Effects of ω-ACTX-Hv1a/GNA, a novel protein biopesticide targeting voltage-gated calcium ion channels, on target and non-target arthropod species

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Abstract

An increasing human population is met with the challenge of feeding over 10 billion people by 2050. Nowadays, over 20% of crop production is lost to insect pests. Chemical control agents, in general, present broad-spectrum activity, killing both pests and beneficial organisms that would contribute to production (i.e. pollinators and natural enemies). Previously, the insecticidal voltage-gated calcium channel blocker peptide ω-ACTX-Hv1a (Hv1a) was linked to the 'carrier' molecule snowdrop lectin (GNA). The resulting fusion protein, Hv1a/GNA, is highly toxic towards lepidopteran and coleopteran pests, presenting potential for use as a biopesticide. Here, the fusion protein was shown to also be toxic to the hemipteran pests *Sitobion avenae* and *Myzus persicae*, via artificial diet and when expressed in transgenic plants. However, its effects on non-target arthropods have not been previously evaluated. Therefore, toxicity of Hv1a/GNA was tested against two beneficial insects, the parasitoid wasp *Eulophus pennicornis* via its host, *Lacanobia oleracea*, and the honeybee *Apis mellifera*. The fusion protein did not present any significant tri-trophic negative effects on *E. pennicornis*, even when injected into host larvae. Honeybee survival was slightly affected when fed on high doses of fusion protein representing a 'worst-case scenario', but lead to no detectable effects when dosed with field-relevant levels. The fact that bees internalized Hv1a/GNA led to the hypothesis that the haemolymph-feeding parasitic mite *Varroa destructor* would be affected by the fusion protein. However, mites were able to digest the protein and hence no effects were recorded. Further attempts to target calcium channels in *M. persicae* and *Tribolium castaneum* via RNAi were made. Whilst there were no phenotypic effects, gene expression was down-regulated in *T. castaneum.* This study shows that Hv1a/GNA is a specific biopesticide, posing low risks against beneficial non-target organisms while being toxic to selected insect pests.

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List of Abbreviations

°C	degrees Celsius
Ace	acetamiprid
ACTX	Atracotoxin
ANOVA	Analysis of Variance
BH	benidipine hydrochloride
BSA	Bovine serum albumine
Bt	*Bacillus thuringiensis*
ca.	circa; around
CaMV	Cauliflower mosaic virus
Cav	Voltage-gated calcium channels
cDNA	complementary DNA
CNS	central nervous system
CS	conditioned stimulus
d.f.	degrees of freedom
Da	Dalton(s)
DAI	days after injection
DDT	dichlorodiphenyltrichloroethane
dsRNA	double-stranded RNA
DUM	dorsal unpaired median
DWV	Deformed wing virus
EC	European Comission
ECL	enhanced luminol-based chemiluminescent
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
EPPO	European and Mediterranean Plant Protection Organization
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
Fera	Food and Environment Research Agency
FP	fusion protein
FW	fresh weight
g	gram(s)
g	gravity
GABA	gamma-Aminobutyric acid
GE	genetically engineered
GNA	*Galanthus nivalis* agglutinin or Snowdrop lectin
GST	Glutathione S-transferase
h	hour(s)
ha	Hectares
HEK	human embryonic kidney
Hv1a	ω-ACTX-Hv1a
HVA	high-voltage activated
IPCC	Intergovernmental Panel on Climate Change
IPM	Integrated Pest Management
K-M	Kaplan-Meyer analysis of survival
kDa	kilodalton(s)

Km	kanamycin
l	litre(s)
L:D	light:dark
LC_{50}	Median lethal concentration
LD_{50}	Median lethal dose
lreg	binary logistic regression
LTM	long-term memory
LVA	low-voltage activated
M	molar
M.p.	*Myzus persicae*
mg	miligram(s)
min	minute(s)
ml	mililitre
µl	microlitre
mm	milimetre(s)
mRNA	messenger RNA
µg	microgram
µM	micromolar
nAChR	nicotinic acetlycholine receptor
ng	nanogram(s)
nM	nanomolar
OECD	The Organisation for Economic Co-operation and Development
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	phosphate buffered saline - Tween
PCR	Polymerase chain reaction
PER	proboscis extension reflex
ppb	parts per billion
ppm	parts per million
PPO	phenylthiocarbamide-phenol oxidase
PVDF	Polyvinylidene fluoride
qPCR	Quantitative real-time PCR
rep	replicate
RH	relative humidity
RISC	RNA-induced silencing complex
RNAi	RNA interference
rpm	rotations per minute
s	second(s)
SDS	Sodium dodecyl sulfate
SE	Standard error
SEM	standard error of the mean
ssRNA	Single-stranded RNA
STM	short-term memory
Suc	sucrose
T.c.	*Tribolium castaneum*
TMX	thiamethoxam
UN	United Nations

USDA	U.S. Department of Agriculture
v	volume
w	weight

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Chapter 1 Introduction

1.1 General Introduction

Food production has doubled in the past four decades due to the use of high-yielding monocultures produced on intensively fertilized and irrigated soils, with pests (weeds, insects and diseases) actively controlled by chemicals (Tilman, 1999). Although poverty has hindered food security in some countries, mainly in sub-Saharan Africa and South Asia, the world is now able to produce enough food for everyone (Alexandratos, 1999). However, according to the United Nations (UN), the world population, currently at 7.2 billion people, is expected to reach 9.6 billion by 2050. Most of this growth will occur in developing countries, from 5.9 billion in 2013 to 8.2 billion in 2050, particularly in Africa, where more than half of the population increase is expected to take place (UN, 2012). Demand for food and fibre will consequently increase; it is projected that feeding 9.1 billion people would need an overall 70% increase in agricultural food production, doubling in developing countries (FAO, 2009). This will be particularly challenging considering soil fertility depletion, limited arable land available for expansion, limited water resources (FAO, 2011) and unsustainable use of fertilizers (Tilman, 1999). Furthermore, extreme temperatures and precipitation as consequences of global warming might negatively interfere with crop production, due to more frequent droughts and floods (EPA, 2013). Increasing temperatures may also help crop pests and diseases to spread (Bebber et al., 2013), and in fact global warming is already affecting food security, according to the Intergovernmental Panel on Climate Change (IPCC, 2014).

Crop production can be significantly increased by pest management practices. Depending on the crop, potential yield losses due to competition or destruction by pests are estimated to range from 50% (e.g. barley) to more than 80% (sugar beet and cotton) (Oerke, 2006). Although weeds can potentially be more destructive to crops than animal pests (~30% against ~20% losses), their control efficacy is higher than that of animal pests and diseases (68% against 39% and 32%, respectively), as weeds can be controlled by both chemical and mechanical methods (Oerke and Dehne, 2004). In 2007, for which the most

recent figures were produced by the Environmental Protection Agency (EPA), the annual pesticide market reached $35.8 billion globally, with expenditures on insecticides reaching $10.25 billion, or 29% of the total (Grube et al., 2011). Although insecticides have contributed to increase food production, their effects on human health (Guillette Jr. and Iguchi, 2012) and on wildlife populations, communities and ecosystems are poorly understood (Kohler and Triebskorn, 2013). Furthermore, the rise of insect resistance to conventional chemical pesticides, combined with more stringent regulations and market growth lead to an increasing demand for safer, specific and more efficient insecticides (Lamberth et al., 2013). In the European Union (EU) in particular, policies were designed to significantly reduce pesticide use while increasing Integrated Pest Management (IPM) strategies (European Commission, 2009). This approach involves the coordinated use of applied ecology concepts on the selection of cultural, chemical and biological methods for suppressing pest populations. Whilst apparently environmentally friendlier than conventional agriculture, it will invariably restrict the number of available active ingredients for pest control and require farmers to have access to information and technical support, causing an increase in production costs (Hillocks, 2011). Nevertheless, chemical or biological insecticides are a vital part of any agricultural system, organic or conventional. The discovery and development of novel compounds, compatible with different production methods, is therefore important for addressing problems relative to insect resistance and thus contribute to food security.

1.2 Molecular targets for insect control

The most commonly used commercial insecticides for agricultural pest control target insect nervous systems (Casida and Durkin, 2013). These chemicals rely on a relatively small set of molecular targets, namely acetylcholinesterase enzymes (organophosphates and carbamates) (Fukuto, 1990); voltage-gated sodium channels (e.g. organochlorides and pyrethroids); and neurotransmitter-gated nicotinic acetylcholine (e.g. neonicotinoids), γ-aminobutyric acid – GABA (e.g. phenylpyrazoles) and L-glutamate (e.g. avermectins) receptors (Raymond-Delpech et al., 2005) (Figure 1.1). In addition to human health and environmental costs involved in the heavy dependence on chemical control methods (Aghabiklooei et al., 2013; Rasmussen et al., 2013; Ali et al., 2014),

the prolonged use of insecticides acting on the same range of molecular targets has consistently led to the evolution of pest resistance within insect populations. Different mechanisms of resistance have been reported, including mutations on sites of interaction, gene duplication/increased expression of detoxifying enzymes (e.g., carboxylesterase and P450) and faster mechanisms of excretion of insecticidal compounds from cells (Heckel, 2012; Ffrench-Constant, 2013). For instance, in the housefly *Musca domestica*, resistance to the organochloride DDT is achieved due to mutations in a sodium channel gene, modifying the amino acid composition of its product. The interaction between DDT and the sodium channel is then compromised, rendering insect resistance to the chemical. In that case, flies presented cross-resistance to pyrethrins and pyrethroids, which also target sodium channels (Davies et al., 2007; Soderlund, 2008). The same mechanism of DDT and pyrethroid cross-resistance has been reported in different strains of the mosquito *Aedes aegypti*, which in some cases have also increased levels of monooxygenase and glutathione S-transferase, increasing the rate of metabolic detoxification (Brengues et al., 2003). At least seven mechanisms of insecticide resistance were described in the aphid *Myzus persicae* (as reviewed by Bass et al., 2014). For example, Field et al. (1998) reported that increased expression of detoxifying carboxylesterases E4 or FE4 results in insect resistance to organophosphates and carbamates. Additionally, changes in the acetylcholinesterase (*AchE*) and sodium channel (*kdr*) genes confer aphid resistance to dimethyl carbamates and pyrethroids, respectively (Devonshire et al., 1998). Puinean et al. (2010) report association between increased expression of another detoxifying enzyme, cytochrome P450, and resistance of *M. persicae* to neonicotinoids.

Detoxification mechanisms generally involve three phases: i) initially, monooxygenases such as P450 make chemicals more water soluble by adding hydroxyl, carboxyl and amino groups, increasing ii) conjugation and processing by other enzymes, such as GSTs; and, finally, iii) the modified compounds can be transported out of the cell (Perry et al., 2011), commonly ABC transporters, which have been associated with approximately 30 cases of insect resistance to pesticides (Dermauw and Van Leewen, 2014).

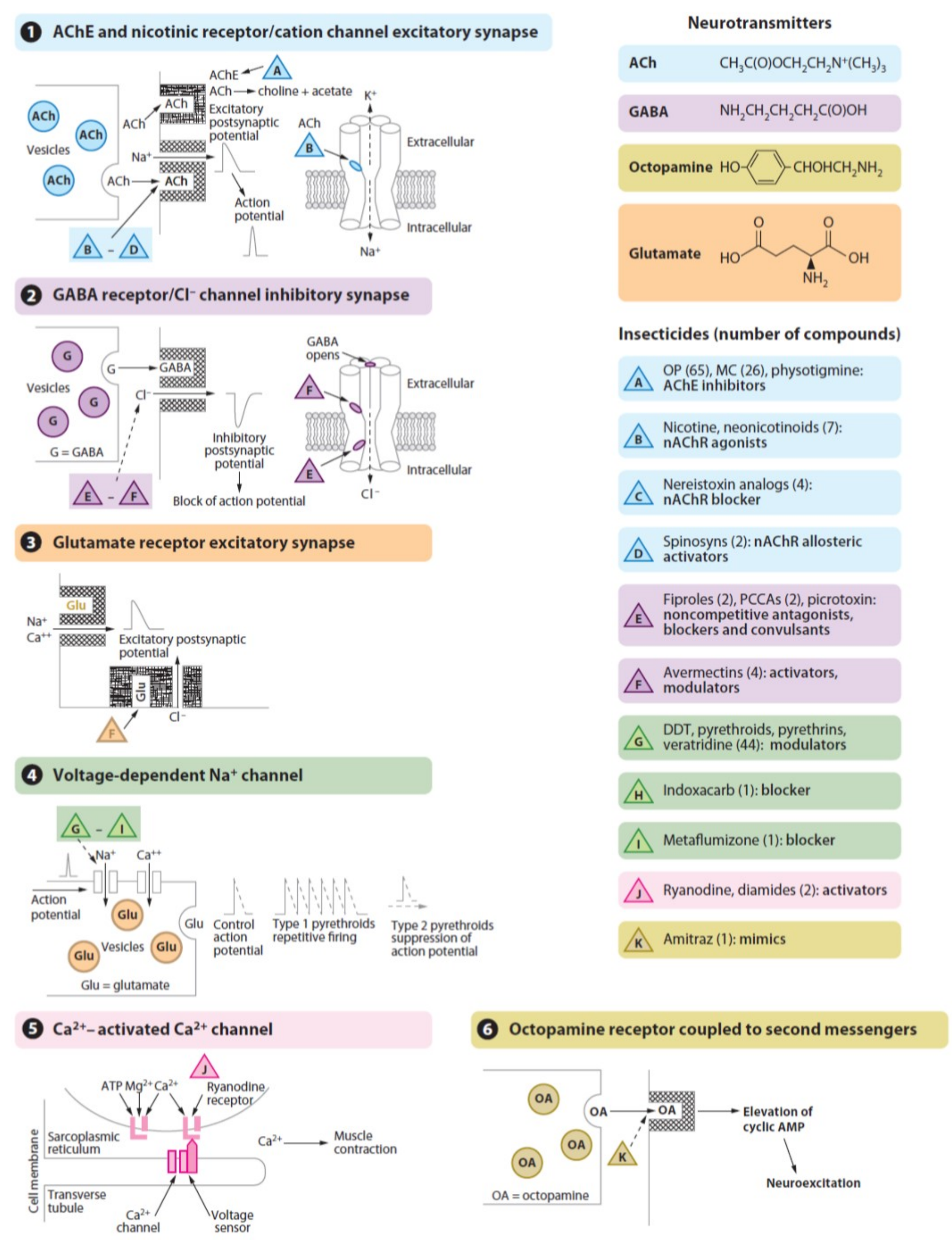

Figure 1.1: Most commercial insecticides are neuroactive agents. Source: Casida and Durkin, 2013.

Biological pesticides, or biopesticides, are derived from natural sources, such as plants, minerals and microorganisms. These include biological agents (e.g.

entomopathogenic fungi, bacteria and viruses) and bioactive compounds (e.g., metabolites) that offer alternatives to chemical control (Glare et al., 2012). One of the most commonly used biopesticides is spinosad, a mixture containing mainly spinosyn A and spinosyn D from the Actinomycete *Saccharopolyspora spinosa* (Kirst et al., 1992). It is a broad-spectrum insecticide, toxic via contact or ingestion and believed to target a subtype of nicotinic acetylcholine receptor (Orr et al., 2009). Although effective in controlling pests from different orders, such as Lepidoptera, Coleoptera, Diptera and Orthoptera, field resistance of *Thrips tabaci* has been recently reported (Lebedev et al., 2013). Furthermore, even though spinosad is widely used in IPM and organic farming systems, it presents high toxicity to insect pollinators (e.g. *Bombus* sp.) and biological control agents, such as parasitoids (Biondi et al., 2012).

The insecticidal crystal proteins (Cry) from the entomopathogenic soil bacterium *Bacillus thuringiensis* (Bt) are the most successful example of a biological insecticide, accountable for around 2% of the insecticidal market (Bravo et al., 2011). These toxins have been used as biopesticides for at least 50 years, including in organic farming systems (Zehnder et al., 2006). They are particularly effective against lepidopteran, coleopteran and dipteran larvae, being grouped according to their amino acid similarities (Crickmore et al., 1998). Contrary to chemical insecticides, Cry proteins are pore-forming toxins, causing midgut lysis and osmotic shock, leading to insect death. The mode of action of Cry toxins is complex, involving i) ingestion by larvae of susceptible insects, ii) toxin interaction with specific receptors in the midgut, facilitating iii) toxin oligomerization, and finally iv) insertion into cell membranes, leading to pore formation and cell death (Bravo et al., 2007).

Since 1996, plants genetically engineered (GE) to express Cry toxins have been employed for insect control. Given its effectiveness and safety to non-target organisms (Romeis et al., 2006), including humans (Mendelsohn et al., 2003), the worldwide area planted with Bt crops, mainly soybean, maize, cotton and canola, increased from 1.1 million hectares (ha) in 1996 to 66 million ha in 2011 (James, 2011). A more recent report by James (2013) shows that 11 million ha of Bt cotton were planted in India (95% adoption rate), against 4.2 million ha in China. Adoption rates are also increasing in Africa (Burkina Faso

and Sudan planting Bt cotton) and Europe, where five countries planted around 150 thousand ha of Bt maize in 2013, 15% more than in 2012 (James, 2013).

Although efficient against their targets, Cry toxins produced by commercial GE crops do not control all insect pests, such as aphids, since these organisms do not have appropriate receptors to trigger the toxin mechanism of action (Porcar et al., 2009). As a consequence, the narrow spectrum of Cry toxins, combined with lower insecticide applications, can contribute to secondary pest outbreaks (Meissle et al., 2011). Furthermore, pests are continuously evolving resistance to Bt crops due to the increase in planted area and constant exposure to Cry-producing plants (Tabashnik et al., 2013). Insects will invariably develop resistance if control methods are not judiciously deployed, and insecticides have been historically losing their effectiveness. Therefore, exploring different molecular targets and strategies for pest management is desirable for guaranteeing sustainable yields.

1.3 Voltage-gated calcium channels as targets for insecticides

Voltage-gated calcium channels (CaV) are heteromeric membrane proteins that mediate the entry of calcium ions (Ca^{2+}) into cells in response to depolarization. In animals, a number of biological processes, including muscular contraction, neurotransmitter release, secretion and gene expression are controlled by CaV (Catterall, 1995). These channels comprise a pore-forming subunit α1 with four repeat domains (I to IV), each containing six trans-membrane segments (S1 to S6) associated with different combinations of a disulfide-linked α2δ dimer subunit, an intracellular ß subunit and a transmembrane γ subunit (Figure 1.2; Catterall, 2011).

