulation by BYDV.

The motivation of an aphid to sit down or initiate movement is dependent on many factors such as population density, host quality, or the presence of predators (Claflin et al., 2017). Since all these factors were ruled out in the experimental arena, Chesnais et al. (2020) hypothesised a lower motivational threshold caused by the virus to initiate foraging movement earlier, when aphids were put into a new

environment. The experiments in this research chapter showed clear evidence for a significantly shorter time until movement initiation in viruliferous aphids, which might be linked to the proposed lower motivational threshold. It was also observed that, when placed into the experimental arena, virus-free aphids showed sensing behaviour by waving the antennae, which was rarely observed in viruliferous aphids. However, these preliminary observations must be quantified in future experiments.

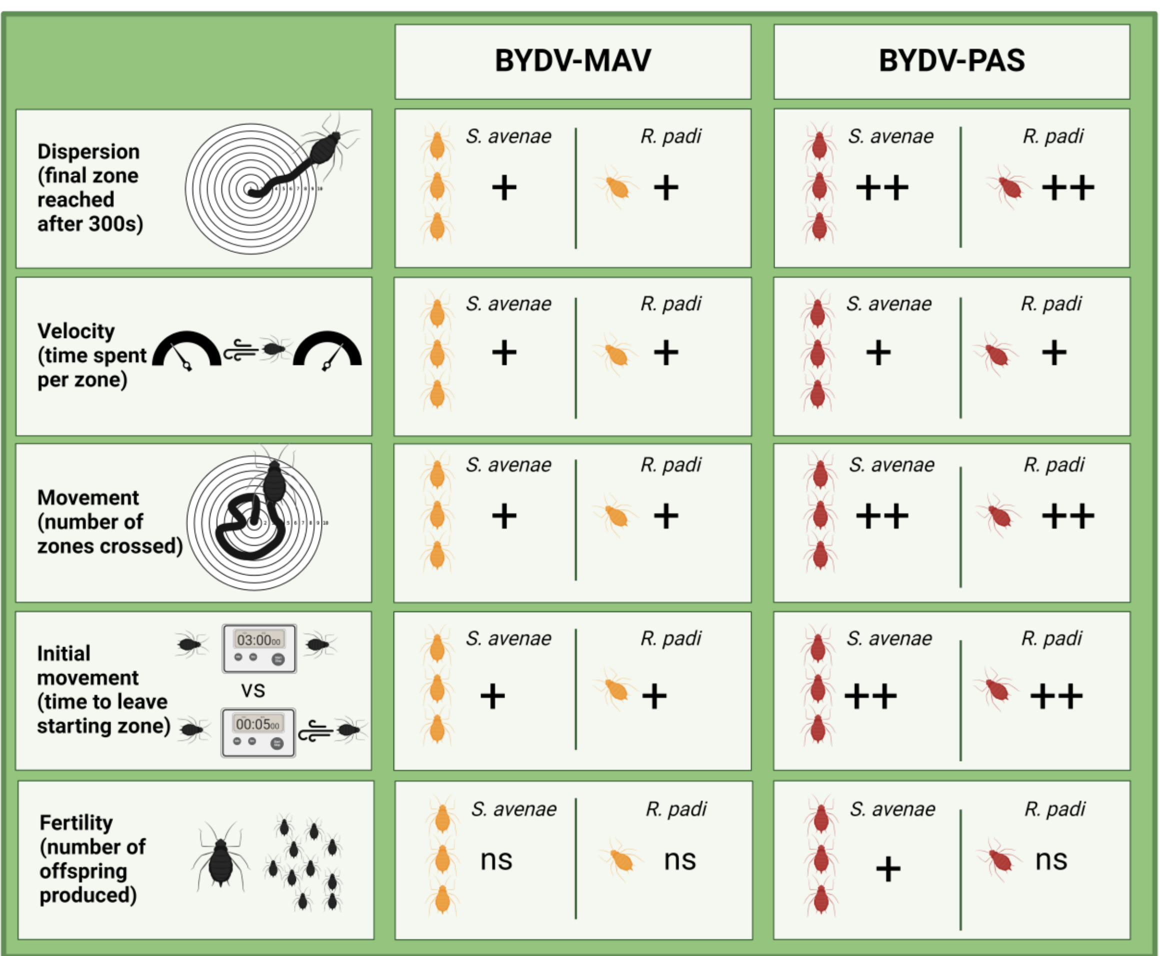

Figure 5.10: Summary of results from the vector manipulation experiments using BYDV-MAV and BYDV-PAS. All experiments were conducted with three *S. avenae* clones and one *R. padi* clone. Symbols: "+" = significant increase compared to virus-free aphids; "++" = significant increase in comparison to virus-free and BYDV-MAV infected aphids; ns = no significant differences.