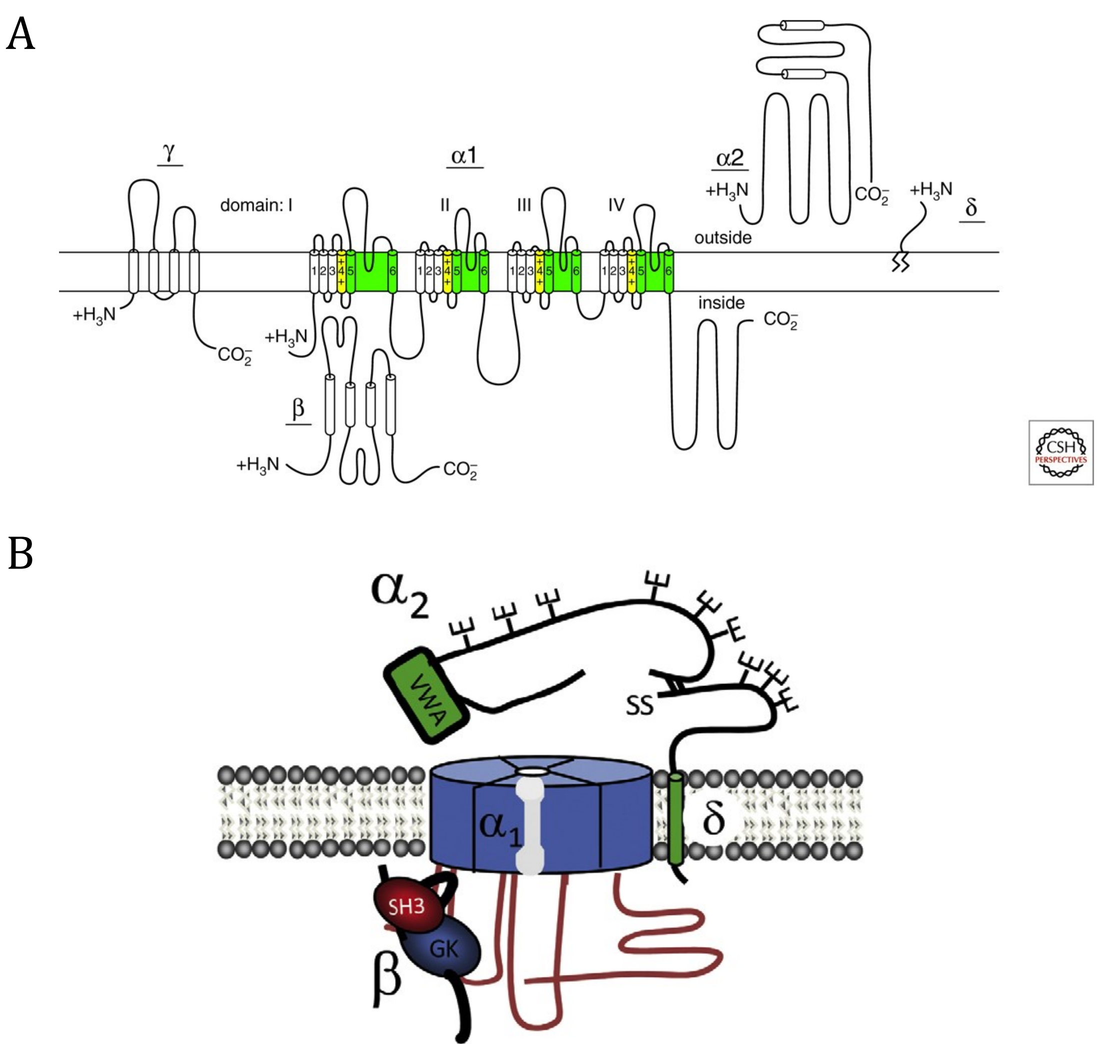

Figure 1.2: Model of a mammalian voltage-gated calcium channel structure, showing its different subunits. A) Two-dimensional structure of CaV. Cylinders represent predicted α helices in α-1 subunit, forming four domains, each composed of six transmembrane segments (1-6); lengths of lines are approximately correlated to the lengths of the polypeptide segments. Source: Catterall, 2011. B) Three-dimensional structure of a calcium channel with its subunits. Source: Dolphin, 2009.

The biophysical and biochemical properties of animal CaV are mainly defined by their α1 subunits, and can be divided in two broad superfamilies: high-voltage activated (HVA) or low-voltage activated (LVA) channels, depending on whether they are activated via a small or larger membrane depolarization, respectively. Additionally, they can be grouped according to one out of three α1 subunit subfamilies, presently CaV1, mediating L-type (long lasting) Ca^{2+} currents; CaV2, mediating P- (Purkinge)/Q-, N- (neuronal) and R- (residual) types; and CaV3, which mediates T-type (transient) Ca^{2+} currents (Catterall et al., 2003) (Table 1.1).

Table 1.1: Nomenclature of vertebrate CaV (King, 2007; Dolphin, 2009).

	Family	A1 subunit	IUP[a] name	Main localization
High-voltage activated (HVA)	L-type	α1S	CaV1.1	Skeletal muscle
		α1C	CaV1.2	cardiac, smooth muscle, neuronal
		α1D	CaV1.3	sinoatrial node, cochlear hair cells, neuronal (dendritic)
		α1F	CaV1.4	Retina
	P/Q-type	α1A	CaV2.1	neuronal (presynaptic)
	N-type	α1B	CaV2.2	neuronal (presynaptic)
	R-type	α1E	CaV2.3	Neuronal
Low-voltage activated (LVA)	T-type	α1G	CaV3.1	neuronal, cardiac
		α1H	CaV3.2	neuronal (+ other tissues)
		α1I	CaV3.3	Neuronal

[a] International Union of Pharmacology nomenclature

Mammalian genes typically code for ten α1 subunits, four ß subunits, four α2δ complexes and seven γ subunits (Dolphin, 2009). In contrast, insects present a lower number of genes coding for α1 subunits; the aphid *A. pisum* has only one gene coding for L-, N- and T-types (Dale et al., 2010), and *Drosophila melanogaster* presents only three, Dmca1D, Dmca1A (or cacophony – cac) and Ca- α_{1T}, generally classified as L-type, N-type and T-type, respectively, or CaV1, CaV2 and CaV3 (Littleton and Ganetzky, 2000; King, 2007). Severe loss of function of Dmca1D or cac in mutant flies leads to embryonic death (Eberl et al., 1998; Kawasaki et al., 2002), which indicates they have unique, non-redundant physiological roles and little functional plasticity. In addition, insect voltage-gated calcium channels are less well conserved than sodium channels and present low similarity to human sequences; as a consequence, developing insecticides that would affect target pests, but not CaV from non-target organisms such as bees, biological control agents and vertebrates would be more feasible (King, 2007).

1.4 CaV blockers – ω-ACTX-Hv1a (Hv1a)

Spiders produce venoms that allow them to incapacitate the nervous system of their preys, commonly insects; their venom is a complex and highly variable mixture of salts, small organic compounds, lytic peptides, proteases, and neurotoxins that affect central and peripheral nervous systems (King and Hardy, 2013). Therefore, they provide a valuable source of insecticidal compounds, including voltage-gated calcium channel blockers (Tedford et al., 2004a). For example, the spider venom toxin ω-ACTX-Hv1a (Hv1a) was isolated from the funnel-web spider *Hadronyche versuta* and initially shown to be acutely toxic to Heliothine insects, but not to newborn mice (Atkinson et al., 1998). Symptoms following injection of Hv1a into susceptible insects typically involve a neuroexcitatory effect, which causes spastic paralysis, followed by flaccid paralysis and death. Hv1a acts in central nervous system (CNS) neurons, not affecting interganglionic neurons or neuromuscular junctions (Fletcher et al., 1997; Bloomquist, 2003). It binds strongly to cockroach (*Periplaneta americana*) neuronal preparations and, at subnanomolar concentrations, induces excitatory responses in *D. melanogaster* CNS, indicating a high affinity for CaV1 (King et al., 2008). Transgenic *D. melanogaster* transformed with a heat-shock inducible Hv1a gene frequently failed to inflate and harden wings after emergence (Tedford et al., 2007), a phenotype resembling that of flies carrying a hypomorfic Dmca1D (the only CaV1 gene in *D. melanogaster*) allele (Eberl et al., 1998).

In *P. americana* dorsal unpaired median (DUM) neurons, Hv1a only moderately and reversibly blocks both mid-low and high-voltage-activated calcium channel currents (Chong et al., 2007), which are putative CaV2 channels. This weak response might be explained by a lack or low concentration of CaV1 channels in DUM neurons, as hypothesized by King et al. (2008). Conversely, Hv1a does not inhibit vertebrate calcium channel currents at high concentrations (10 μM), although at extremely high concentrations (30 μM), it partially antagonizes rat CaV1.2 and also very weakly against CaV2.1, CaV2.2 in human embryonic kidney (HEK) cells transiently expressing those channels (Tedford et al., 2004b).

The Hv1a peptide consists of 37-residues, displaying structural homology with the vertebrate calcium channel antagonists ω-agatoxins and ω-conotoxins, despite their considerably different sequences (Fletcher et al., 1997). Interestingly, the funnel-web spider is able to produce five other ω-atracotoxins, four of them differing by 1-3 amino acids and showing similar insecticidal activities to Hv1a (ω-ACTX-Hv1b to Hv1e), and another, ω-ACTX-Hv1f, that differs by 10 residues and shows reduced insecticidal activity (Wang et al., 1999). Structurally, Hv1a encompasses a disordered N-terminal (residues 1-3), a globular core rich in disulfide bonds (residues 4-21) and a protruding finger-like ß-hairpin (residues 22-37), with a cysteine knot motif formed by three disulfide bonds (Fletcher et al., 1997). Protein engineering and site-directed mutagenesis studies by Tedford et al. (2001) evidenced the importance of residues Asn^{27} and Arg^{35} in Hv1a activity, proposing that their presence in the ß-hairpin is essential for interaction between toxin and calcium channels. A complete Hv1a alanine mutants panel was subsequently constructed, demonstrating that Pro^{10} also plays an important role in insecticidal activity against *M. domestica* (houseflies) and *Acheta domestica* (crickets) (Figure 1.3; Tedford et al., 2004b).

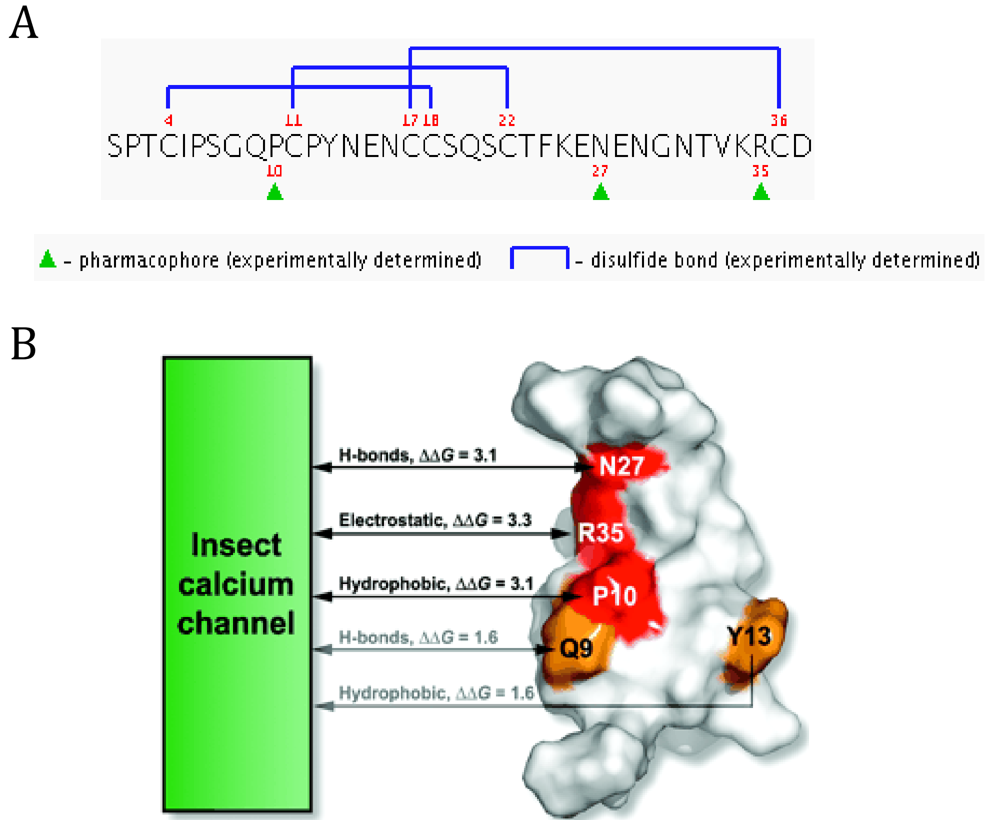

Figure 1.3: Sequence and structure of ω-ACTX-Hv1a. A) Amino acid sequence of ω-ACTX-Hv1a, with disulphide bridges and sites of interaction with calcium channels shown. Adapted from arachnoserver.org/toxincard.html?id=193, accessed on 02/04/2014. B) Three-dimensional structure of Hv1a, and major sites of interaction with CaV depicted in red; minor sites of interaction depicted in orange. Source: Tedford et al., 2004b.

Khan et al. (2006) have claimed that Hv1a was toxic to *Helicoverpa armigera* and *Spodoptera littoralis* when expressed in *E. coli* and applied topically on larvae. However, the peptide is unlikely to cross the insect exoskeleton and reach its targets on the central nervous system (King and Hardy, 2013). It is more plausible that the toxic effect observed occurred because imidazole was not removed following protein purification, leading to insect mortality (Pence, 1965). Except for the tick *Amblyomma americanum* (Mukherjee et al., 2006), Hv1a does not present oral activity against arthropods, namely aphids and lepidopterans (Fitches et al., 2012; Pal et al., 2013).

1.5 **Galanthus nivalis *Agglutinin – GNA***

Lectins comprise a group of proteins that can reversibly bind to specific carbohydrates. In plants, it is believed that they serve as nitrogen-storage

proteins and, given their deleterious effects in several herbivorous invertebrates, are also involved in plant defense against biotic stresses (Peumans and van Damme, 1995).

The snowdrop lectin or *Galanthus nivalis* agglutinin (GNA) is a 50 kDa tetrameric protein composed of 12.5 kDa subunits that shows specificity to α-D-mannose, being able to agglutinate rabbit erythrocytes (Figure 1.4; van Damme et al., 1987). Although initially isolated from *G. nivalis* bulbs, GNA can be found in other plant parts, including leaves and ovaries. Transcripts typically code for a 157 amino acids polypeptide, with 23 residues composing an N-terminal signal sequence and 29 residues composing a C-terminal extension, yielding a 105-residues mature lectin (van Damme et al., 1991a). However, different isoforms have been isolated and cloned (van Damme et al., 1991b).

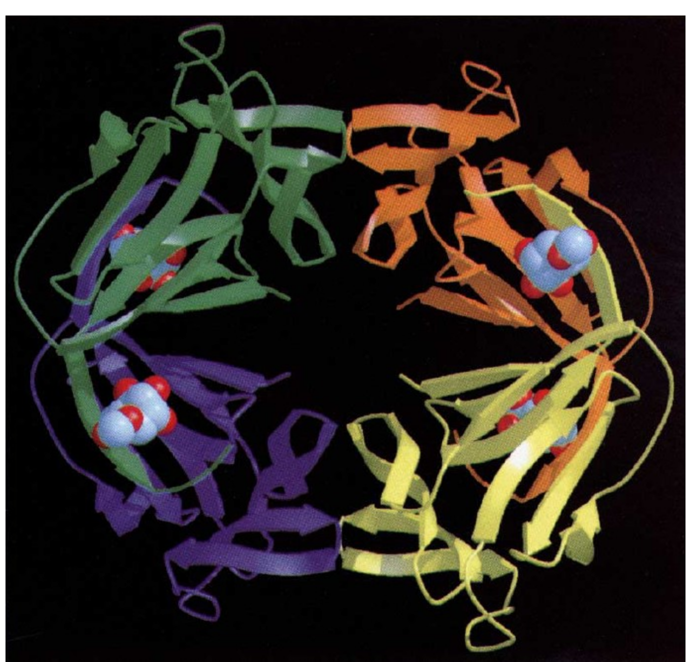

Figure 1.4: Structure of GNA tetramer; each subunit is represented by a different colour (green, orange, yellow and purple). Source: Hester et al., 1995.

GNA presents moderate insecticidal activity against lepidopteran (Fitches et al., 1997) and hemipteran pests (Powell et al., 1996; Sauvion et al., 1996), negatively affecting biological parameters such as weight gain and fecundity, rather than survival. Nevertheless, the gene coding for GNA has been inserted into potato (Gatehouse et al., 1996), wheat (Stoger et al., 1998), rice (Rao et

al., 1998) and papaya (McCafferty et al., 2008), generally rendering plants with increased resistance to different pests (Table 1.2).

Table 1.2: Effects of GNA on arthropod pests via transgenic plants.

Organism	Plant	Effects	Reference
Aulacorthum solani	Potato	Reduced fecundity reduced population growth	Down et al., 1996
Myzus persicae	Potato	Reduced fecundity reduced population growth	Gatehouse et al., 1996
Lacanobia oleracea	Potato	Reduced leaf damage insect biomass reduction slight survival reduction (20%)	Gatehouse et al., 1997
Nilaparvata lugens	Rice	decreased survival Reduced fecundity retarded insect development anti-feedant effect	Rao et al., 1998
Sitobion avenae	Wheat	Reduced fecundity	Stoger et al., 1999
Nephotettix virescens	Rice	anti-feedant effect decreased survival	Foissac et al., 2000
Nilaparvata lugens	Rice	anti-feedant effect decreased survival	Foissac et al., 2000
Nephotettix virescens	Rice	anti-feedant effect decreased survival	Nagadhara et al., 2003
Nilaparvata lugens	Rice	anti-feedant effect decreased survival	Nagadhara et al., 2003
Lacanobia oleracea		Increased consumption increased weight Increased survival	Wakefield et al., 2006
Tetranychus cinnabarinus	Papaya	Reduced fecundity anti-feedant effect	McCafferty et al., 2008
Sitobion avenae	Wheat	anti-feedant effect Reduced fecundity	Miao et al., 2011
Schizaphis graminum	Wheat	anti-feedant effect Reduced fecundity	Miao et al., 2011
Rhopalosiphum padi	Wheat	no effects	Miao et al., 2011

Although the mechanism of action is still unknown, GNA is able to cross the gut barrier following ingestion by insects. In the brown planthopper *Nilaparvata lugens* (Hemitera), GNA binds to the luminal surface of midgut epithelial cells and effectively crosses the midgut, being subsequently observed in the insect haemolymph, fat bodies and ovarioles (Powell et al., 1998). GNA transport to the haemolymph has also been reported in other insect orders, including Coleoptera, Neuroptera and Lepidoptera (Hogervorst et al., 2006; Fitches et al., 2004a).

1.6 GNA as delivery agent for insecticidal peptides

The ability of GNA to cross the midgut barrier following ingestion opened the possibility of delivering insecticidal molecules that would otherwise not be toxic via an oral route by fusing them to GNA. For example, the spider toxin SFI1 from *Segestria florentina* induces paralysis after injection into *Heliothis virescens* (Lepidoptera) larvae (Lipkin et al., 2002). As the toxin is unlikely to be absorbed by the insect cuticle and does not display toxic activity via ingestion, Fitches et al. (2004a) fused SFI1 to GNA. The resulting fusion protein induced 100% mortality in *Lacanobia oleracea* after six days when incorporated into artificial diet at 2.5% dietary protein, also inducing mortality in the hemipteran pests *M. persicae* and *N. lugens* (Down et al., 2006). This strategy gave rise to a novel approach for the development of biopesticides. So far, five different insecticidal peptides, including SFI1, have been fused to GNA. Initially, a fusion between GNA and allatostatin from *Manduca sexta* suppressed growth and feeding of *L. oleracea* when incorporated into artificial diets (Fitches et al., 2002). Trung et al. (2006), fusing the toxin ButaIT from the scorpion *Mesobuthus tamulus* to GNA, generated an orally active molecule that was toxic not only against lepidopteran *L. oleracea*, but also to the dipteran *M. domestica* and coleopteran *Tribolium castaneum* (Fitches et al., 2010). Fitches et al. (2012) have fused the insect calcium channel blocker ω-ACTX-Hv1a (Hv1a) to GNA, showing that GNA delivered Hv1a to its target in the central nervous system (CNS) of *Mamestra brassicae* via ingestion (Figure 1.5). Leaf disks coated with 0.1-0.2% of this fusion protein, denominated Hv1a/GNA, caused more than 80% mortality in *M. brassicae* larvae, whereas those coated with GNA alone presented no effects on survival. Further improvements on the

Hv1a/GNA fusion protein were made by Pyati et al. (2014), who enhanced protein stability by amino acid substitution (K34 to Q34, thus removing a potential Kex2 cleavage site). Additionally, protein production was increased by inserting multiple gene copies in *Pichia pastoris* and a C-terminal his-tag to the protein, allowing single-step purification by using affinity chromatography techniques.

Similar to Fitches et al. (2012), Bonning et al. (2014) fused Hv1a to another carrier, a coat protein from luteovirus, which is an aphid-vectored plant virus. The fusion protein was active against four aphid species, *Acyrthosiphon pisum*, *Rhopalosiphum padi, Aphis glycines* and *M. persicae*. Transgenic plants expressing this fusion protein were also effective against *M. persicae.* These studies validate the feasibility of using spider venom toxins for developing orally active biopesticides. More recently, Yang et al. (2014) combined GNA with the sodium channel blocker δ-amaurobitoxin-Pl1a, yielding an effective pesticide against *M. domestica*, *M. brassicae* and the aphid *A. pisum*.

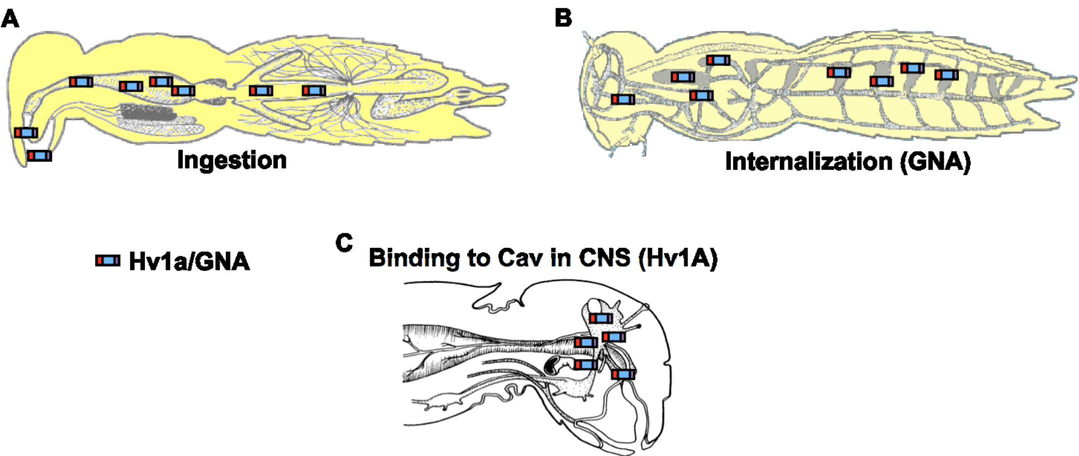

Figure 1.5: Mode of action of the fusion protein Hv1a/GNA. A) Hv1a/GNA is ingested by insect, B) internalized in the midgut by the GNA portion, being transported through the haemolymph to C) Hv1a receptors voltage-gated calcium channels (CaV) in the central nervous system (CNS) of the insect.

1.7 Effects of GNA and fusion proteins on non-target organisms

Pest control by natural enemies (predators and parasitoids) is an important ecological service, capable of delaying both, insect pest outbreaks (Schmidt et

al., 2003; Snyder and Ives, 2003) and development of resistance to other pest control agents such as transgenic Bt-expressing plants (Liu et al., 2014). Whilst research on the safety of lectin-based fusion proteins to beneficial species is scarce (Wakefield et al., 2010a), effects of GNA on non-target arthropods have been extensively studied (Table 1.3), often with contrasting results. For example, GNA has a direct, toxic effect on growth of the parasitoid wasp *Eulophus pennicornis* when hosts feed on/are injected with GNA (Wakefield et al., 2010a), or when parasitoid adults directly feed on diets containing the lectin (Bell et al., 2004). However, if expressed in transgenic potato, GNA has no detrimental effects on *E. pennicornis* (Bell et al., 1999), and can actually augment biological control of *L. oleracea* by the parasitoid (Bell et al., 2001), demonstrating the compatibility between the use of insect-resistant transgenic plants and biological control. These results indicate that, although GNA can have detrimental effects on beneficial insects under worst-case scenarios, it is unlikely to disrupt ecological services, such as parasitism/predation, if levels taken up by biological control agents are low. This is generally the case under more realistically designed experiments, which mimic conditions encountered in the field (e.g. Bell et al., 2001). Nevertheless, it is important to expose non-target organisms to high doses of test proteins in laboratory tests, so as to increase the chances of detecting potential deleterious effects. If any effects are encountered, then the margins of safety of these novel proteins to non-target organisms can be determined.

Table 1.3: effects of GNA on non-target beneficial insects.

Organism	Functional group	System	Effects	Reference
Eulophus pennicornis	parasitoid	host fed on transgenics	No effect	Bell et al., 1999
Eulophus pennicornis	parasitoid	host reared on transgenic potato	Beneficial	Bell et al., 2001
Podisus maculiventris	predator	injected prey; prey reared on transgenics	Negative indirect	Bell et al., 2003
Eulophus pennicornis	parasitoid	artificial diet and via host	Negative direct	Bell et al., 2004
Adalia bipunctata	predator	transgenic potato (tritrohic) and bitrophic	No effect	Birch et al., 1999
Aphelinus abdominalis	parasitoid	artificial diet & transgenic plants	Negative indirect	Couty et al., 2001a
Aphelinus abdominalis	parasitoid	GNA-dosed or GNA-fed aphids	Negative direct/indirect	Couty et al., 2001b
Aphelinus abdominalis	parasitoid	intoxicated host	No effect	Couty & Poppy, 2001
Aphidius ervi	parasitoid	intoxicated host; tritrophic	Negative direct	Couty et al., 2001c
Adalia bipunctata	predator	intoxicated prey	Negative indirect	Down et al., 2000
Adalia bipunctata	predator	transgenic potato (tritrophic)	No effect	Down et al., 2003
Chrysoperla carnea	predator	sucrose+GNA	Negative direct	Hogervorst et al., 2006
Adalia bipunctata	predator	sucrose+GNA	Negative direct	Hogervorst et al., 2006
Coccinella septempunctata	predator	sucrose+GNA	Negative direct	Hogervorst et al., 2006
Aphidius colemani	parasitoid	sucrose+GNA	Negative direct	Romeis et al., 2003
Trichogramma brassicae	parasitoid	sucrose+GNA	Negative direct	Romeis et al., 2003
Cotesia glomerata	parasitoid	sucrose+GNA	Negative direct	Romeis et al., 2003
Cotesia flavipes	parasitoid	transgenic sugarcane (tritrophic)	Negative indirect	Setamou et al., 2002
Parallorhogas pyralophagus	parasitoid	transgenic sugarcane (tritrophic)	Negative indirect	Tomov et al., 2003
Meteorus gyrator	parasitoid	host that ingested or injected with GNA	No effect	Wakefield et al., 2006
Eulophus pennicornis	parasitoid	host fed on transgenics and diet, artificial diet	Negative direct	Wakefield et al., 2010a

1.8 Aims and Objectives

The present project seeks to investigate the suitability of a fusion protein comprised of *Galanthus nivalis* agglutinin and ω-ACTX-Hv1a (with amino acid substitution K34Q) for use as either biopesticide or for expression in transgenic crops, for safe and sustainable control of insect pests (Figure 1.6). This was evaluated through the following specific objectives:

(1) To produce the fusion protein Hv1a/GNA by fermentation using recombinant *P. pastoris* for evaluation of its biopesticide potential.
(2) To determine the fusion protein efficacy against both target insect pests (Hemiptera) and non-target beneficial insects (natural enemies and pollinators).
(3) To express the fusion protein in a model plant (*Arabidopsis thaliana*) as 'proof of concept' of its efficacy against target pests.
(4) RNAi techniques were employed in order to silence voltage-gated calcium channel genes in *M. persicae* and *T. castaneum*, as these are putative molecular targets for the Hv1a peptide.

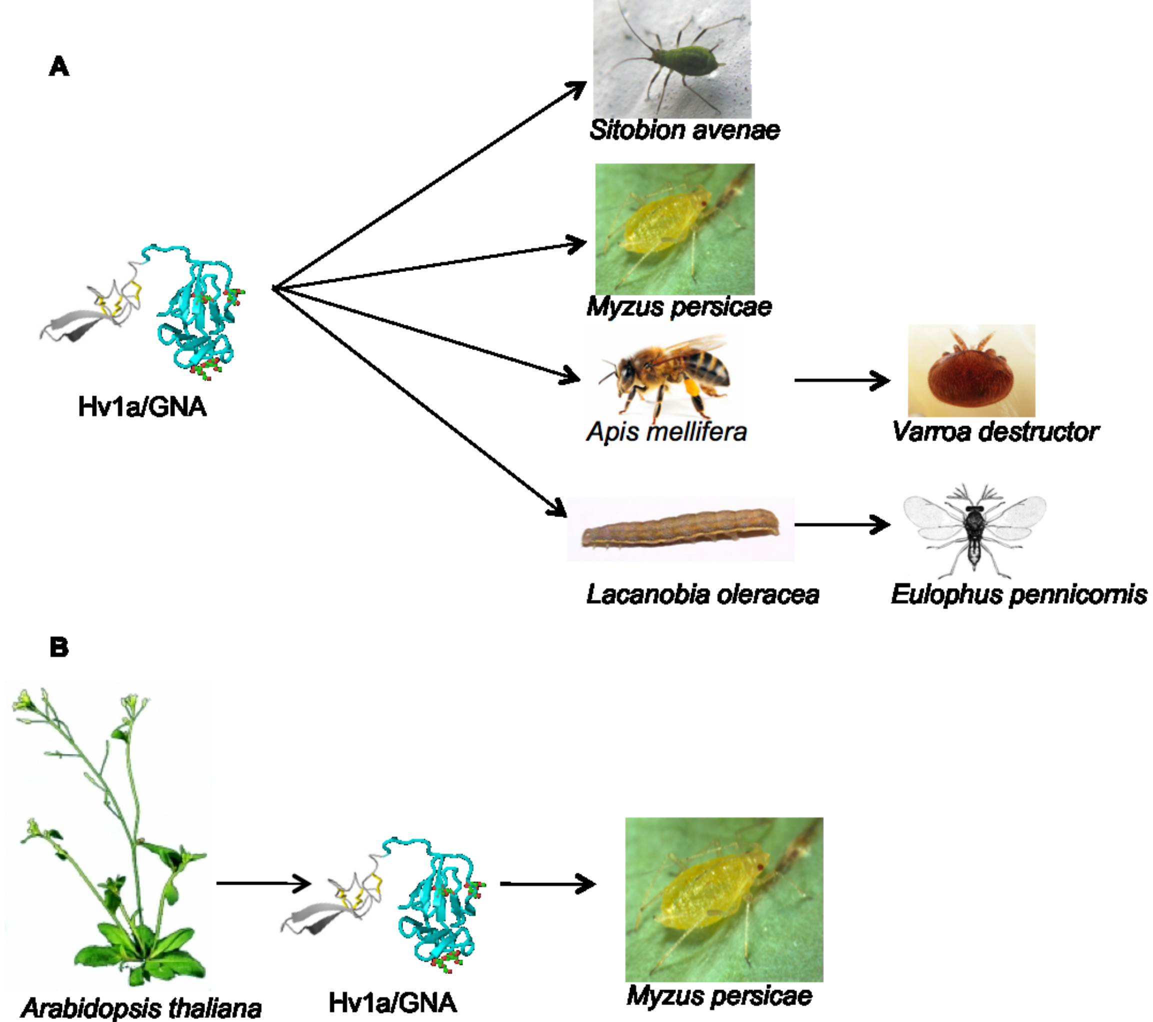

Figure 1.6: Experimental framework of this thesis. A) The fusion protein Hv1a/GNA produced in *P. pastoris* was tested for its toxicity against the aphids *S. avenae* and *M. persicae*. Effects on honeybees and their parasite *Varroa destructor* and on *Lacanobia oleracea* and its parasitoid *E. pennicornis* were also evaluated. B) Transgenic Arabidopsis plants expressing Hv1a/GNA were generated, and their efficacy against *M. persicae* was evaluated.

1.9 Synopsis of chapters

Chapter 2 examines toxicity of Hv1a/GNA against aphids (Hemiptera: Aphididae) and the viability of using transgenic plants expressing fusion proteins for insect control. A version of this chapter has been submitted to the journal Frontiers in Plant Science.

Chapter 3 assesses effects of the fusion protein against a biological control agent, the parasitoid wasp *E. pennicornis* (Hymenoptera: Eulophidae) via its host, *L. oleracea* (Lepidoptera: Noctuidae).

Chapter 4 assesses the non-target effects of Hv1a/GNA on a pollinator, the honeybee *A. mellifera mellifera* (Hymenoptera: Apidae). This chapter has been published in the Proceedings of the Royal Society B, vol 281, 20140619 (2014).

Chapter 5 investigates the effectiveness of Hv1a/GNA against the honeybee parasite *Varroa destructor* (Acari: Varroidae).

Chapter 6 utilises RNA interference techniques against putative receptors for ω-ACTX-Hv1a, namely voltage-gated calcium channels, in the aphid *M. persicae* and the coleopteran *T. castaneum*, to verify the molecular targets for the biopesticide.

Chapter 7 is a general discussion about the results obtained from the results derived from this work.

Chapter 2 Transgenic plants expressing ω-ACTX-Hv1a and snowdrop lectin (GNA) fusion protein show enhanced resistance to aphids

A version of this chapter has been submitted for publication: Nakasu EYT, Edwards MG, Fitches EC, Gatehouse JA, Gatehouse AMR. (2014) Transgenic plants expressing ω-ACTX-Hv1a and snowdrop lectin (GNA) fusion protein show enhanced resistance to aphids. *Frontiers in Plant Science*. I have contributed with >80% of the experimental design and have conducted all experimental work and drafting of the manuscript. I would like to thank the other authors for allowing me to use this version of the paper as a chapter for my thesis.

2.1 Abstract

The present work demonstrates the efficacy of a recombinant fusion protein (Hv1a/GNA) comprising of the spider venom toxin, ω-ACTX-Hv1a (Hv1a), and the snowdrop lectin (GNA) to reduce survival of the peach-potato aphid *Myzus persicae* via artificial diet bioassays. Although the fusion protein was shown to be rapidly degraded by the activity of proteases, Hv1a/GNA oral toxicity to *M. persicae* was significantly greater than when compared to that of GNA alone ($P<0.05$). Compared to nontransformed plants, transgenic Arabidopsis expressing Hv1a/GNA under CaMV35S (25.6±4.1 ng/mg FW) promoter induced up to ~40% mortality after seven days in detached leaf bioassays, and ~35% after 14 days in whole plant bioassays. The use of transgenic plants to deliver fusion proteins to aphids has been therefore proven to be effective. Additional studies with grain aphids, *Sitobion avenae*, showed that they were more susceptible to 0.1% fusion protein in artificial diet bioassays (90% mortality vs 75% in *M. persicae* after four days), as they were not able to hydrolyze the fusion protein as readily as *M. persicae.* It is therefore anticipated that expression of this fusion protein in suitable host plants for the grain aphid will confer higher levels of resistance than that shown with the Arabidopsis to *M. persicae* model.

Keywords: *Myzus persicae*, Arabidopsis, Hv1a/GNA, fusion proteins, *Sitobion avenae*

2.2 Introduction

Aphids significantly impact agricultural and horticultural crops, either by causing direct damage to plants through feeding on the phloem, or indirectly by acting as vectors for plant pathogenic viruses. Aphid control relies heavily on the use of synthetic insecticides. Intensive pesticide use has positively selected aphid genotypes that are resistant to carbamates and organophosphates, which inhibit the enzyme acetylcholinesterase, and pyrethroids, which target sodium channels (Devonshire et al., 1998). More recently, aphid resistance to neonicotinoids, nicotinic acetlycholine receptor (nAChR) agonists, has also been reported (e.g. Puinean et al., 2010). Therefore, alternatives for chemical control and the development of insecticides with different modes of action are needed.