Interestingly, direct vector manipulation was observed independently of aphid species or clonal lineage, even showing manipulation effects by viruses in aphid species that are not the main vector species or cannot transmit the virus at all (Fig. 5.10). For example, *S. avenae* was shown to be a poor transmitter of BYDV-PAS

and *R. padi* cannot transmit BYDV-MAV (see results Chapter 4), yet both BYDV species directly manipulate the respective vector. This experiment is the first to examine non-transmitter manipulation, suggesting that the manipulation mechanism is independent of the ability of the aphid to transmit the virus.

Additionally, the results showed that BYDV-PAS significantly increased the locomotive activity when compared to the vector manipulation by BYDV-MAV. Previously, BYDV-PAS has been described as a more severe virus species than BYDV-MAV or the closely related BYDV-PAV because it causes stronger symptoms and therefore higher yield losses (Van den Eynde et al., 2020). Here, evidence was found that BYDV-PAS might spread faster in the field than other BYDVs, because of its ability to stronger manipulate the aphid vector. However, further experiments, including the behavioural manipulation of BYDV-PAV, are required to test this hypothesis.

Evidence for indirect virus manipulation leading to a higher aphid fecundity was found in *S. avenae* and BYDV-PAS, but not in the other experiments. In comparison to other studies, the indirect effects of fecundity manipulation by BYDV in *S. avenae* and *R. padi* remain unclear; for example, in one study with wheat, BYDV-PAV and BYDV-MAV infected plants had a negative impact on aphid fecundity (Fiebig et al., 2004). However, in a different experiment on wheat and BYDV-GAV, positive fecundity effects were found (Liu et al., 2014). These results are in contrast to other species of Luteoviruses, where a positive effect on the fecundity of viruliferous aphids has been reported (Chesnais et al., 2020). Therefore, effects of BYDV-induced indirect changes on aphid fertility may depend on the study system.

The results of these experiments support the hypothesis of increased BYDV transmission due to manipulated locomotive activity in viruliferous aphids. However, to show that this manipulation has a significant impact on virus spread and yield, experiments under field conditions, which differ from controlled laboratory conditions at 20 °C, are needed. For example, it has been shown that temperatures below 12 °C inhibit virus transmission; however, no study has shown differences in vector manipulation under varying temperatures. As aphid mobility and fecundity correlate with outside temperature, virus-vector manipulation could play an important role in enhancing the spread of BYDV in crops, especially if temperatures are around the

limits of transmission. The virus would even benefit from manipulations below the limits of transmission (which for winter barley occurs for months), as virus-manipulated aphids would still spread further and faster from infected to healthy plants, increasing the chances of successful transmission on a warmer day. Through climate change, milder winters with more days above 12 °C, are expected in the future, meaning that manipulated aphids do potentially have larger impacts on BYDV spread in winter barley (García et al., 2020).

6

General discussion and conclusion

6.1 Insecticide resistant *Sitobion avenae* have not become predominant in Ireland

The main hypothesis of this thesis was that the incidence of pyrethroid resistant *S. avenae* in Ireland would rise following the neonicotinoid seed treatment ban in 2018, due to the favourable selection of resistant aphids after pyrethroid spray applications and the lack of alternative chemicals. Throughout the fieldwork (Chapter 2) and suction tower monitoring (Chapter 3), no higher levels of resistant aphids were found in Ireland relative to previous studies (Walsh, Schmidt, et al., 2020). Instead, overall resistance levels have decreased in comparison to years in which neonicotinoid seed treatments were still available, which was unexpected (Walsh, Schmidt, et al., 2020). Therefore, concerns about the loss of neonicotinoids and the overuse of pyrethroids, which were thought to lead to an increase in insecticide resistant *S. avenae*, cannot be confirmed for Ireland, to date (Dewar & Foster, 2017; Mc Namara et al., 2020). However, slightly higher levels of resistance, and lower levels of aphids carrying BYDV in the suction tower network were found, in comparison to fieldwork, showing that there were differences between the monitoring methods, which should be taken into account when optimising decision support tools, as suggested by White et al. (2023). Overall, fieldwork and suction tower data (Chapters 2 & 3) showed that numbers, resistance levels and BYDV levels in aphids fluctuated significantly between the sampling years. The results from this thesis confirm that spatial and temporal distributions of aphids follow environmental factors such as local climate and temperature, rather than local insecticide spray applications (Ciss et al., 2014).

Fieldwork carried out between 2021 and 2023 (Chapter 2), showed that insecticide spray applications in winter barley significantly reduced the overall numbers of cereal aphids (*S. avenae* and *M. dirhodum*). This suggests that pyrethroid applications, besides the evolution of partial insecticide resistance in *S. avenae*, remain an effective tool for controlling cereal aphids in Irish barley crops. Interestingly, results from the fieldwork also showed that other cereal aphids like *M. dirhodum* (a known vector of BYDV-MAV/PAS/PAV) might play an important role in BYDV epidemiology in Ireland. For example, in two out of three years of fieldwork, *M. dirhodum* was found in 2.7 and 6.2 times higher numbers than *S. avenae*. However, more long term monitoring data will be needed to study the relative

importance of *S. avenae*, in comparison to other cereal aphids like *M. dirhodum* and *R. padi* in Ireland.