Spider venom neurotoxins offer a high degree of biological activity, providing an attractive source for novel pest management strategies (King, 2007). However, there are major drawbacks to the use of these peptides, particularly as topical sprays, as they are unlikely to be rapidly absorbed through the insect cuticle to reach their site of action and are prone to degradation in the environment (Fitches et al., 2004a). Should they survive the application process and be taken up by the insect, they are then unlikely to survive the conditions of the insect gut (Fitches et al., 2004a) or be delivered across the midgut epithelium to the correct targets within the insect (Tedford et al., 2004a). The discovery that snowdrop lectin *Galanthus nivalis* agglutinin (GNA) remains stable and active within the insect gut after ingestion, and that it is able to cross the midgut epithelium (Powell et al., 1998), provided an opportunity for its use as a ‘carrier molecule’ to deliver other peptides to the circulatory system of target insect species (Fitches et al., 2002).

The venom peptide ω-ACTX-Hv1a (Hv1a) from the Australian funnel web spider *Hadronyche versuta* (Rainbow) acts as a calcium channel blocker in the insect central nervous system (CNS) (Bloomquist, 2003). It has proven to be lethal to a broad range of insects (Atkinson et al., 1998), but causes no inhibition to mammalian voltage-gated calcium channel currents (Fletcher et al., 1997). However, since the peptide on its own does not show oral toxicity to insects (Tedford et al., 2004a), Fitches et al. (2012) fused it to the carrier molecule

GNA. The authors were able to demonstrate effective delivery of the peptide to *Mamestra brassicae* haemolymph when ingested and that it reached the Hv1a site of action in the central nerve cord. Furthermore, the neurotoxin portion of the Hv1a/GNA fusion protein was modified in order to improve stability during yeast expression (Pyati et al., 2014).

The peach-potato aphid, *Myzus persicae*, is a cosmopolitan, generalist species that feeds on more than thirty different plant families, including commercially important crops, being capable of transmitting more than 100 viral diseases (van Emden et al., 1969). Additionally, this species is able to infest *Arabidopsis thaliana* plants, thus providing a valuable model for the proof of concept of using transgenic plants expressing fusion proteins for insect control. The present study demonstrates that the fusion protein Hv1a/GNA is toxic towards the peach-potato aphid. Furthermore, transgenic Arabidopsis plants expressing the fusion protein were effective at controlling *M. persicae,* thus demonstrating the potential of Hv1a/GNA for aphid control.

2.3 Material and Methods

2.3.1 Protein Expression and purification

Pichia pastoris (SMD1168H strain) was transformed with genes encoding GNA or Hv1a/GNA and fermentation carried out in a Bio Console ADI 1025 (Applikon) fermenter (2 l vessels), with a continuous 50% glycerol feed. After expression, cultures were centrifuged at 7500 *g* for 30 min and the supernatant collected. Recombinant GNA was purified by hydrophobic interaction chromatography on a phenyl-sepharose resin packed into a Pharmacia XK16 column. Fractions containing GNA were reloaded onto a size-exclusion column (HiPrep™ 16/60 Sephacryl S-100, GE-Healthcare). Following purification, recombinant proteins were dialyzed, freeze-dried and stored at -20 °C. For His-tagged Hv1a/GNA purification, supernatants were diluted in binding buffer (0.02 M sodium phosphate, 0.4 M NaCl, pH 7.4). Samples were loaded onto a HisTrap™ (GE Healthcare) column and then eluted with binding buffer containing 0.2 M imidazole. After purification, samples were extensively dialyzed in water and freeze-dried. The concentration of Hv1a/GNA was estimated by comparing band intensities with known amounts of GNA on SDS-

PAGE, as described (Down et al., 2006).

2.3.2 Artificial diet bioassays

M. persicae were kept on Chinese cabbage plants (*Brassica rapa*) at 25 °C, 16:8 (L:D), whereas *S. avenae* were reared on wheat (*Triticum aestivum*), at 20°C, 16:8 (L:D). Prior to bioassays, adult aphids were transferred from plants to 90 mm diameter Petri dishes containing artificial diet (Febvay et al., 1988) in Parafilm sachets as described by Down et al. (1996), and allowed to reproduce for 24 h. Neonate aphids were collected and exposed to one of the four treatments in artificial diet: i) artificial diet alone (negative control), ii) 0.1% GNA, iii) 0.05% Hv1a/GNA, or iv) 0.1% Hv1a/GNA. Mortality was recorded daily for eight days and diets were changed every 48 h. Thirty aphids per treatment were used for *S. avenae* bioassays, and 70 aphids/treatment were used for *M. persicae* bioassays. Survival analysis was carried out using Sigmaplot 11 (2008).

Fecundity of *M. persicae* was evaluated by continuously feeding aphids with 0.025% Hv1a/GNA or GNA for eight days. Three cages containing 10 aphids were used for each treatment, and the cumulative number of nymphs produced/day/adult was recorded. For evaluating the effects of GNA or Hv1a/GNA on *M. persicae* development, three replicates of ten 2-days old aphids were given artificial diet, 0.1% GNA or 0.1% Hv1a/GNA. Aphid lengths (from head to cauda) were measured on the first three days by using a graticule. Data recorded for fecundity and sizes on each treatment were compared by one-way ANOVA and corrected mortalities were calculated using Abbott's formula (1925). The median lethal concentrations (LC_{50}) were calculated by plotting log dose vs probit of corrected mortalities (Randhawa, 2009; Miller and Tainter, 1944).

2.3.3 Uptake of Hv1a/GNA by aphids

Neonate *M. persicae* and *S. avenae* were fed for 24 h on artificial diet containing Hv1a/GNA at 0.05% or 0.1% (w:v). Insects (10-15) were either collected, flash frozen in liquid nitrogen and macerated in SDS sample buffer for protein extraction, or transferred to Petri dishes containing artificial diet without added proteins for a pulse-chase experiment. After 24 h, those aphids were

collected and their proteins extracted as described above. Samples were heat-denatured and separated in 15% SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and the uptake of fusion proteins evaluated by western blot using anti-GNA antibodies (1:5000 dilution) and ECL as substrate, as previously described (Fitches et al., 2012).

2.3.4 Plant transformation

A sequence coding for Hv1a/GNA was synthesized with *A. thaliana* codon usage for optimal plant expression (ShineGene Molecular Biotech, Inc.). Primers containing attB1 and attB2 sites (Table 2.1) were used to amplify the gene via PCR (30 cycles of 98 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, with a final extension step of 7 min), which was then transferred to pDONR vectors using BP clonase reaction (Gateway®, invitrogen™). Constructs (Figure 2.1) were electroporated into *Escherichia coli* Top10 and plasmids extracted from positive colonies. In a subsequent step, the gene coding for the fusion protein was transferred from the pDONR to pK2GW7 vector (Karimi et al., 2002) via LR clonase using Gateway® technology (invitrogen™).

Table 2.1: Primers used to add *att*B sites (in **bold**) to Hv1a/GNA coding sequence.

Primer	Sequence
Sense	5’**GGGGACAAGTTTGTACAAAAAAGCAGGCT**ATGGCTAAGGCAAGTCTCCT3’
Antisense	5’**GGGGACCACTTTGTACAAGAAAGCTGGGT**TTACTTTGCCGTCACAAGC3’

Figure 2.1: Structure of plant constructs in pK2GW7 vector.

Expression constructs were finally electroporated into *Agrobacterium tumefaciens* C58C1, and antibiotic resistance was used to screen transformed colonies. *A. thaliana* (var. Columbia) were transformed with *A. tumefaciens* following the floral dip method described by Clough and Bent (1998). Seeds were harvested, surface-sterilized and spread on plates with Murashige-Skoog medium containing 50 µg/mL kanamycin. Plates were kept at 4 °C for 48 h in order to break seed dormancy and then transferred to environmentally controlled growth rooms (16:8 h L:D, 22 °C day and 17 °C night). Putative transformed plantlets were transferred to plastic pots containing soil (John Innes No. 2). Transformation was confirmed via PCR using the same conditions described above and by western blots. Protein expression was estimated by extracting a known amount of leaf tissue in 1.5x SDS loading buffer containing 2-mercaptoethanol (1 mg/10 µl). Samples were macerated, boiled for 5 min and centrifuged at 13,000 *g* for 2 min. Supernatants (20 µl) and GNA standards (25, 50 and 100 ng), used to estimate Hv1a/GNA concentrations, were loaded onto 15% SDS-PAGE. After electrophoresis, proteins were transferred to nitrocellulose membranes. Fusion proteins and GNA standards were probed with anti-GNA antibody as described above and quantified by densitometry.

2.3.5 Bioassays with transgenic plants

Transgenic Arabidopsis plants homozygous for the gene expressing Hv1a/GNA were used in two different assays with *M. persicae* only, as *S. avenae* does not feed on crucifers. In the first assay, leaves from two homozygous transgenic lines (1.2a and 1.3b) and non-transgenic Arabidopsis (negative control) were detached from approximately five-week-old plantlets. Their petioles were immersed in 0.5% agar contained in 1.5 ml plastic tubes, which were then individually placed in 450 ml plastic boxes. Six replicates of five aphids were used for each treatment. Leaves were replaced every two days and the number of alive aphids recorded daily for six days.

In the second assay, whole plants expressing Hv1a/GNA (line 1.2a) and untransformed plants were used. Ten neonate aphids were placed in 5- to 6-week old Arabidopsis plants and monitored for 14 days. A total of six and four replicates were used for negative control and Hv1a/GNA-expressing plants, respectively. For both bioassays, aphids were kept at 25 °C, 12:8 h (L:D).

2.4 Results

2.4.1 Demonstration of insecticidal activity of Hv1a/GNA against peach-potato aphid M. persicae

The toxicity of Hv1a/GNA was assayed using neonate (<24 h) *M. persicae* nymphs fed recombinant fusion protein Hv1a/GNA at 0.05% or 0.1% (w:v) in artificial diet. Aphids presented increased mortality on fusion protein treatments from the second and third days after the start of experiments. Survival curves differed from each other (χ^2=138.684, 3 d.f., P<0.001), and pairwise multiple comparisons showed significant differences between all treatments (P<0.05). Hv1a/GNA showed higher levels of toxicity towards *M. persicae* than that of GNA alone, demonstrating its increased toxicity against this species. When fed at a concentration of 0.1%, the fusion protein Hv1a/GNA resulted in more than 90% decrease in survival after 8 days, whereas 0.1% GNA alone resulted in less than 35% reduction (Figure 2.2). Subsequently, a dose/response assay was carried out using five different protein concentrations of either GNA or Hv1a/GNA. When continuously feeding on diets with test proteins, aphids were once more shown to be significantly more susceptible to the fusion protein than to GNA (P<0.05, Figure 2.3), with an estimated LC_{50} of 0.058% (0.58 µg/µl) after four days. It was not possible to reliably calculate the LC_{50} for GNA alone with the concentrations used, as mortalities did not always increased linearly with increased concentrations of the lectin.

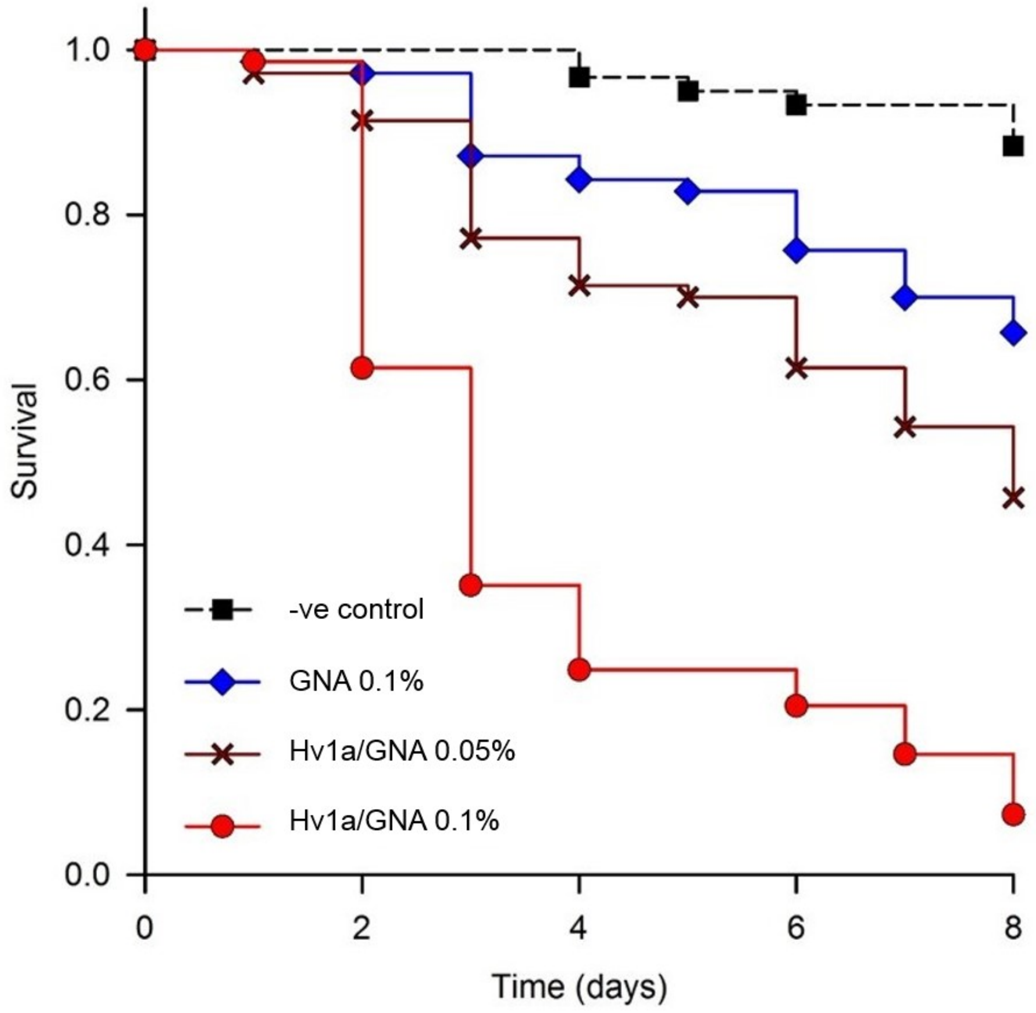

Figure 2.2: Survival of *M. persicae* on artificial diet bioassays. Hv1a/GNA is significantly ($P<0.05$) more toxic than GNA alone in artificial diet bioassays ($n=70$ aphids per treatment), as shown by Kaplan-Meier survival analysis.

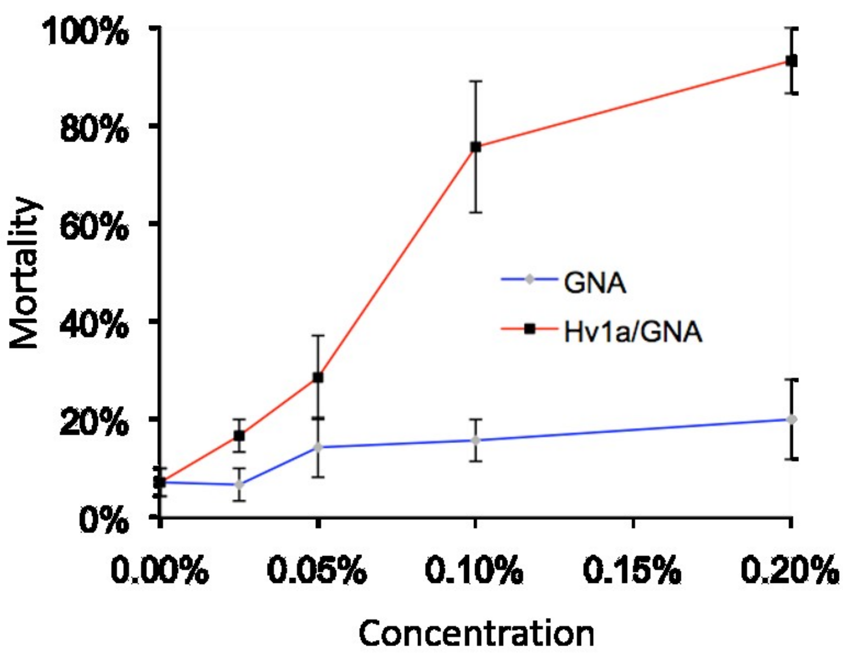

Figure 2.3: Dose-response curves for GNA and Hv1a/GNA of *M. persicae* in artificial diet bioassays after four days (means ± SEM).

2.4.2 Effects of Hv1a/GNA on aphid development and fecundity

M. persicae nymphs were significantly smaller than controls (H=15.291, 2 d.f., P<0.001) following two days continuously feeding on diet containing 0.1% Hv1a/GNA, but not on 0.1% GNA, although they presented similar sizes at the beginning of the experiments (H=0.04, 2 d.f., P=0.98). After three days, insects fed on 0.1% Hv1a/GNA or GNA were approximately 30% and 20% smaller than controls, respectively (H=58.761, 2 d.f., P<0.001) (Figure 2.4A). Additionally, when compared to controls, the cumulative number of nymphs produced per adult was significantly reduced on aphids fed on 0.025% GNA (ca. 69%, t=5.157, P=0.002) or 0.025% Hv1a/GNA (>90% reduction, t=6.915, P<0.001) after nine days from the start of the experiment (Figure 2.4B).