Even though no increased levels of resistance in *S. avenae* in Ireland were found in this study, it is likely that new mutations and resistances will occur in the future (Bass & Nauen, 2023). A possible resistance mechanism that could arise in *S. avenae* is the development of homozygous *kdr*, which has not yet been detected during fieldwork or in the suction tower network (Chapter 2 & 3), but can develop through sexual reproduction (Ffrench-Constant, 2013). Previous studies have shown that the resistant SA3 clone retained its capacity to reproduce sexually after field rate exposures to pyrethroids and that homozygous *kdr* led to greater resistance in other aphids (Martinez-Torres et al., 1999; Walsh et al., 2019). However, sexual reproduction in aphids, by the production of female and male adults following oviposition by females, is a strategy to survive harsh winter conditions (Dedryver et al., 2001). Given the oceanic climate in Ireland, in combination with expected warmer winters due to climate change, the likelihood of the emergence of full resistance in *S. avenae* is therefore expected to be at low levels, at least in Ireland (García et al., 2020). Another important aspect to consider is that the situation could worsen if new resistances in other cereal aphids like *M. dirhodum* or *R. padi* evolve. For example, there was evidence that *R. padi* had evolved resistance to pyrethroids in Ireland (Walsh, Ferrari, et al., 2020), yet further research did not find a mutation in the VGSC gene and concluded that the reduced sensitivity observed in a single *R. padi* clone may have been due to metabolic tolerance (George et al., 2022). Nonetheless, the selection pressure of insecticides, may also lead to new mutations that confer resistance in the future. Therefore, ongoing monitoring of resistances needs to continue to be expanded, especially, as climate change will lead to higher aphid population pressure and therefore increased insecticide applications are expected in the future (Peters et al., 2022).

6.2 Insecticide resistant *Sitobion avenae* are not better BYDV transmitters

In the UK in 2016, reports of widespread pyrethroid spray failures of *S. avenae* were linked with high BYDV outbreaks, which led to the hypothesis that the partially resistant *S. avenae* clone (SA3) might be a more efficient BYDV transmitter than other clones of the population (Dewar & Foster, 2017). The results from Chapters 2 and 3 of this thesis showed that resistant *S. avenae* collected from the field and in the Oak Park suction tower in the Republic of Ireland are not more likely to carry BYDV than other susceptible *S. avenae* clones. Additionally, there was no direct link between treated fields showing higher levels of resistance; therefore, BYDV outbreaks, during this study could not be linked to the presence of insecticide resistant *S. avenae*.

Chapter 4 of this thesis focused on a direct comparison of the transmission efficiencies of two BYDV species (BYDV MAV and BYDV-PAS) found in Irish barley, between the resistant *S. avenae* clone (SA3) and other susceptible *S. avenae* clones. For both BYDV-MAV (found in 100% of sequenced field samples (Byrne et al. 2024)) and BYDV-PAS (found in 29% of sequenced field samples (Byrne et al. 2024)), there was no evidence that the resistant SA3 clone was a better BYDV transmitter than other susceptible *S. avenae* clones. Therefore, the main hypothesis that the resistant SA3 is a better BYDV spreader than susceptible clones could not be confirmed in this study. This, in combination with the results from Chapters 2 and 3 and other fitness deficits in the resistant clonal lineage (Jackson et al., 2020), forms the conclusion that the evolution of insecticide resistant *S. avenae*, at least in Ireland, is currently not as impactful as feared for BYDV management in winter barley (Dewar & Foster, 2017).

Another potential method of BYDV management in the near future could be the sowing of BYDV tolerant or resistant varieties, such as tolerant winter barley varieties Joyau or Molly. Preliminary results from this thesis, investigating BYDV transmission efficiencies from a BYDV susceptible barley variety onto a BYDV tolerant variety (Chapter 4), showed a decreased transmission efficiency of BYDV-PAS. However, while it is promising to see new tolerant varieties for BYDV control emerging, it remains unclear how these new varieties perform under Irish conditions,

which consist of a different climate, different endemic aphid vectors, and BYDV species. Therefore, future IPM tools might need to be adjusted for managing BYDV and resistant aphids, especially in light of the possible evolution of new resistances due to a lack of insecticide alternatives, the development of BYDV tolerant/resistant barley varieties, and in a changing climate (Mc Namara et al., 2020).

6.3 BYDV manipulates aphid behaviour to enhance its own spread

All *S. avenae* clones tested in the transmission experiments of this thesis were found to be efficient spreaders of BYDV-MAV, while BYDV-PAS transmission efficiencies were found to be surprisingly low in *S. avenae* (Chapter 4), in comparison to previous studies (Dedryver et al., 2005; Van den Eynde et al., 2020). The virus transmission results confirmed that *S. avenae* was a main vector of BYDV-MAV, which was found in 100% of the fields sampled in Ireland (Chapter 4). However, BYDV-PAS (for the first time sequenced in Ireland (Byrne et al. 2024) was also found in almost a third of all fields sampled, even though *S. avenae* was a poor vector (Chapter 4), and the main vector *R. padi* was almost absent in all fields tested (Chapter 2). Chapter 5 therefore investigated whether there was a potential mechanism for BYDV to manipulate aphids, to enhance its own spread (Chesnais et al., 2020). For this, a large-scale vector manipulation experiment was conducted, including three clones of *S. avenae* and one *R. padi* clone, to investigate fecundity and behavioural manipulation by both BYDV-MAV and BYDV-PAS. The results showed, that BYDV-MAV carrying aphids (both *S. avenae* and *R. padi*) showed a significantly increased dispersion, velocity, movement, and initial movement compared to virus-free aphids. In addition, BYDV-PAS carrying aphids showed a significantly stronger increase in behavioural manipulation than BYDV-MAV carrying aphids, and increased fecundity in *S. avenae*. Therefore, as BYDV-PAS is still widely spread throughout Ireland (Byrne et al. 2024), behavioural manipulation of aphids could be a potential mechanism for BYDV-PAS to compensate for poor transmission efficiencies (Chapter 4, Mauck and Chesnais (2020)). However, this will need to be confirmed in further experiments, as results from the fieldwork (Chapter 2) indicate that *M. dirhodum*, which transmits both BYDV-MAV and BYDV-

PAS, might also play a yet to be investigated major role in BYDV epidemiology in Ireland.

Aphid populations are closely linked to weather conditions and show a high yearly variation throughout a year (Chapter 2 & 3, Bell et al. (2015)). However, there was no direct link between high aphid numbers resulting in high levels of BYDV, showing that aphid presence in a field does not automatically lead to increased virus presence (Chapter 2 & 3). To increase our understanding of BYDV epidemiology, it will be necessary to further understand its life cycle, movement of cereal aphids in the field and aphid migration patterns (Van den Eynde et al., 2020). Behavioural vector manipulation of aphids carrying BYDV may therefore play an important role in virus epidemiology, although any existing decision support tool (DST) has not yet considered this.