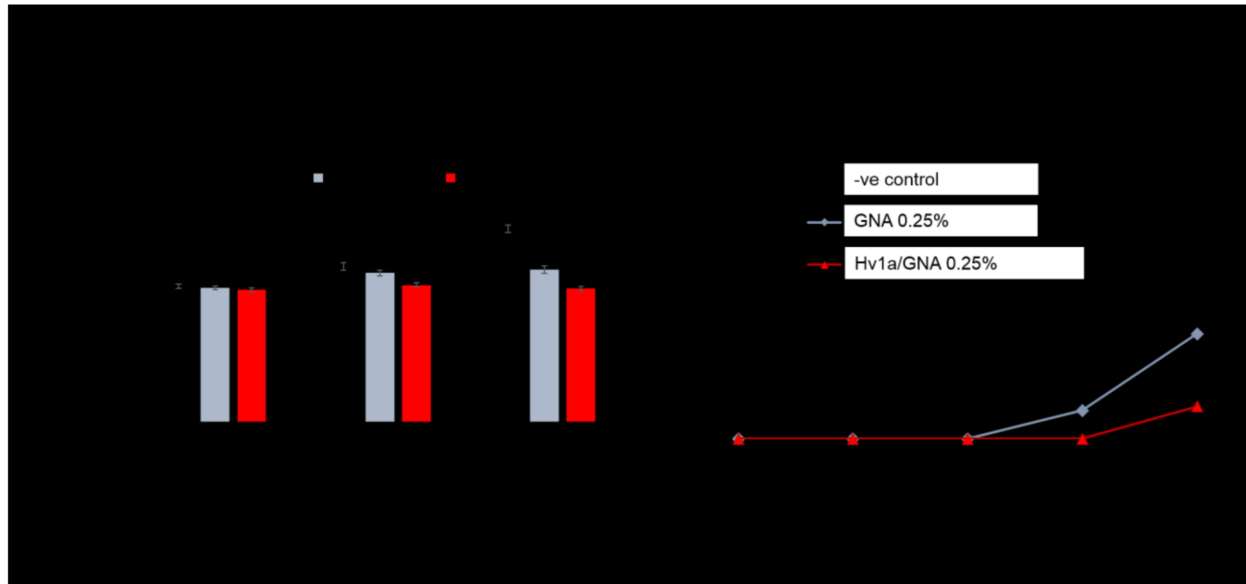

Figure 2.4: Hv1a/GNA and GNA significantly affect *M. persicae* development and fecundity (P<0.05). A) Aphid length after feeding on artificial diets containing 0.1% (w:v) of either GNA or Hv1a/GNA (n=30 aphids/treatment). B) Cumulative number of nymphs/adult is negatively affected by GNA and Hv1a/GNA. For both graphs, different letters represent significant difference between treatments (P<0.05); bars represent means±SEM.

2.4.3 Uptake of Hv1a/GNA by M. persicae

Immunoassays by western blot analysis of aphids fed on artificial diet containing Hv1a/GNA demonstrated that fusion proteins were rapidly digested by *M. persicae.* Anti-GNA antibodies recognized a single band of around 10 kDa (Figure 2.5) in extracts from whole aphids fed with Hv1a/GNA in a pulse-chase experiment, 24 h after exposure. Furthermore, the ~10 kDa band was also detected in the honeydew, suggesting that it is cleaved in the gut, and no evidence of intact Hv1a/GNA was observed. Although GNA and fusion proteins are internalized by homopterans (Down et al., 2006), it was not transmitted to nymphs descended from aphids feeding on Hv1a/GNA.

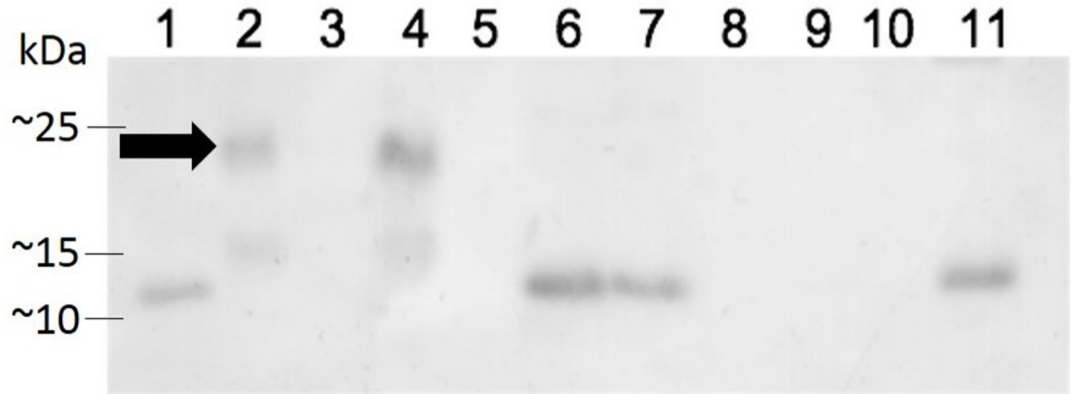

Figure 2.5: *M. persicae* is able to rapidly digest Hv1a/GNA. 1) GNA 100 ng; 2) Hv1a/GNA 100 ng; 3) Aphid diet (negative control); 4) Aphid diet + Hv1a/GNA 0.05%; 5) Adult aphids (negative control); 6) Adult aphids after feeding for 24 h with Hv1a/GNA; 7) Adult aphids chase experiment; 8) Aphid nymphs (negative control); 9) Aphid nymphs from adults fed with Hv1a/GNA; 10) Honeydew (negative control); 11) Honeydew from aphids feeding on Hv1a/GNA 0.05%. Arrow shows migrating pattern of intact Hv1a/GNA.

2.4.4 Expression of Hv1a/GNA in Arabidopsis

Transgenic *A. thaliana* plants harbouring the pK2GW7 vector carrying the sequence for Hv1a/GNA under the control of the CamV35S promoter were generated using the *A. tumefaciens*-mediated floral dip technique. After selection of T_0 seeds on plates containing kanamycin, a transformation efficiency of 2.67±0.46% (average number of kanamycin-resistant seeds ± SEM) was obtained from seven independent events. Integration of the transgene cassette was investigated by PCR (Figure 2.6A) and positive plants were self-pollinated in order to generate homozygous lines for the Hv1a/GNA fusion protein.

Western blot of leaf extracts from plants carrying Hv1a/GNA gene demonstrate that the fusion protein was expressed in T_0 and homozygous F_3 plants (Figure 2.6B and C). The ~25 kDa band corresponding to the intact fusion protein is detected along with another lower molecular weight protein that also reacts with anti-GNA antibody. The lower molecular weight cleavage product was also present when the fusion protein is expressed in *P. pastoris*. This result indicates that the plant cleaves Hv1a/GNA following translation, and further improvements and alterations to the peptide structure would benefit its expression in heterologous systems. Quantification of expression was carried out by comparing intensity of Hv1a/GNA bands from known amounts of leaf

extracts compared to GNA standards in western blots. It was estimated that the fusion protein was being expressed at 25.6 ± 4.1 ng/mg fresh weight (F.W.) leaf tissue.

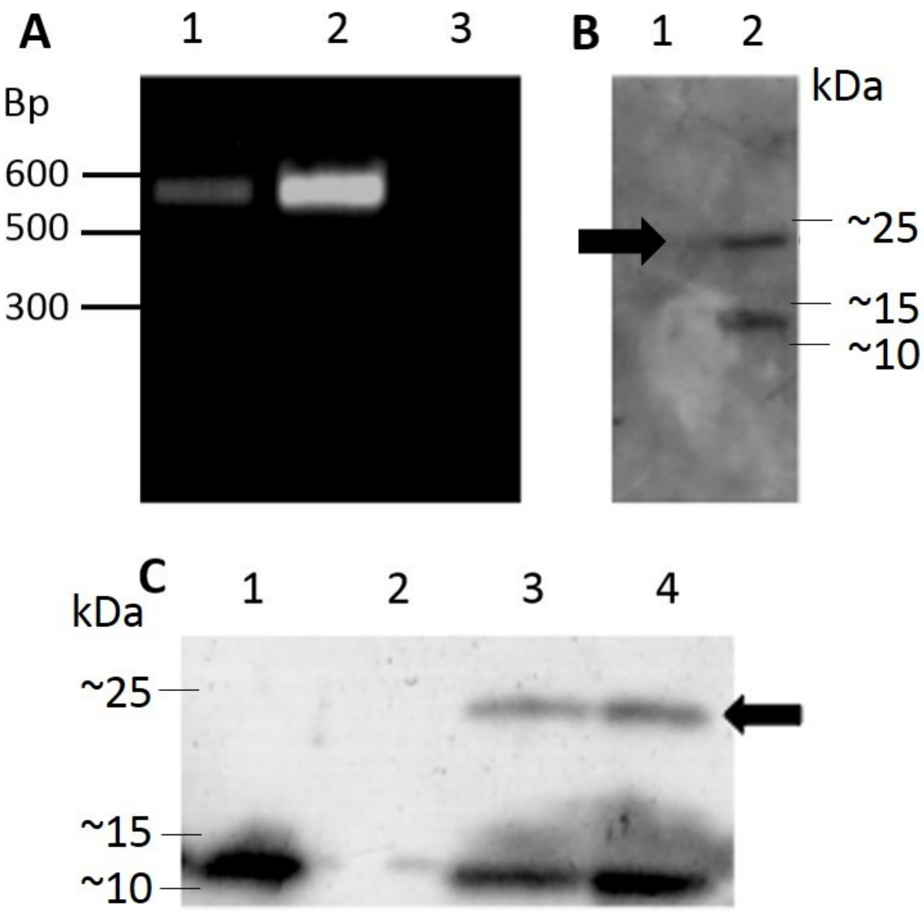

Figure 2.6: Genomic integration of a coding sequence for Hv1a/GNA in Arabidopsis and expression analysis. A) PCR-positive plants; lanes 1 and 2, transformed plants, lane 3, untransformed plant (negative control). B) Western blot showing expression of Hv1a/GNA (as shown by arrow) in F_0 plants; lane 1, negative control (untransformed plant), lane 2, PCR-positive plant C) expression of Hv1a/GNA (arrow) in homozygous plants. Lane 1, positive control (100 ng GNA), lane 2, negative control (untransformed plant), lanes 3 and 4, two different homozygous events.

2.4.5 Performance of **M. persicae** *in* **planta***: detached leaves*

In order to test the efficacy of fusion proteins expressed in plants against aphids, a bioassay with transgenic Arabidopsis was carried out. Two homozygous lines (designated 1.2a and 1.3b) from independently transformed plants were assayed for aphid resistance. Leaves were detached from plants and their petioles immersed in 0.5% agar. When compared to controls, aphids feeding on both events showed similar survival patterns, with significantly

increased levels of aphid mortality (K-M, P=0.014; control vs 1.2a, P=0.01; control vs 1.3b, P=0.003; 1.2a vs 1.3b, P=0.691); aphid survival was reduced to around 60% after seven days (Figure 2.7). The corrected mortality using Abbott's formula for 1.2a was 29.6% and 37% for 1.3b.

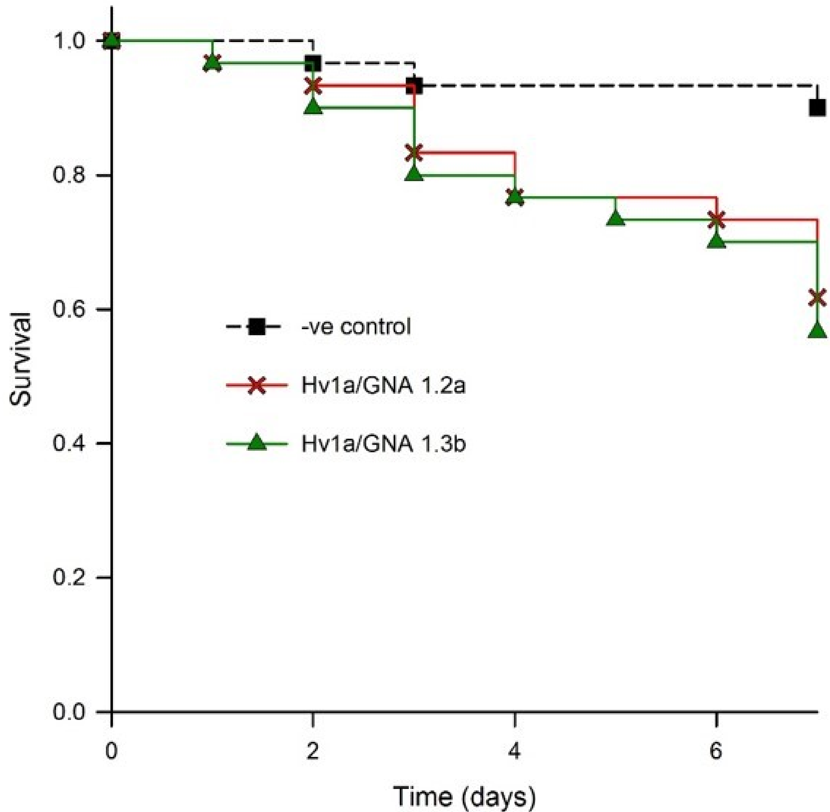

Figure 2.7: Evaluation of biological activity of Hv1a/GNA expressed in Arabidopsis leaves. Two different homozygous lines were assayed against *M. persicae* in a bioassay using detached leaves. Both transformed lines induced a significant mortality (n=30 aphids/treatment; K-M survival, P=0.01) when compared to the non-transformed controls.

2.4.6 Performance of M. persicae *in planta: whole plants*

As both homozygous transformants showed similar levels of toxicity to *M. persicae*, only one of the events (1.2a) was used for whole plant bioassays. After 14 days, aphid survival was reduced to 40% on plants expressing Hv1a/GNA, differing significantly (P<0.05) from control treatment, in which survival was reduced to 63% (Figure 2.8). Compared to control, corrected mortality in Hv1a-GNA-expressing plants was 36.51% using Abbott's formula.

Although subsequent nymph production per adult was numerically higher on plants expressing Hv1a/GNA, the difference when compared to control was not significant (P>0.05, data not shown).

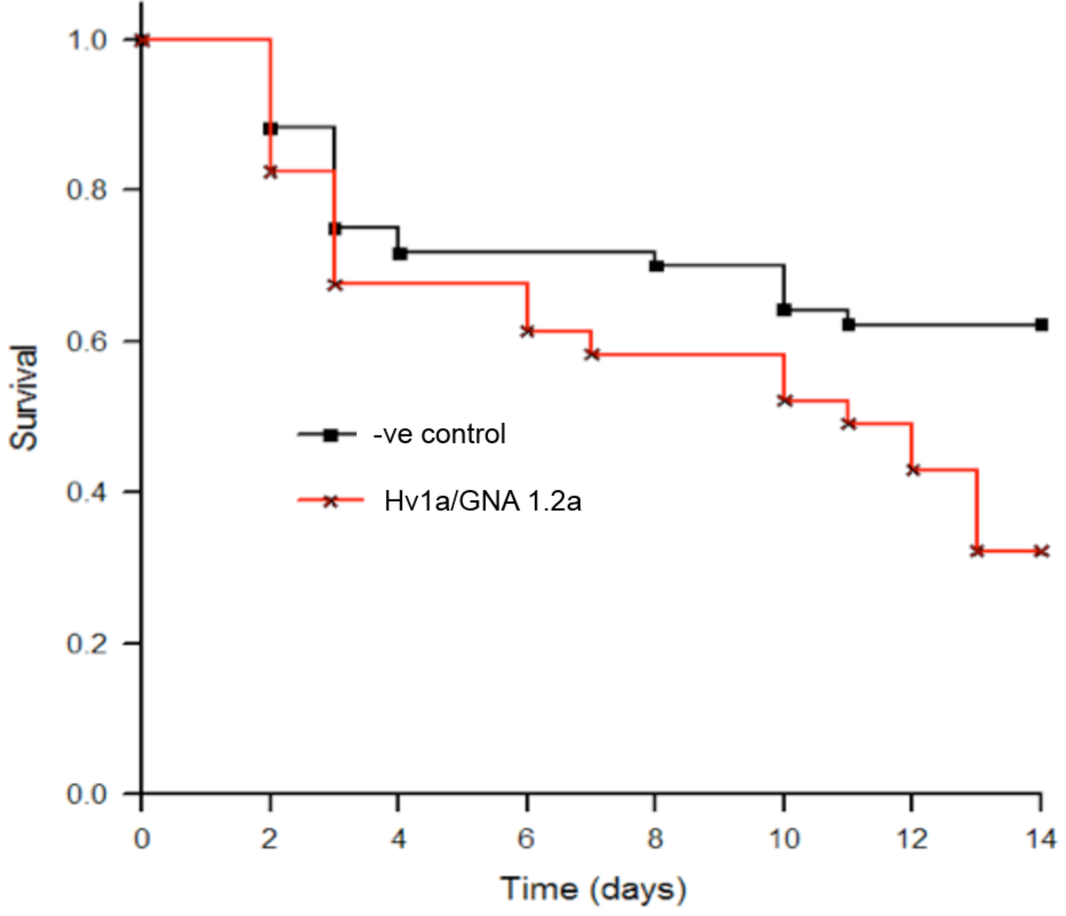

Figure 2.8: Effects of Hv1a/GNA expressed in Arabidopsis. Expression of Hv1a/GNA in Arabidopsis induced increased aphid mortality ($n_{Control}$=60 aphids; $n_{Hv1a/GNA}$=40 aphids; K-M survival, P=0.02).

2.4.7 Effects of Hv1a/GNA fusion protein on Sitobion avenae *survival*

A bioassay with a semi-specialist aphid species, the grain aphid *S. avenae*, was carried out to test the efficacy of Hv1a/GNA against this important pest. Following Kaplan-Meier Survival analysis, significant differences between survival curves were found with bioassays using *S. avenae* (χ^2=116.486, 3 d.f., P<0.001). Pairwise multiple comparisons (Holm-Sidak) revealed non-significant differences between GNA and control treatments (χ^2=1, P=0.317), while 0.05% and 0.01% (w:v) Hv1a/GNA treatments differed from all other treatments (P<0.05) (Figure 2.9). These results demonstrate that *S. avenae* is more susceptible to Hv1a/GNA than *M. persicae*, and while GNA alone did not

significantly affect survival, the fusion protein rapidly induced mortality, with LC_{50} of 0.073% (0.73 µg/µl) after two days. As concentrations higher than 0.1% induced 100% mortality after day three (Figure 2.10), the LC_{50} was calculated two days after the beginning of the assay, whereas the LC_{50} for *M. persicae* was estimated after four days.