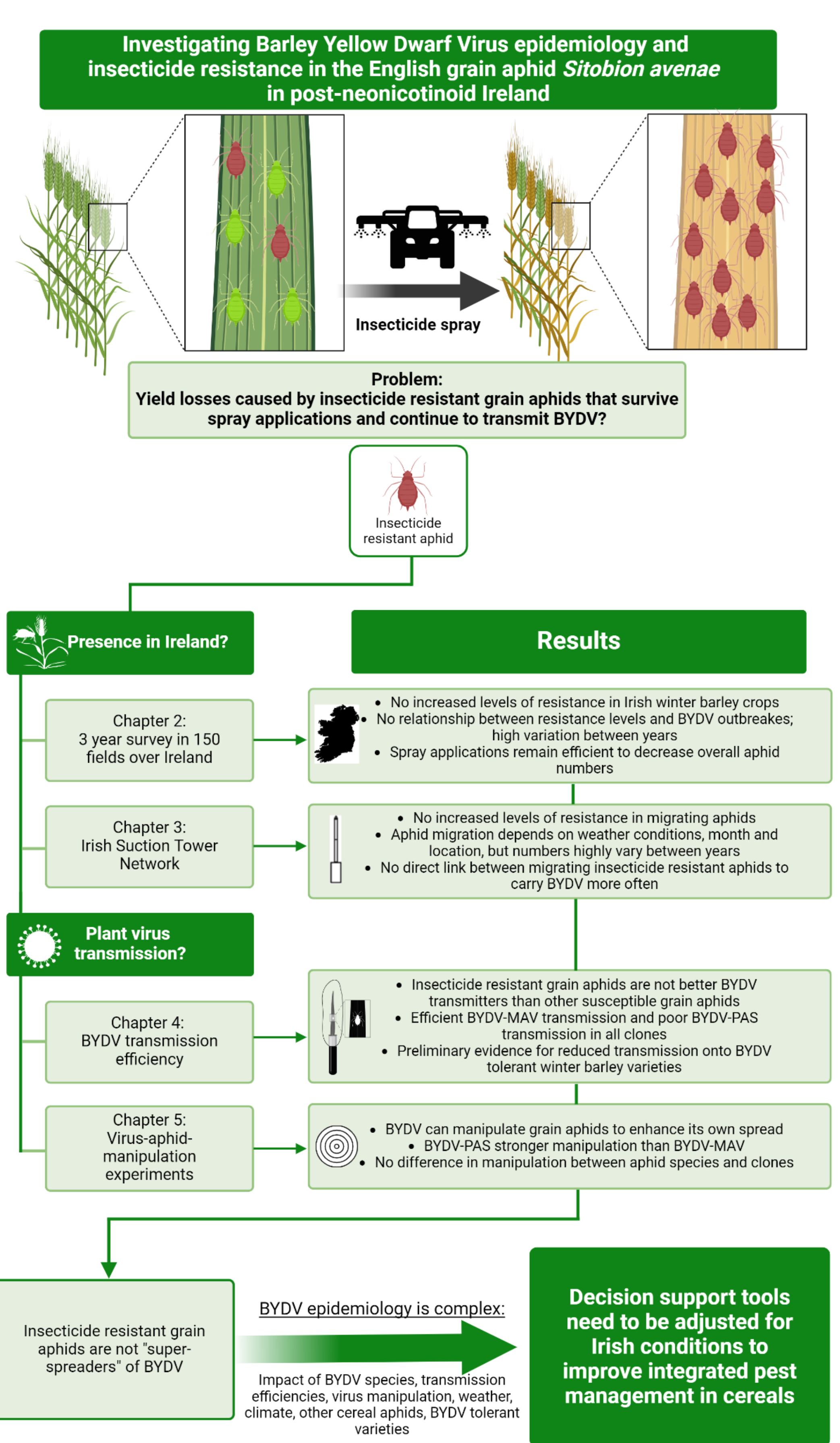

Figure 6.1: Summary of this research thesis

6.4 Next generation DST for BYDV management need to be adjusted for Ireland

The relatively mild Irish climate allows for some of the highest winter barley yields in the world but may also lead to strong disease pressure, including BYDV outbreaks caused by cereal aphids (Teagasc, 2023). To manage aphid pressure, farmers can consult decision support tools (DST). However, Ireland currently lacks an accurate DST, as this thesis showed that Irish cereal aphid populations and BYDV epidemiology differ from the UK or continental Europe, for which most DST were designed (Kennedy & Connery, 2001; Walls III et al., 2019).

In this thesis for example, *S. avenae* and *M. dirhodum* were found to be the predominant aphids in Irish winter barley fields, while almost no *R. padi* (for which the newly developed ACroBAT (ADAS) and other DSTs were designed) were found in Irish winter barley fields (Chapter 2 + 4, White et al. (2023)). Additionally, BYDV-MAV, which can only be transmitted by *S. avenae* and *M. dirhodum*, was found to be the predominant BYDV species in Irish winter barley fields, while BYDV-PAS, which was shown to be poorly transmitted by *S. avenae*, was less frequent (Byrne et al. 2024, Chapter 4). However, BYDV-PAS showed a higher capability to manipulate aphid behaviour, which could compensate for the poor transmission (Chapter 5). All of the above could be an important mechanism in BYDV epidemiology in Ireland and therefore should be taken into account in the development of a future DST.

Another important aspect in the development of an accurate DST is monitoring aphid migration through a network of suction towers (Harrington & Hullé, 2017). The installation of a Republic of Ireland suction tower network showed that *S. avenae* migration followed the expected environmental patterns, but numbers, resistance and virus levels varied between the years (Chapter 3). Although, the suction tower data is still limited. For this thesis, only *S. avenae* was analysed, while *R. padi* and *M. dirhodum* were also collected throughout the years. Testing other aphid species for resistance and BYDV, in combination with predicting aphid migration patterns based on future long-term data, will be crucial for the development of a future DST (Bell et al., 2015).

In conclusion, the results from this thesis highlight further aspects that should be considered when developing an Irish DST (White et al., 2023). A solution for the development of an Irish model should be based on the aggregation of existing models, which additionally include Irish-specific differences such as different predominant BYDV species and their transmission efficiencies and vector manipulation effects, weather and climate differences between years, and the impact of other aphid species and their spatio-temporal population dynamics. Although this potential model would be computationally challenging, AI and machine learning tools using real-time weather data or aphid numbers should make it possible to create a living model which would be an important step into an accurate DST for Ireland (Holloway et al., 2018; White et al., 2023).

6.5 Impact of research findings onto Irish IPM

The current IPM advice to manage *S. avenae* and BYDV in winter barley in Ireland is based on cultural control, biological and chemical pest control, and, most recently, the inclusion of BYDV tolerant cultivars (DAFM, 2024; Teagasc, 2023). This research thesis found that the relative importance of insecticide resistant *S. avenae* on BYDV spread was lower than expected, and that chemical control, for susceptible *S. avenae* and other cereal aphids, although limited to pyrethroids, currently remains an important control tool. It was also shown that BYDV outbreaks are highly variable in location and between years. Nonetheless, new resistances to insecticides are likely to emerge, which will challenge their efficacy and increase their reliance on other IPM tools in the near future (Bass & Nauen, 2023). Therefore, it is rational to assume that current IPM tools are efficient for managing BYDV and resistant aphids, yet this may change with the evolution of new resistances due to a lack of insecticide alternatives, the development of BYDV tolerant/resistant barley varieties, or in a changing climate (Mc Namara et al., 2020). Ongoing national monitoring of key BYDV vectors and their resistance status will be crucial for a comprehensive understanding of events on a yearly basis, so that future IPM advice can be updated to include these dynamic challenges.

6.6 Conclusion

By incorporating field monitoring, the suction tower network, BYDV sequencing, BYDV transmission experiments, and vector manipulation experiments, this thesis showed that the importance of the insecticide resistant grain aphid (*S. avenae*) for BYDV spread in Ireland is likely no greater than that of susceptible aphids, and that current IPM measures will protect yields (Fig. 6.1). Additionally, the results highlighted the complexity of tripartite interactions between aphids, viruses, and plants, all of which affect BYDV epidemiology, and should be taken into consideration when developing the next generation of DST for Ireland.

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Appendices

Table A1
Leaf samples collected from Irish winter and spring barley fields that were selected for virus sequencing based on the serological testing results (ELISA, Type B, Type F and CYDV-RPV) (Byrne et al., 2024)

Sample ID	Year Sampled	County	Crop	Type B (PAV-like)	Type F (MAV-like)	CYDV-RPV
M3	2021	Meath	Winter Barley	+	+	-
SAC[1]	2021	NA	NA	+	+	-
Wi1	2021	Wicklow	Winter Barley	+	+	-
Ti5	2021	Tipperary	Winter Barley	+	+	-
Ki2	2021	Kildare	Winter Barley	+	+	-
SBKST	2021	Kilkenny	Spring Barley	+	+	-
La2a	2021	Laois	Winter Barley	+	+	-
C6	2021	Cork	Winter Barley	+	+	-
RPC[1]	2021	NA	NA	+	+	-
SBKnST	2021	Carlow	Spring Barley	+	+	-
FC[1]	2021	NA	NA	+	+	-
Ki5	2021	Kildare	Winter Barley	+	+	-
C4	2021	Cork	Winter Barley	+	+	-
C7	2021	Cork	Winter Barley	+	+	-
Ki1	2021	Kildare	Winter Barley	+	+	-
KK3	2021	Kilkenny	Winter Barley	+	+	-
La1a1c	2021	Laois	Winter Barley	+	+	-
La3a	2021	Laois	Winter Barley	+	+	-
M5	2021	Meath	Winter Barley	+	+	-
SBK	2021	Kilkenny	Spring Barley	+	+	-
SBW	2021	Wexford	Spring Barley	+	+	-
WB20	2020	Carlow	Winter Barley	+	+	-
Ti3	2021	Tipperary	Winter Barley	+	+	-
Wi6	2021	Wicklow	Winter Barley	+	+	-
C1	2022	Cork	Winter Barley	+	+	-
C10	2022	Cork	Winter Barley	-	+	-
Ki4	2022	Kildare	Winter Barley	+	+	-
Ki9	2022	Kildare	Winter Barley	+	+	-
Wi6RD3	2022	Wicklow	Winter Barley	+	+	-
Wi2J	2022	Wicklow	Winter Barley	+	+	-
Ti4J	2022	Tipperary	Winter Barley	+	+	-
Ti2J	2022	Tipperary	Winter Barley	+	+	-
La3RD3	2022	Laois	Winter Barley	+	+	-
La11J	2022	Laois	Winter Barley	+	+	-
Kk3RD3	2022	Kilkenny	Winter Barley	+	+	-
M2	2022	Meath	Winter Barley	-	+	-
P3F2	2022	Wexford	Spring Barley	+	+	+
P3F9	2022	Waterford	Spring Barley	+	+	-
P4A8	2022	Laois	Spring Barley	+	+	-
P1A7	2022	Carlow	Spring Barley	+	+	-
P4E2	2022	Kilkenny	Spring Barley	+	+	-
P4D1	2022	Cork	Spring Barley	+	+	+
P3E1	2022	Louth	Spring Barley	+	+	-
P3H3	2022	Tipperary	Spring Barley	+	-	-
P3G3	2022	Wexford	Spring Barley	+	+	-
P4B3	2022	Kilkenny	Spring Barley	+	+	-

P4B9	2022	Meath	Spring Barley	+	+	-
P1H1	2022	Kildare	Spring Barley	-	+	-

[1] *Leaf samples were taken from colonies maintained in laboratories at Oak Park – these colonies were SAC (S. avenae colony positive for BYDV-MAV); RPC (R. padi colony positive for PAS) and FC (S. avenae colony positive for PAS).*

Table A2

High throughput sequencing results and identification of yellow dwarf viruses (Byrne et al., 2024)