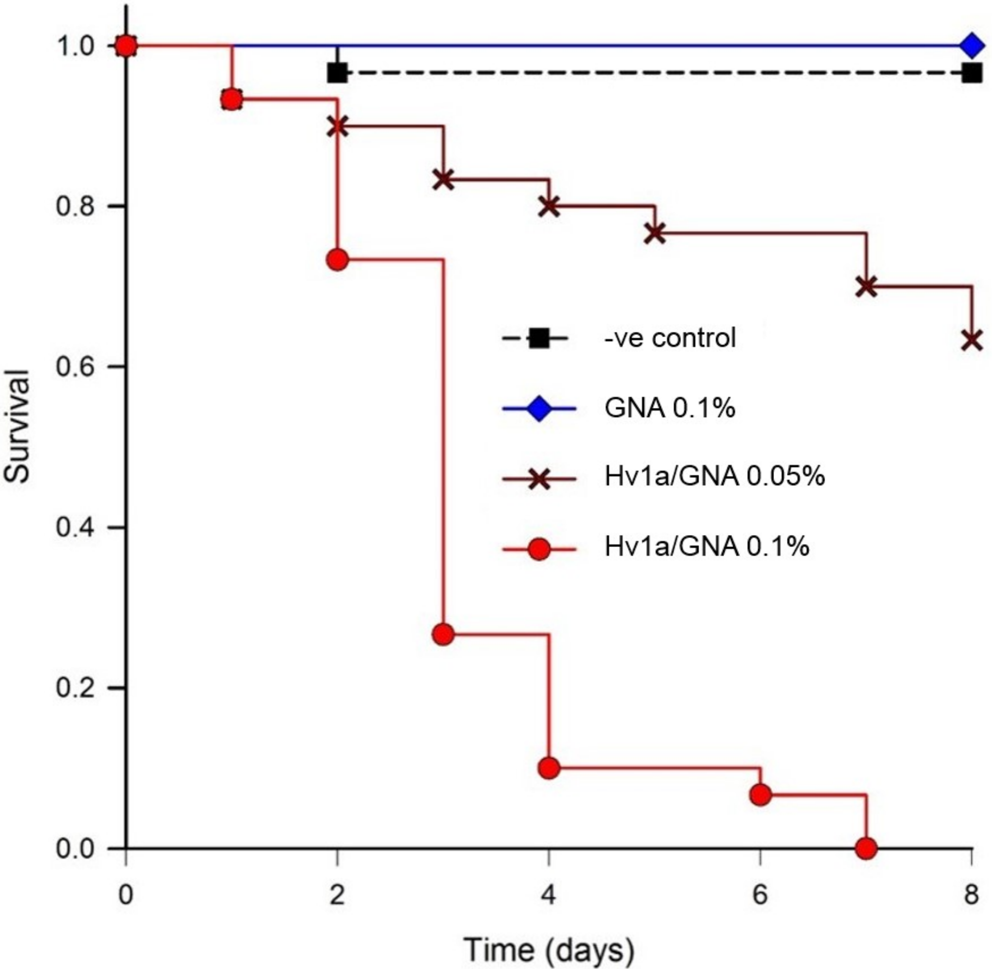

Figure 2.9: Biological activity of Hv1a/GNA against *S. avenae.* The fusion protein rapidly induced mortality in the grain aphid following artificial diet bioassays, as shown by the survival curves (K-M survival, $P<0.05$; n=30 aphids/treatment).

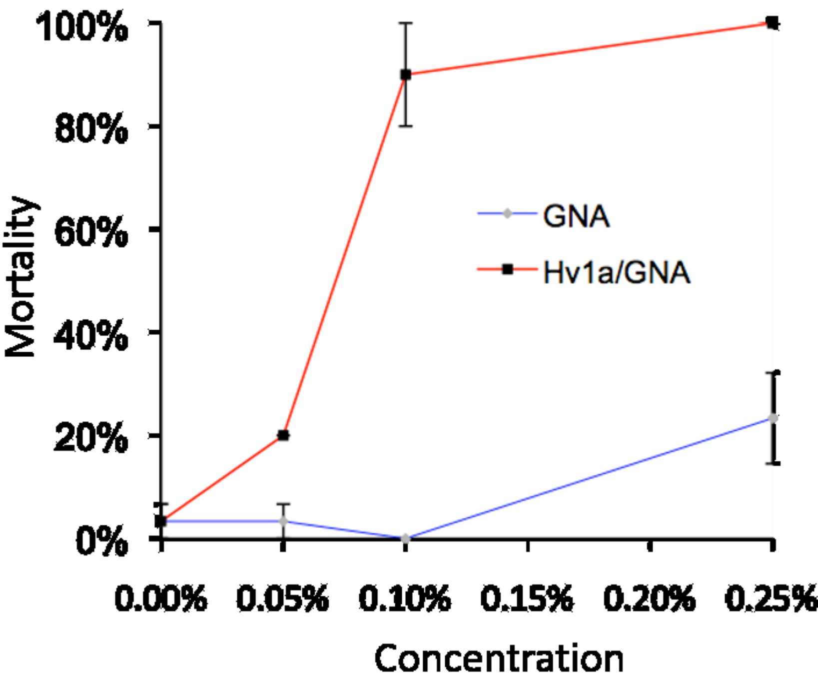

Figure 2.10: Dose-response bioassay for *S. avenae* after four days feeding on artificial diet containing different concentrations of Hv1a/GNA or GNA. Mortalities are shown as means ± SEM.

Western blot analyses show that the grain aphid, as opposed to *M. persicae*, does not readily cleave the fusion protein. In fact, Hv1a/GNA was detected intact in whole aphids feeding on fusion protein and also in their honeydew. Only after 24 h, as shown in the chase experiment, is the fusion protein cleaved (Figure 2.11).

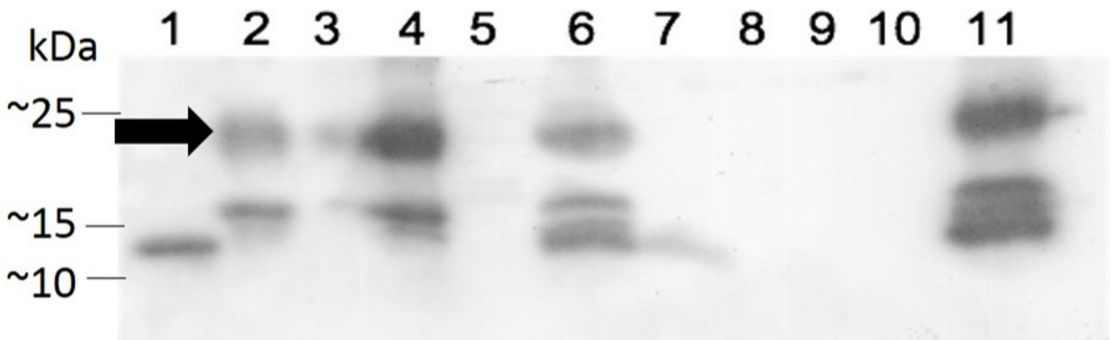

Figure 2.11: The fusion protein is not degraded in the gut of *S. avenae* as readily as in *M. persicae.* Western blot analysis shows that the fusion protein is found intact in the honeydew, being degraded only 24 h after aphids were fed with Hv1a/GNA. 1) GNA 100 ng; 2) Hv1a/GNA 100 ng; 3) Aphid diet (negative control); 4) Aphid diet + Hv1a/GNA 0.05%; 5) Adult aphids (negative control); 6) Adult aphids after feeding for 24 h with Hv1a/GNA; 7) Adult aphids chase experiment; 8) Aphid nymphs (negative control); 9) Aphid nymphs from adults fed with Hv1a/GNA ; 10) Honeydew (negative control); 11) Honeydew from aphids feeding on Hv1a/GNA 0.05%. Arrow indicates position of intact Hv1a/GNA.

2.5 Discussion

Aphids are important crop pests that are difficult to control, as they possess high rates of reproduction and generally feed on plant parts that are inaccessible to insecticide applications. Therefore, transgenic plants expressing genes conferring aphid resistance would be valuable tools for managing their populations. To this end, different strategies, including expression of lectins (Chang et al., 2003; Down et al., 1996), proteinase inhibitors (Rahbé et al., 2003; Carrillo et al., 2011, Zhang et al., 2012) and alarm pheromones (Beale et al., 2006) have been investigated. These approaches commonly result in plants presenting modest effects on aphid survival, having greater outcomes on fitness parameters, such as size and fecundity, or behaviour.

The fusion protein presented significant levels of toxicity when compared to GNA alone in artificial diet bioassays. Sub-lethal effects of Hv1a/GNA on aphid size and fecundity, which have previously been reported for GNA (Down et al., 1996, Sauvion et al., 1996), were also recorded in the present study. Toxicity of GNA to the peach-potato aphid has been previously assayed (Sauvion et al., 1996), and transgenic plants expressing this particular lectin generally offer low levels of insect control (Hilder et al., 1995; Down et al., 1996; Stoger et al., 1999). The other component of the fusion protein, Hv1a, is highly toxic towards

M. persicae when injected into the haemocoel, but innocuous when ingested (Pal et al., 2013). The high levels of toxicity of the fusion protein obtained in the present study following ingestion can be attributed to the transport of the intact and functionally active Hv1a peptide to its sites of action within the insect's body by the GNA carrier.

Even though other fusion proteins encompassing GNA as the carrier molecule have been tested against homopterans via artificial diet (e.g. Trung et al., 2006 tested ButaIT/GNA against *Nilaparvata lugens*; Down et al., 2006 tested SFI1/GNA against *M. persicae* and *N. lugens*), this is the first time a representative of these biopesticides is delivered to insects via transgenic plants. *M. persicae* was targeted not only because of its status as a pest for several crop species, but also because it feeds on Arabidopsis plants, thus providing a valuable proof of concept of expressing GNA-based fusion proteins for insect control. Regarded as a generalist, this aphid can infest several plant species, being able to cope with different diet regimes. Consequently, the observed increased proteolytic activity when compared to *S. avenae* might play an important role for the resilience of this species and extended host range. In the present study, Hv1a/GNA was readily cleaved by *M. persicae* gut proteases, as demonstrated by western blots of honeydew material. It has been previously demonstrated that proteolysis can significantly impact the effectiveness of fusion proteins (Fitches et al., 2004b), as the venom peptide on its own, without a carrier molecule, is not transported to its sites of action within the insect's body. However, as aphids fed continuously on diets and plants containing Hv1a/GNA, minute amounts of undigested fusion protein would have crossed the gut, reaching Hv1a sites of action in the CNS. This can be ascertained by two observations. Firstly, the increased toxicity of the fusion protein was markedly higher than GNA alone. Secondly, expression of GNA in transgenic potatoes can affect fecundity, but not survival of *M. persicae* (Gatehouse et al., 1996) and *Aulacorthum solani* (Down et al., 1996). In this work, although transgenic plants caused aphid mortality, expression levels were still insufficient to significantly influence reproduction. Stoger et al. (1999) report that expression of GNA in wheat plants only affects *S. avenae* fecundity at expression levels greater than 0.04% total soluble protein (TSP). A more

efficient expression system would therefore benefit aphid control using fusion proteins.

As aphids feed on the phloem sap, the use of a tissue-specific promoter would be desirable, avoiding unnecessary expression and preventing non-target organisms from being exposed to the fusion protein. However, previous work has shown that GNA expression in wheat under constitutive promoters was considerably higher than when using phloem-specific promoters, and the control of *S. avenae* comparatively more efficient (Stoger et al., 1999). Surprisingly, expression in chloroplasts proved to be effective in delivering *Pinellia ternate* agglutinin to *M. persicae*, reducing its growth rate by up to 90% (Jin et al., 2012). In the present study, the fusion protein was expressed under the control of the CaMV 35S promoter. A western blot-based quantification had to be performed, as two bands react with anti-GNA antibody, Hv1a/GNA and a ~10 kDa degradation product at an approximate proportion of 1:1. Therefore, results based on another commonly used method for protein quantification, ELISA, could be misleading in this case, as antibodies would recognize both, intact and degraded protein. Further improvements on protein stability would be necessary to prevent degradation following plant expression and ingestion by the aphid, enhancing activity.

In contrast to *M. persicae*, *S. avenae* is a semi-specialist species and although it possesses proteolytic activity in the gut (Pyati et al., 2011), this aphid is not able to cleave the fusion protein as effectively. As a consequence, levels of Hv1a/GNA toxicity towards the grain aphid were higher than in *M. persicae* and also more evident, as GNA by itself did not affect its survival in artificial diet bioassays. The grain aphid infests plants from the Poaceae family, being an important pest of wheat (*T. aestivum*) in China (Wang et al., 2011) and Western Europe (Larsson, 2005). It is therefore likely that expression of Hv1a/GNA in plants compatible with *S. avenae* feeding habits would render them significantly more resistant to aphid infestation.

Recently, Bonning et al. (2014) fused the same spider venom peptide, Hv1a, to a luteovirid coat protein that is internalized by aphids following ingestion. The resulting fusion, CP-P-Hv1a, was toxic to four different hemipteran species:

Acyrthosiphon pisum, *Rhopalosiphum padi*, *Aphis glycines* and *M. persicae*. These results indicate that Hv1a/GNA might also be effective against those aphids, as contrary to the Hv1a peptide, the viral protein is innocuous to the insects. Compared to Hv1a/GNA, CP-P-Hv1a yielded apparently higher mortality to *M. persicae* when expressed in Arabidopsis, but with the drawback of not being effective against other major insect pests, such as *Heliothis virescens* larvae. This is because the viral coat protein is only likely to cross the gut barrier in insects that can act as vectors of luteoviruses, i.e., aphids. The outcome is that even though CP-P-Hv1a potentially poses lower risks of affecting non-target insect species, to which GNA can often be detrimental, it will also have a very limited spectrum of activity. On the other hand, Hv1a/GNA was previously shown to also be effective against the coleopterans *Tribolium castaneum* (Back, 2011) and *Leptinotarsa decemlineata* (EC Fitches 2012, unpublished), and the lepidopteran *M. brassicae* (Fitches et al., 2012), whilst presenting little hazard to honeybees (Nakasu et al., 2014). It is clear, however, that the levels of aphid control by Hv1a/GNA when expressed in transgenic plants are currently not sufficiently high to maintain aphid populations under economic thresholds.

Improvements in the fusion protein stability in the plant and after ingestion, coupled with increased expression in the phloem sap would potentially be beneficial for achieving this goal. Expressing Hv1a/GNA in suitable plant hosts for lepidopteran and coleopteran pests, e.g. *H. virescens* and *L. decemlineata*, might further expand the range of insects that could be controlled by this biopesticide.

Chapter 3 Exposure of the parasitoid wasp *Eulophus pennicornis* to the insecticidal fusion protein Hv1a/GNA via its lepidopteran host *Lacanobia oleracea*

3.1 Abstract

Fusion proteins present potential for insect control as biopesticides or expression in transgenic plants. Consequently, a thorough evaluation needs to be performed in order to assure their safety towards non-target invertebrates. Previously, the neurotoxin peptide ω-ACTX-Hv1a, active only when injected into the haemolymph of insects, has been fused to the carrier molecule *Galanthus nivalis* agglutinin (GNA). The resulting fusion protein was orally toxic against several insect pests. The present study evaluates the effects of this novel biopesticide on the parasitoid *Eulophus pennicornis* via its host *Lacanobia oleracea*. Hv1a/GNA did not cause mortality when injected or fed to 5th stage *L. oleracea*, but caused up to 39% reduction in mean larval weight ($P<0.05$) and increased developmental time when compared to controls. When fed to insects, GNA, but not Hv1a/GNA, induced ~35% reduction in larval weight, indicating that host quality was not affected by the fusion protein. Although GNA and Hv1a/GNA were internalized by the hosts following ingestion and thus made available to higher trophic levels, no significant changes on the rate of *E. pennicornis* parasitism were observed. Furthermore, the number of parasitoid pupae/host, emergences and sex ration were unaffected by GNA- or Hv1a/GNA-treated hosts ($P>0.05$). Western blot analyses demonstrated that the fusion protein was digested by parasitoid larvae, preventing it from producing a toxic effect. These results indicate that the fusion protein has negligible effects on the parasitoid under worst-case scenarios. The low toxicity to these insects is of interest in terms of biopesticide specificity and safety to non-target organisms.

Keywords: Fusion protein, *Eulophus pennicornis*, *Lacanobia oleracea*, *Galanthus nivalis* agglutinin, ω-ACTX-Hv1a

3.2 Introduction

Neurotoxins derived from spider venoms have the potential to effectively target different insect species whilst being innocuous to vertebrates (Tedford et al., 2004a). However, there are major drawbacks on their practical use as topical insecticides, including inability to be absorbed by the insect cuticle, degradation in the environment (Fitches et al., 2004a), and lack of insecticidal activity via oral route (e.g. Pal et al., 2013).

The demonstration that the *Galanthus nivalis* agglutinin (GNA) is able to cross the insect midgut and reach the haemolymph following ingestion (Powell et al., 1998) opened the possibility of using it as a carrier molecule for insecticidal peptides. For example, the spider venom peptide *Segestria florentina* toxin 1 (SFI1) is structurally similar to other small spider neurotoxins that target voltage-dependent Ca^{2+} channels, causing flaccid paralysis when injected into *Heliothis virescens* larvae, but inactive when injected into mice (Lipkin et al., 2002). As it is orally inactive against insects, Fitches et al. (2004) have engineered a fusion protein comprising of the spider venom toxin SFI1 and GNA. The resulting fusion protein presented a high oral toxicity to *Lacanobia oleracea,* which was not observed for its components alone. The oral biological activity of the novel protein was due to the GNA transporting the SFI1 peptide to the haemolymph, from where it would be carried to its site of action in the central nervous system (CNS). More recently, Fitches et al. (2012) fused the calcium channel blocker ω-ACTX-Hv1a (Hv1a) from the funnel-web spider *Hadronyche versuta* to GNA. Once again, the fusion protein was effective in controlling a lepidopteran pest, *Mamestra brassicae* and the Colorado potato beetle, *Leptinotarsa decemlineata* (EC Fitches, personal communication).

Although insecticidal fusion proteins are effective, their use in the field as either a biopesticide or when expressed in transgenic plants should ideally be compatible with other pest management strategies, including that of biological control. As a consequence, their potential effects on beneficial non-target organisms, such as parasitoids, have to be evaluated.

Previous work has demonstrated that parasitoids respond differently to exposure to GNA alone. For instance, this lectin can have beneficial effects on

biological control when expressed in GE plants. Bell et al. (2001) demonstrated that the damage caused by *L oleracea* to transgenic potato plants expressing GNA was further reduced (ca. 21%) when *E. pennicornis* wasps were used for their biological control. On the other hand, indirect deleterious effects of GNA in parasitoids, such as decreased lifespan and fecundity as a consequence of reduced host quality, were observed by other authors (e.g. Couty et al., 2001a, Sétamou et al., 2002, Tomov et al., 2003). GNA can also induce direct insecticidal effects when delivered via artificial diet to parasitoid adults (Bell et al., 2004, Romeis et al., 2003), affect parasitoid fecundity when administered via dosed hosts (Couty et al., 2001b), or even present no effects at all when hosts are fed with artificial diets based on transgenic maize or potato expressing GNA (Bell et al., 1999). On the other hand, only limited information is available on the impacts of insecticidal fusion proteins against parasitoids (Wakefield et al., 2010a).