Sample	Read pairs (M)	Non-host read pairs (M)	Percentage non-host reads	NCBI Blastn Yellow Dwarf Virus Hits[1]	Reads mapped to each reference genome (as percentage)	Mean Coverage (percentage coverage)[2]
M3	58.6	37.1	63	BYDV-PAV (KY593458.1)	37,365 (3.7%)	931.0 (99.6%)
				BYDV-GAV (MK012662.1)	157,141 (15.7%)	3,924.1 (100%)
SAC	31.7	19.2	61	BYDV-GAV (MK012662.1)	629,207 (62.9%)	16,672.3 (99.9%)
Wi1	37.1	19.7	53	BYDV-GAV (MK012662.1)	31,554 (3.2%)	144.0 (97.6%)
Ti5	46.4	24.7	53	BYDV-GAV (MK012662.1)	101,387 (10.1%)	2516.1 (100%)
Ki2	31.6	16.1	51	BYDV-PAV (EF521844)	10,312 (1.0%)	268.1 (67.7%)
				BYDV-GAV (MK012662.1)	66,106 (6.6%)	1,663.1(98.0%)
				BYDV-PAS (MN128940)	1,036 (0.1%)	27.6 (78.7%)
SBKST	29.8	15.5	52	BYDV-GAV (MK012662.1)	435,882 (43.6%)	11,715.6 (99.5%)
				BYDV-PAV (MK962883.1)	87,438 (8.7%)	2,405.1 (95.2%)
La2a	29.3	22	75	BYDV-GAV (MK012662.1)	220,844 (22.1%)	5,507.1 (99.8%)
C6	28.7	14.5	51	BYDV-GAV (MK012662.1)	144,142 (14.4%)	3,804.1 (99.2%)
				BYDV-PAS (MK012654.1)	1,888 (1.9%)	49.7 (97.9%)
Ki5	33.3	23.2	70	BYDV-GAV (MK012662.1)	222,197 (22.2%)	5,542.8 (100%)
SBKnST	32.9	23.8	72	BYDV-PAV (MK962883.1)	22,832 (2.3%)	568.8 (81.8%)
				BYDV-GAV (MK012662.1)	222,642 (22.3%)	5,571.6 (97.9%)
FC	36.3	28.3	78	BYDV-PAS (MK012654.1)	447,490 (44.7%)	11,358.2 (100%)
RPC	28.2	6.8	24	BYDV-GAV (MK012662.1)	26,657 (2.7%)	701.6 (98.9%)
				BYDV-PAS (MK012652.1)	15,773 (1.6%)	415.2 (99.4%)
C4	34.5	13.8	40	BYDV-GAV (MK012662.1)	200,316 (20.0%)	4,865.4 (99.9%)
				BYDV-PAS (MK012654.1)	1,808 (1.8%)	42.2 (94.4%)
C7	33.1	16.5	50	BYDV-GAV (MK012662.1)	118,980 (11.9%)	2,863.9 (100%)
				BYDV-PAS (MK012654.1)	1,378 (1.4%)	32.9 (76.4%)
Ki1	44.8	21.4	48	BYDV-PAV (KY621333)	26,870 (2.7%)	664.7 (99.9%)
				BYDV-GAV (MK012662.1)	187,854 (18.8%)	4,644.6 (100%)
KK3	29.1	18	62	BYDV-GAV (MK012662.1)	88,380 (8.8%)	2,148.8 (100%)
La1a1c	32.5	19.3	59	BYDV-GAV (MK012662.1)	14,614 (1.5%)	363.0 (96.4%)