The present study evaluates the effects of a fusion protein containing GNA and a modified version of Hv1a (K34Q) (Pyati et al., 2014) on *Eulophus pennicornis* Nees (Hymenoptera: Eulophidae), a gregarious ectoparasitoid of the tomato moth *L. oleracea.*

3.3 Material and Methods

3.3.1 Protein expression and purification

Proteins were produced by heterologous expression in *Pichia pastoris* (SMD1168H strain) carrying sequences encoding GNA or Hv1a/GNA. Fermentations were carried out in Bio Console ADI 1025 (Applikon) fermentors (2 l vessels), with a continuous 50% glycerol feed for 72 h. Supernatants from the cultures were collected by centrifugation after expression. GNA was purified by hydrophobic interaction chromatography on a phenyl-sepharose resin packed onto a Pharmacia XK16 column. Fractions containing GNA were reloaded onto a size-exclusion column (HiPrep™ 16/60 Sephacryl S-100, GE-Healthcare). Following purification, recombinant proteins were dialyzed, freeze-dried and stored at -20 °C. Supernatants containing his-tagged Hv1a/GNA were diluted in binding buffer (0.02 M Sodium phosphate, 0.4 M NaCl, pH 7.4). Samples were then loaded onto a HisTrap™ (GE Healthcare) column and then

eluted with binding buffer containing 0.2 M imidazole. After purification, samples were extensively dialyzed in water and freeze-dried.

3.3.2 Bioassays with L. oleracea

L. oleracea were derived from a laboratory culture, reared in artificial diet at 25 °C and 16:8 h (L:D) (Corbitt et al., 1996). All bioassays with *L. oleracea* were performed using 450 ml transparent plastic cages. Larval stages were determined by measuring the head capsules, as described by Corbitt et al. (1996).

Initially, toxicity of Hv1a/GNA was assayed against *L. oleracea* via injection bioassays. Newly molted 5th stage larvae were anesthetized with CO_2 and injected with 15 µg (in 5 µl PBS) of BSA (n=37 larvae) or Hv1a/GNA (n=35 larvae) on the ventral side of their abdomen using a Hamiltonr® syringe (model 25F, needle gauge 25). Larval weight and mortality were recorded daily. Comparisons between treatments were made using Kaplan-Meyer Survival analysis for mortality data and one-way ANOVA for larval weight and time taken to reach 6th instar.

3.3.3 Internalization of GNA and Hv1a/GNA

The presence of Hv1a/GNA or GNA in *L. oleracea* haemolymph was verified by western blot using anti-GNA as primary antibody and enhanced luminol-based chemiluminescent (ECL), as previously described (Wakefield et al., 2010a). As wasp eggs would take on average 2.7 days to hatch (Marris and Edwards, 1995), haemolymph was collected four days after hosts had moulted to 6th stage, i.e., after eggs were laid, hatched and parasitoid larvae started feeding on host larvae.

It was not possible to immuno-detect the fusion protein on parasitoids feeding on hosts that were exposed to GNA or fusion proteins by ingestion. Therefore, in order to verify the fate of Hv1a/GNA following ingestion by *E. pennicornis*, parasitized *L. oleracea* larvae were injected with 15 µg of Hv1a/GNA. Parasitoid larvae feeding on injected larvae were then collected and subjected to western blot as described above.

3.3.4 Tri-trophic bioassays with L. oleracea *and the parasitoid* E. pennicornis

As the fusion protein did not affect *L. oleracea*, a tri-trophic bioassay was carried out in order to assess direct effects of Hv1a/GNA on the parasitoid *E. pennicornis* using the method described by Wakefield et al. (2010a). Fifth instar *L. oleracea* larvae were fed with 5 µl of a 5% sucrose solution containing 50 µg of BSA (negative control), Hv1a/GNA or GNA, for a minimum of three and a maximum of four consecutive days. Larvae were weighed daily in order to assure that hosts were of comparable quality to parasitoids. After moulting to 6th stage, caterpillars were individually exposed to one newly emerged, fecundated female of *E. pennicornis*. Adult female parasitoids were removed after 24 h, freeze-killed and screened for the presence of mature eggs. Parasitized *L. oleracea* larvae were kept until emergence of *E. pennicornis* at 25 ˚C and 16:8 h (L:D). The rate of parasitism, number of *E. pennicornis* pupae/host, sex ratio and parasitoid emergence rate were assessed.

3.3.5 Effects of GNA and Hv1a/GNA on E. pennicornis *development on injected hosts*

Fifth instar *L. oleracea* were exposed to fecundated female *E. pennicornis*, in a proportion of two larvae per parasitoid, for up to four days. After this period, larvae were screened for the presence of parasitoid eggs, anaesthetized with CO_2 and injected with 15 µg of BSA (used as negative control, n=34), GNA (n=34) or Hv1a/GNA (n=50), as described above. Host survival, parasitism, number of pupae per host and rate of *E. pennicornis* emergence were recorded.

3.4 Results

3.4.1 *Effects of Hv1a/GNA when injected into* L. oleracea

Fifth instar *L. oleracea* larvae were injected with recombinant fusion protein (Figure 3.1). Survival analysis (log-rank) of injected larvae resulted in no significant differences on mortality between treatments (P=0.149). However, a significant reduction in mean weight was observed in Hv1a/GNA-treated larvae from the second (P = 0.043) to the 10th day (P=0.006). After this period, larvae have recovered from the effects of the fusion protein, since no differences in mean weights were found from the 11th day onwards (P=0.067) (Figure 3.2).

These results also show that there was a decline in larval weight in both treatments from day 12, as insects were reaching the end of the larval stage and starting to pupate. Additionally, a significant increase in development time from 5th to 6th stage was observed in the Hv1a/GNA treatment (BSA n=20, 7.4±1.53 days to moult; Hv1a/GNA n=15, 8.66±1.87 days to moult; T=331.00, P=0.039).

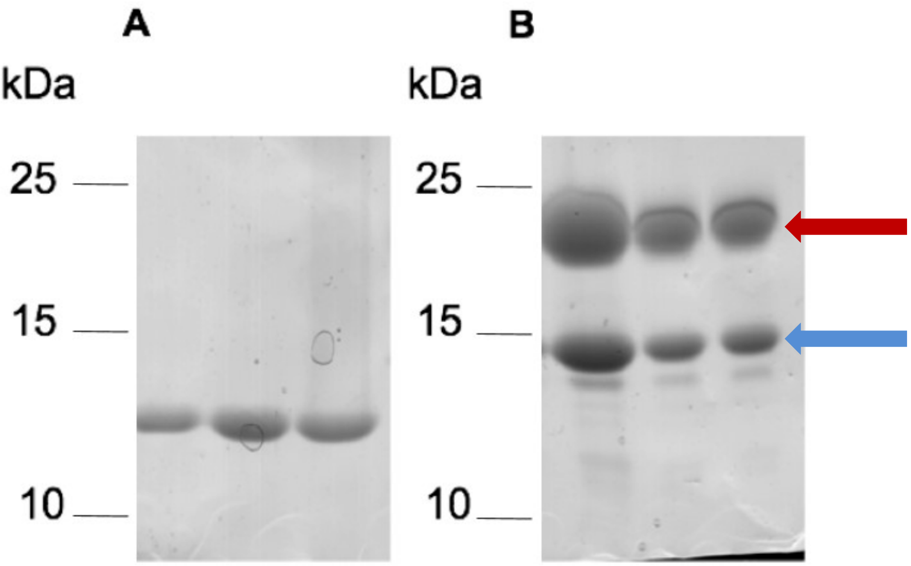

Figure 3.1: Profiles of recombinant proteins after expression in *P. pastoris* and purification using liquid chromatography techniques. SDS-PAGE showing A) purified GNA and B) purified Hv1a/GNA. A ~15 kDa degradation product (blue arrow) is co-expressed and co-purified with the intact fusion protein (red arrow).

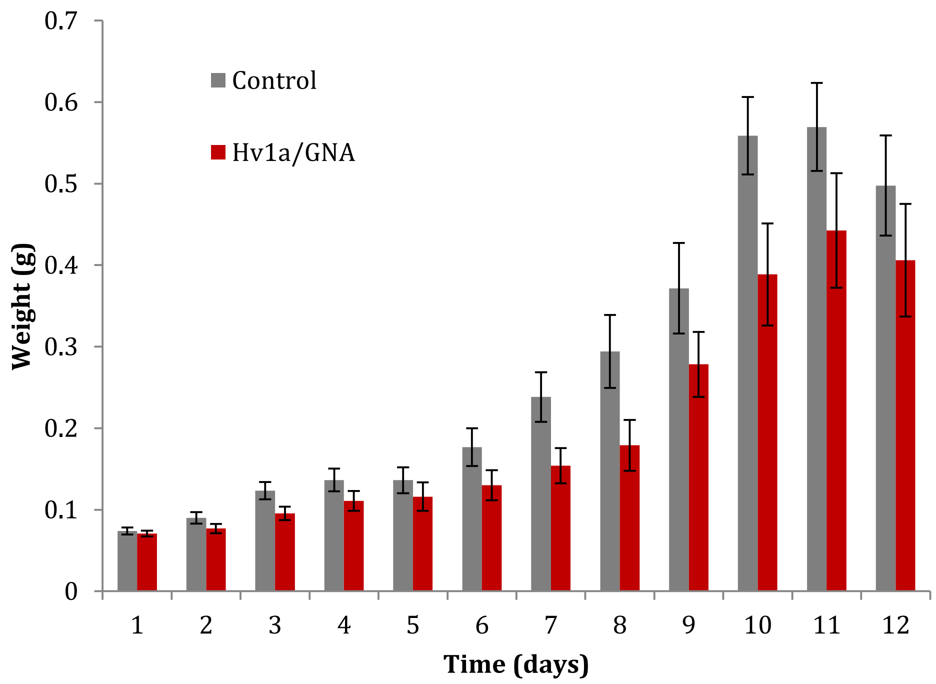

Figure 3.2: Effects of Hv1a/GNA (15 µg/larva) on *L. oleracea* via injection, compared with the negative control (BSA). A significant reduction in mean weight (± SEM) was observed in Hv1a/GNA treatment from day 2 to day 11, when larvae then recovered from the effects of the fusion protein. Pairwise comparisons are significant at $P<0.05$.

3.4.2 Effects of Hv1a/GNA on the host **L. oleracea** *via ingestion*

After ingesting droplets containing Hv1a/GNA or GNA, *L. oleracea* larvae were shown to internalize the proteins, as detected in haemolymph samples by western blot (Figure 3.3). Even though the fusion protein band at around 25 kDa appears to be fainter than its degradation products, it would still be made available to higher trophic levels, i.e., parasitoid wasps feeding on the haemolymph would also ingest the fusion protein or GNA.

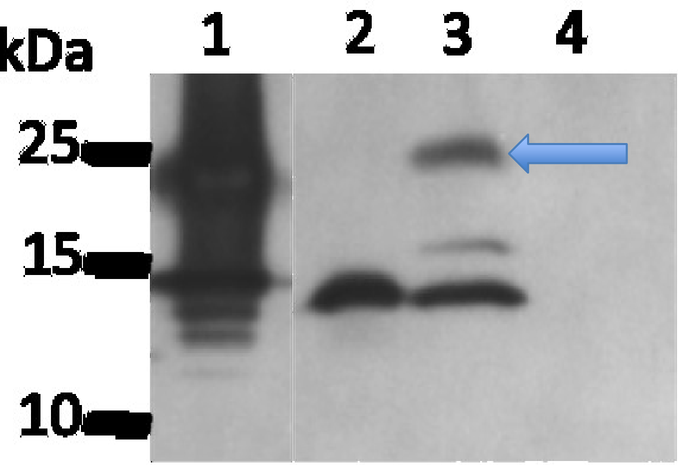

Figure 3.3: Western blot showing internalization of Hv1a/GNA by *L. oleracea* larvae. 1) positive control (Hv1a/GNA); 2) haemolymph from larva fed with droplets containing GNA; 3) haemolymph from larva fed with droplets containing Hv1a/GNA. Intact Hv1a/GNA is indicated by the arrow; 4) Negative control (haemolymph from larva fed on droplets containing BSA).

As with the injection bioassays, droplet feeding of the recombinant Hv1a/GNA had no effect on mortality of *L. oleracea* ($P>0.05$, data not shown). In contrast with injection bioassays (Figure 3.2), droplet feeding of Hv1a/GNA lead to no effect on weight on the host larvae, although GNA induced a significant reduction on this parameter ($P< 0.05$, Figure 3.4). Even though differences in weight were detected on the droplet feeding bioassay, only *L. oleracea* larvae of similar masses were offered to *E. pennicornis* adult females ($P=0.394$).

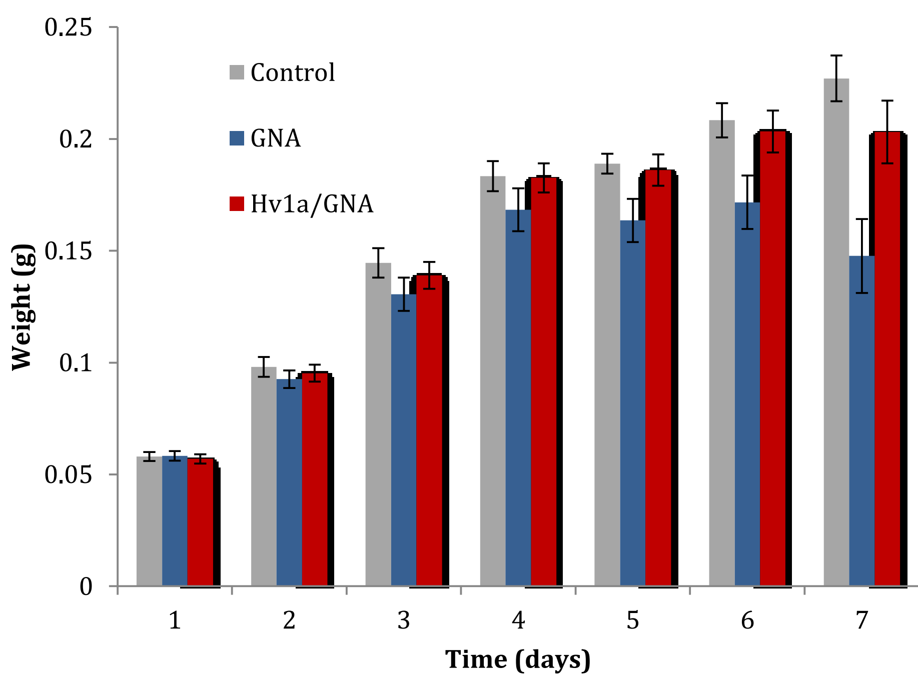

Figure 3.4: Average weight (g) per day of development of 5th stage *L. oleracea*. Only GNA treatment differed from the others from the 5th to the 7th day. One-way ANOVA followed by pair wise comparisons, significant differences if P<0.05.

3.4.3 Effects of Hv1a/GNA performance on **E. pennicornis** *when hosts were dosed orally*

The rate of parasitism of *E. pennicornis* on *L. oleracea*, even though slightly higher in the control, did not differ between treatments (P=0.378, Figure 3.5). Dissections of parasitoid females that did not oviposit demonstrated that they all carried mature eggs when in contact with *L. oleracea* (data not shown).

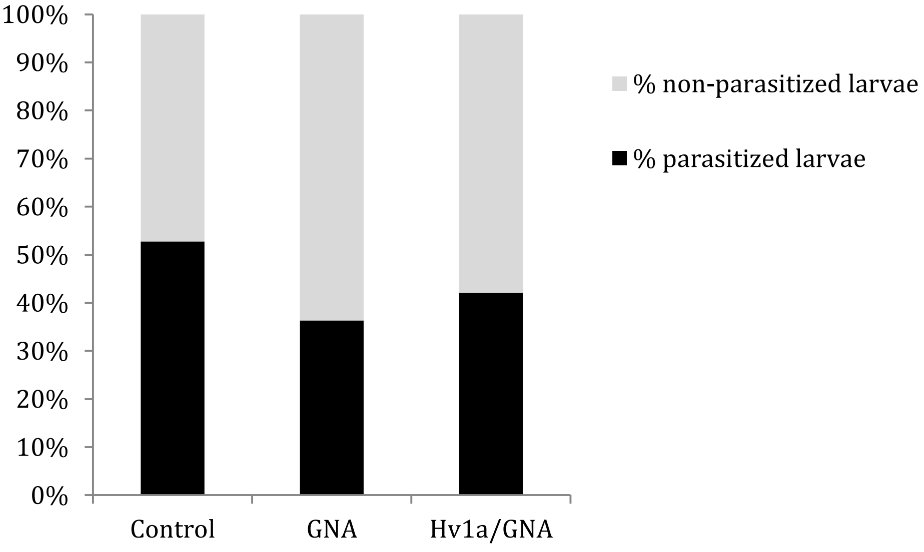

Figure 3.5: Percentage of *E. pennicornis* parasitism on *L. oleracea*, per treatment. Difference between treatments is not significant (P = 0.378).

Furthermore, no differences were found in the mean number of *E. pennicornis* pupae/host larva (ANOVA; P = 0.889), emergences over time (P = 0.114), or sex ratio (P = 0.570, Table 3.1). Although non-significant, control pupae started emerging 13 days after parasitoids deposited eggs on *L. oleracea*, whereas first emergences occurred 15 and 16 days after parasitoid exposure for GNA and Hv1a/GNA treatments, respectively. These results indicate that Hv1a/GNA does not affect any of the life parameters analyzed on the parasitoid *E. pennicornis.* Neither the fusion protein nor GNA were detected in parasitoid larvae samples that were feeding on *L. oleracea* hosts that were previously exposed to those proteins (data not shown).

Table 3.1: Comparison of the exposure of *E. pennicornis* larvae to hosts that ingested BSA (negative control), GNA or Hv1a/GNA.

Parameter	**BSA**	**GNA**	**Hv1a/GNA**
Mean number of pupae (n)	26.25±3.62(16)[a]	23.72±3.35(11)[a]	25.5±3.62(14)[a]
Mean number of adults emerged (n)	20±3.59(11)[a]	15.25±1.96(8)[a]	17.3±1.96(10)[a]
Sex ratio (males:females±SE)	0.18±0.03[a]	0.17±0.03[a]	0.11±0.02[a]
Emergences rate (n)	65% (482)[a]	68% (269)[a]	71% (368)[a]

Same superscript level letters mean that there are no significant differences between treatments (P>0.05).