La3a	34.5	16.7	48	BYDV-PAV (AJ810418)	46,196 (4.6%)	141.7 (98.4%)
				BYDV-GAV (MK012662.1)	5,050 (0.5%)	125.7 (97.1%)
				BYDV-PAS (MK012654)	90,342 (9.0%)	2,217.7 (100%)
				CYDV-RPS (MK012664)	55,998 (5.6%)	1,399.7 (98.4%)
M5	37.9	22.5	59	BYDV-GAV (MK012662.1)	427,060 (42.7%)	10,455.7 (100%)
SBK	36	23	64	BYDV-GAV (MK012662.1)	483,390 (48.3%)	12,012.8 (100%)
SBW	33.1	18.6	56	BYDV-GAV (MK012662.1)	356,072 (35.6%)	8,847.9 (99.9%)
				BYDV-PAS (MK012652.1)	926 (0.09%)	22.7 (93.3%)
WB20	34.4	21.9	64	BYDV-GAV (MK012662.1)	436,058 (43.6%)	10,832.4 (99.9%)
Ti3	41.7	18.8	45	BYDV-GAV (MK012662.1)	60,690 (6.1%)	1,464.3 (98.2%)
Wi6	42.8	21.6	50	BYDV-GAV (MK012662.1)	49,042 (4.9%)	1,226.3 (99.4%)
C1	32.8	27.3	83	BYDV-PAV (MK012661)	24,802 (2.5%)	622.8 (91.3%)
				BYDV-GAV (MK012662.1)	145,552 (14.6%)	3,585.1 (99.7%)
				BYDV-PAS (MK012654)	28,582 (2.9%)	707.0 (100%)
C10	34.3	25.4	74	BYDV-PAV (EF521841)	16,803 (1.7%)	421.0 (82.1%)
				BYDV-GAV (MK012662.1)	157,831 (15.8%)	3,910.1 (99.7%)
Ki4	34.1	28.6	84	BYDV-PAV (AJ810418)	76,134 (7.6%)	1,840.4 (93.9%)
				BYDV-GAV (MK012662.1)	70,896 (7.1%)	1,759.6 (100%)
				BYDV-PAS (MK012654)	52,654 (5.3%)	1,315.4 (100%)
Ki9	35.2	29.6	84	BYDV-PAV (AJ810418)	18,178 (1.8%)	453.7 (100%)
				BYDV-GAV (MK012662.1)	157,342 (15.7%)	3,978.3 (99.9%)
Wi6	40.8	31.7	78	BYDV-PAV (AJ810418)	25,316 (2.5%)	636.1 (99.4%)
				BYDV-GAV (MK012662.1)	127,630 (12.8%)	3,142.4 (100%)
Wi2J	35.3	27	76	BYDV-PAV (AJ810418)	15,919 (1.6%)	389.2 (100%)
				BYDV-GAV (MK012662.1)	137,605 (13.8%)	3,407.9 (100%)
Ti4J	37.1	27.3	74	BYDV-PAV (AJ810418)	26,334 (2.6%)	674.7 (100%)
				BYDV-GAV (MK012662.1)	149,650 (15.0%)	3,712.4 (99.8%)
Ti2J	35.5	29	82	BYDV-PAV (AJ810418)	42,994 (4.3%)	1,024.9 (95.6%)
				BYDV-PAS (MK012654)	89,330 (8.9%)	2,205.0 (98.7%)
				BYDV-GAV (MK012662.1)	65,700 (6.6%)	1,653.1 (99.6%)
La3RD3	38.7	30.9	80	BYDV-GAV (MK012662.1)	229,153 (22.9%)	5,674.2 (100%)
La11J	31.5	24.3	77	BYDV-GAV (MK012662.1)	189,643 (19.0%)	4,668.2 (100%)

Kk3-RD3	37.3	30.1	81	BYDV-PAV (AJ810418)	49,536 (4.9%)	1,184.1 (97.8%)
				BYDV-GAV (MK012662.1)	121,220 (12.1%)	3,005.5 (99.9%)
				BYDV-PAS (MK012654)	63,956 (6.4%)	1,583.8 (99.6%)
M2	31.6	15.8	50	BYDV-GAV (MK012662.1)	74,947 (7.5%)	1,855.9 (100%)
P3F2	33.7	18.1	54	BYDV-GAV (MK012662.1)	111,529 (11.2%)	2,765.4 (99.9%)
P3F9	30	18.3	61	BYDV-PAV (AJ810418)	21,590 (2.2%)	535.1 (99.9%)
				BYDV-GAV (MK012662.1)	135,124 (13.5%)	3,430.8 (98.1%)
P4A8	32.8	24.1	73	BYDV-GAV (MK012662.1)	165,271(16.5%)	4,122.2 (99.9%)
P1A7	34.7	27.4	79	BYDV-PAV (KY593458.1)	23,026 (2.3%)	598.4 (76.9%)
				BYDV-GAV (MK012662.1)	146,078 (14.6%)	3,728.9 (100%)
P4E2	29.7	20.7	70	BYDV-GAV (MK012662.1)	126,848 (12.7%)	2,863.9 (100%)
				BYDV-PAS (MK012654.1)	1,038 (0.1%)	32.9 (76.4%)
P4D1	37.8	31	82	BYDV-PAV (AJ810418)	16,041 (1.6%)	410.1 (99.6%)
				BYDV-GAV (MK012662.1)	110,331 (11.0%)	2,740.3 (100%)
P3E1	34.5	21.2	61	BYDV-GAV (MK012662.1)	128,212 (12.8%)	3,220.3 (99.9%)
				BYDV-PAS (MK012654.1)	870 (0.09%)	22.2 (94.2%)
P3H3	30.3	22.9	76	BYDV-PAV (AJ810418)	45,100 (4.5%)	1,113.9 (93.6%)
				BYDV-GAV (MK012662.1)	45,694 (4.6%)	1,145.9 (100%)
				BYDV-PAS (MK012654)	55,682 (5.6%)	1,462 (100%)
P3G3	33.8	18.8	56	BYDV-PAV (AJ810418)	19,856 (2.0%)	510.3 (92.4%)
				BYDV-GAV (MK012662.1)	112,472 (11.2%)	2,820.4 (97.3%)
P4B3	33.1	26.6	80	BYDV-GAV (MK012662.1)	195,187 (19.5%)	4,820.7 (100%)
P4B9	35.4	24.2	68	BYDV-PAV (MK962883)	18,192 (1.8%)	462.1 (86.5%)
				BYDV-GAV (MK012662.1)	132,626 (13.3%)	3,290.0 (99.2%)
P1H1	37.9	24.6	65	BYDV-GAV (MK012662.1)	124,593 (12.5%)	3,150.7 (99.9%)