3.4.4 Effects on parasitoid performance when hosts were injected with Hv1a/GNA

As no effects were detected on parasitoids developing on hosts that were orally exposed to GNA or Hv1a/GNA, *L. oleracea* hosts were injected with 15 µg of BSA, GNA or Hv1a/GNA after they were parasitized by *E. pennicornis*, representing a 'worst-case scenario' bioassay. Protein injections following parasitism resulted in high *L. oleracea* mortality, particularly in the fusion protein treatment, in which only two hosts in 50 survived. No differences between control and GNA treatments were found on the number of *E. pennicornis* pupae or emergences per host (Table 3.2). Comparisons between these two

treatments and Hv1a/GNA treatment were not made due to the low number of surviving hosts injected with fusion protein.

Table 3.2: Comparison of the exposure of *E. pennicornis* larvae to hosts injected with 15 μg of BSA (negative control), GNA or Hv1a/GNA.

	BSA	GNA	Hv1a/GNA
Number of injected hosts	34	34	50
Dead hosts 48 h after injection	22	22	48
Surviving hosts	12	12	2
Mean number of pupae/host (n)	3.8±1.5 (12)[a]	8.8±3.4 (12)[a]	2±2 (2)
Mean number of emergences/host (n)	6.6±1.8 (6)[a]	12.5±4.8 (7)[a]	4 (1)
% emergence rate (n)	91.6±8.3 (6)[a]	79±6.1 (7)[a]	100 (1)

Same superscript level letters mean that there are no significant differences between treatments (P>0.05). Due to low number of viable hosts, no comparisons were made between Hv1a/GNA and other treatments.

Even though the injection of Hv1a/GNA yielded low survival rates for both the host and *E. pennicornis*, parasitoid larvae feeding on *L. oleracea* injected with the fusion protein were collected and subjected to immunoassays. Hv1a/GNA is digested following ingestion by parasitoid larvae, as the ~25 kDa band corresponding to the intact fusion protein is not seen on the western blot (Figure 3.6).

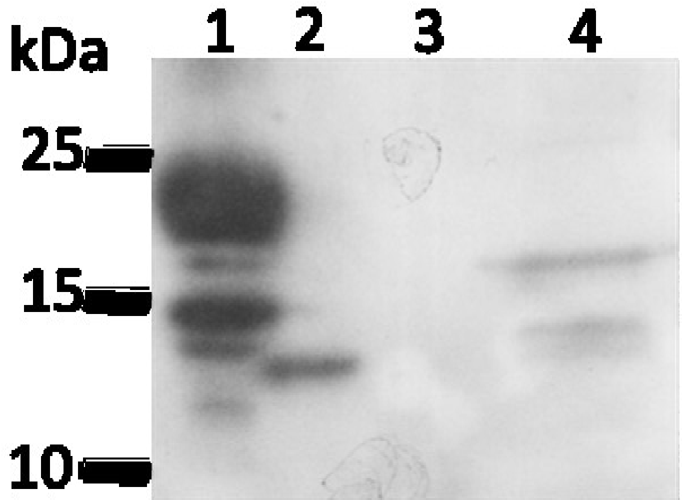

Figure 3.6: Hv1a/GNA is degraded following ingestion by *E. pennicornis*. Lanes were loaded as follows: 1 and 2) positive controls (Hv1a/GNA and GNA, respectively), 3) Negative control (samples of parasitoid larvae feeding on hosts injected with BSA); 4) samples of parasitoid larvae feeding on hosts injected with the fusion protein, showing degradation of Hv1a/GNA.

3.5 Discussion

The fusion protein Hv1a/GNA is currently being developed as a biopesticide for controlling important lepidopteran (Fitches et al., 2012) and coleopteran pests (EC Fitches, personal communication). However, it is important that this new biopesticide is also compatible with other pest management strategies, such as biological control. Commonly used neuroactive insecticides such as pyrethroids, organophosphates, carbamates and carbamyltriazole can be highly toxic to parasitoid wasps at field application rates (Desneux et al., 2004). Furthermore, some insecticides (e.g. malathion, etofenprox and methomyl) can also have strong, sub-lethal negative effects on the foraging behaviour (Kawazu et al., 2010), while others (e.g. chlorpyrifos) can reduce the sex ratio in parasitoid progenies (Delpuech and Meyet, 2003). It is not expected that Hv1a/GNA would have contact toxicity against insects, as it is an orally-active biopesticide. Other biopesticides, however, might present contact toxic effects against parasitoids. For example, Spinosad causes high acute mortality on adults and pupae of *Bracon nigricans*. Neurotoxic biopesticides emamectin benzoate and abamectin induce sub-lethal effects on this parasitoid, affecting its biocontrol activity, whereas Bt is relatively safe (Biondi et al., 2013).

In order to test effects of a fusion protein against beneficial arthropods, a system that mimics a relevant interaction was selected, since *E. pennicornis* is an effective biological control agent against the tomato moth *L. oleracea* (Marris and Edwards, 1994). Additionally, a host that would not be negatively affected by the fusion protein was deliberately used, reducing potential effects due to host quality, rather than direct toxicity (as suggested by Romeis et al., 2011). Injection of Hv1a/GNA into fifth stage larvae of *L. oleracea* caused a significant delay in developmental time, but only a temporary weight reduction, as larvae recovered their weight loss before moulting into the sixth stage. In contrast, when fed to *L. oleracea*, the fusion protein did not cause any measurable detrimental effects on the larvae, despite being internalized following ingestion. The lack of Hv1a/GNA toxicity when orally administered to *L. oleracea* could be explained by relatively small quantities of fusion protein being internalized in comparison to the amount injected. However, the low toxicity was unexpected, as at similar doses this fusion protein induces mortality via droplet feeding to larvae of *M. brassicae* (Fitches et al., 2012), another polyphagous pest of the

same family as *L. oleracea* (Noctuidae). Differences in susceptibility may be due to variations in the target site of action of Hv1a, voltage-gated calcium channels (Fletcher et al., 1997), or inability of the fusion protein to reach the CNS, where those channels are expressed. On the other hand, larvae fed GNA exhibited significant weight reduction, as previously reported by Fitches et al. (1997), thus demonstrating that the lectin was biologically active.

Exposure routes are a major consideration in the experimental design, as parasitoids can be exposed to the biopesticide in many different ways, particularly via its hosts. Therefore, in order to represent a field-relevant scenario, a tri-trophic system via host larvae was used, as it enabled an investigation as to whether: (i) ovipositing parasitoid females would avoid contaminated hosts and (ii) *E. pennicornis* larvae would be negatively affected by the recombinant proteins. Furthermore, if the fusion proteins were to be applied on the crops or expressed in transgenic plants, adult parasitoids would have minimal exposure, as they are unlikely to feed on plant parts other than pollen and nectar (Wakefield et al., 2010b).

The environmentally safe use of Hv1a/GNA as a biopesticide for the control of *M. brassicae* in Brassicaceae, tomatoes and a wide range of plants, which are also attacked by *L. oleracea*, should exclude any effect of the fusion protein on the pest's natural enemies, which play an important role in biological control. The use of a non-sensitive host, *L. oleracea*, provided an effective system to test direct effects of Hv1a/GNA on the parasitoid *E. pennicornis*, due to the fact that host quality, when considering size and weight, could be excluded as variables explaining potential differences between treatments. Furthermore, administering the fusion protein to parasitoids via hosts provides a realistic scenario, to some extent mimicking the route by which *E. pennicornis* would be exposed to Hv1a/GNA in crop systems. Although *L. oleracea* larval weight was affected by the GNA treatment, this difference in host quality did not influence any of the parameters evaluated on the development of *E. pennicornis*. This is consistent with previous results with hosts feeding on GNA-containing diets. For example, Bell et al. (1999) showed that maize-based and potato leaf-based diets containing GNA, and transgenic potato leaves expressing GNA fed to host *L. oleracea* did not have negative effects on *E. pennicornis*. Conversely,

Wakefield et al. (2010a) reported a direct effect of GNA on *E. pennicornis* larvae, as none of the eggs deposited on GNA-fed or injected *L. oleracea* developed to the adult stage. The inconsistency between the present study and the results presented by Wakefield et al. (2010a) remain unclear, as in both studies *L. oleracea* larvae were dosed with the same amounts of GNA.

The rate of parasitism of *E. pennicornis* adult females was not affected by treatment. Since Hv1a/GNA and GNA were present in *L. oleracea* haemolymph, it is reasonable to assume that parasitoid larvae that developed on those hosts were exposed to test proteins. However, attempts to detect the fusion protein in parasitoid larvae feeding on orally dosed hosts have failed. Therefore, parasitized *L. oleracea* hosts were injected with high ammounts (15 µg/larva) of the fusion protein in order to facilitate Hv1a/GNA immuno-detection on parasitoid larvae. Following western blot analysis of those parasitoid samples, none of the bands that reacted with anti-GNA antibodies presented the correct molecular weight of intact Hv1a/GNA (ca. 25 kDa). This result indicates that the fusion protein was being digested by *E. pennicornis* larvae, which might explain the lack of toxicity when parasitoids were exposed to orally dosed hosts. Hosts that were injected after being parasitized presented high mortality in all treatments, particularly in the fusion protein treatment. Even though only a small number of parasitized *L. oleracea* survived, *E. pennicornis* pupae were still able to emerge in all treatments.

Following Regulation (EC) 1107/2009 and Directive 2009/128/EC (European Parliament and the Council of the European Union, 2009) concerning the registration and sustainable use of pesticides in the EC, member States should reduce the risks and impacts of pesticide use on human health and the environment (Barzman, 2011). If proven to be effective in field trials, fusion proteins that target insect pests while being innocuous to non-target, beneficial arthropods provide a promising step towards novel environmentally friendly pest control strategies. Results from the present study demonstrate that the fusion protein Hv1a/GNA does not affect important life history parameters of the parasitoid *E. pennicornis* and is thus unlikely to compromise this particular parasitoid as a biological control agent.

Chapter 4 Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees

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4.1 Abstract

Evidence is accumulating that commonly-used pesticides are linked to decline of pollinator populations; adverse effects of three neonicotinoids on bees have led to bans on their use across EU. Developing insecticides that pose negligible risks to beneficial organisms such as honeybees is desirable and timely. One strategy is to use recombinant fusion proteins containing neuroactive peptides/proteins linked to a "carrier" protein that confers oral toxicity. Hv1a/GNA, containing an insect-specific spider venom calcium channel blocker (ω-hexatoxin-Hv1a) linked to snowdrop lectin (GNA) as a "carrier", is an effective oral biopesticide towards various insect pests. Effects of Hv1a/GNA towards a non-target species, *Apis mellifera*, were assessed through a thorough early-tier risk assessment. Following feeding, honeybees internalized Hv1a/GNA, which reached the brain within one hour after exposure. However, survival was only slightly affected by ingestion (LD_{50}>100 μg/bee) or injection of fusion protein. Bees fed acute (100 μg/bee) or chronic (0.35 mg/ml) doses of Hv1a/GNA and trained in an olfactory learning task had similar rates of learning and memory to no-pesticide controls. Larvae were unaffected, being able to degrade Hv1a/GNA. These tests suggest that Hv1a/GNA is unlikely to cause detrimental effects on honeybees, indicating that atracotoxins targeting calcium channels are potential alternatives to conventional pesticides.

Keywords: insecticidal fusion proteins, biopesticide, honeybees, ω-hexatoxin-Hv1a, snowdrop lectin (GNA), pollinator decline

4.2 Introduction

Pest control is an essential component of food security and agricultural productivity, as herbivorous pests, weeds, and pathogens can cause significant losses in staple food crops unless control measures are in place (Oerke, 2006). Since the 1940s, crop protection from insect pests has been reliant on synthetic chemical insecticides such as DDT and organophosphates (Casida and Quistad, 1998); these chemicals improved yields, but with a cost of negative consequences for non-target organisms, including humans (Carson, 1962). To overcome this, industrial producers have designed pesticides such as synthetic pyrethroids, neonicotinoids, and growth regulators with greater specificity for targeted pests that are now used worldwide (Elbert et al., 2008). Neonicotinoids are general agonists of insect nicotinic acetylcholine receptors, but bind only weakly to homologous receptors in higher animals (Tomizawa, 2004). Their efficacy and low mammalian toxicity have led to their widescale adoption, and they currently make up 24% of the world insecticide market (Jeschke et al., 2011). However, several reports of adverse effects of neonicotinoids on beneficial pollinating insects (Decourtye et al., 2004a; Williamson and Wright, 2013) have recently resulted in a controversial ban of the use of three neonicotinoid pesticides (clothianidin, thiamenthoxam, imidacloprid) by the European Commission. Insect pollination is an important ecosystem service, but it is also essential for fruit set in many crop species, contributing to 35% of global food production in approximately 70% of crops (Klein et al., 2007). Sub-lethal exposure to nectar-relevant doses of neonicotinoids impairs the function of Kenyon cells in the honeybee's mushroom bodies (Palmer et al., 2013) and reduces olfactory learning and memory (Decourtye et al., 2004a; Decourtye et al., 2004b) and homing ability (Bortolotti et al., 2003). In bumblebees, field-relevant, sublethal doses of these pesticides reduce foraging success and cause failure of bee colonies (Whitehorn et al., 2012). While neonicotinoids and other chemical pesticides clearly have negative impacts on pollinating bee species (Whitehorn et al., 2012; Henry et al., 2012), banning them without more appropriate alternatives could have significant consequences for food production or biodiversity, if less specific pesticides are used to replace them. Potential alternatives to neonicotinoids and other chemical pesticides include the development and use of biopesticides: biological agents or bioactive compounds that often have high specificity for target pest species (Glare et al.,

2012). Examples of currently used biopesticides include entomopathogenic fungi (Shah and Pell, 2003), and toxins derived from the entomopathogenic bacterium *Bacillus thuringiensis* (Bravo et al., 2011). Biopesticide candidates such as the venom of predatory arthropods that target the voltage-gated calcium ion channels (CaV) are very potent and selective (Tedford et al, 2004a). Since CaV channels are not highly conserved in insects, this makes them attractive alternatives and represents a novel mode of action to conventional pesticides.

Fusion protein technology, in which insecticidal peptides are linked to a plant lectin "carrier" protein, has been developed to allow proteins such as spider venom toxins to act as orally delivered biopesticides. For example, ω-hexatoxin-Hv1a (Hv1a; also referred to elsewhere as ω-atracotoxin-Hv1a or ω-ACTX-Hv1a) from the Australian funnel web spider *Hadronyche versuta* acts on CaV channels in the insect central nervous system (CNS), causing paralysis (Bloomquist, 2003). This toxin is lethal to many insect species when injected, but does not affect mammals (Fletcher et al., 1997). When delivered orally it is essentially non-toxic to insects, as it is unable to reach its site of action in the CNS. Fusion of this insecticidal molecule to the carrier protein snowdrop lectin (GNA), allows Hv1a to traverse the insect gut epithelium and access its sites of action, producing an orally active insecticidal protein (Fitches et al, 2012). The Hva1/GNA fusion protein has oral insecticidal activity against insects from a range of orders, including Lepidoptera, Coleoptera, Diptera and Hemiptera. Fusion protein biopesticides have the potential to improve pest management strategies, but they have not yet been tested on important insect pollinators such as bee species. In Europe, laboratory risk assessments of pesticides on bees currently include determination of acute contact and oral toxicity on adult honeybees, following the guidelines from EPPO 170 (2010) and OECD 213 and 214 (1998a; 1998b). Despite conforming to these criteria for assessing pesticide toxicity to bees, pesticides can also exert a range of effects on pollinator behavior at sub-lethal and field-realistic concentrations that are not detectable by current guidelines (Decourtye and Devillers, 2010; Schneider et al., 2012). For example, subtle aspects of bee behavior important for foraging and survival, such as learning and memory, can be impaired after prolonged exposure to pesticides (Decourtye et al., 2004a; Williamson and Wright, 2013). It is therefore sensible to assume that more rigorous testing of pesticide toxicity

to pollinating insects should be implemented alongside the development of new biopesticide products, to identify risks prior to their implementation in the field and to reduce environmental impact.

Here we report the testing of the insecticidal fusion protein Hv1a/GNA for toxicity to honeybees including the recommended acute toxicity tests from the OECD guidelines and in a test of cognitive function under both acute and long-term exposure. We also address the issues involved in testing pesticides on pollinators, suggesting that additional toxicity tests, such as a chronic toxicity assay, and an evaluation of any potential effects which pesticides may have on honeybee behaviour should be adopted to assess critical factors for bee viability and their role as pollinators.

4.3 Material and Methods

4.3.1 Honeybees

Honeybee colonies (*Apis mellifera mellifera*) were originally obtained from the National Bee Unit, York, UK, and were then maintained at Newcastle University. During the summer months (April – October 2012) bees were kept outdoors and allowed to fly and forage freely. During the winter months (November 2012 – March 2013) bees were maintained indoors, but were still allowed to fly freely via a plastic pipe connecting the hive entrance to the outdoors.

4.3.2 Pesticides and toxins

Recombinant *Galanthus nivalis* agglutinin (GNA), and the fusion protein Hv1a/GNA were produced in the yeast expression system *Pichia pastoris* as previously described (Raemaekers et al., 1999; Fitches et al., 2012). The pesticide thiamethoxam (Sigma Aldrich, 99% purity) and the CaV channel blocker benidipine HCl (Tocris Bioscience) were dissolved directly in 1 M sucrose solution for oral administration to adult forager bees. Acetamiprid (Scotts®) was obtained as a liquid formulation (0.5% acetamiprid, 1-5% ethanol, <1% of aqueous dipropylene glycol solution of approx. 20% 1,2-benzisothiazolin-3-one, 5-10% glycerol).

4.3.3 Acute toxicity tests of Hv1a/GNA

Acute toxicity was assessed by injection, and by oral and contact bioassays, using adult forager honeybees. Bees were collected from outside the hive in small plastic vials and then cold anaesthetised to allow manipulation or transference to containers.

After all acute toxin administration regimes (see below), bees were kept in 650 ml plastic storage containers fitted with 2 ml microcentrifuge tubes that had four holes drilled in for bee access. Bees were kept at 25 °C in the dark and allowed to feed *ad libitum* on 50% w/v sucrose solution. Mortality was recorded at 4, 24 and 48 h after exposure to the test compound.

Acute oral and contact toxicity assays were performed according to the Organisation for Economic Co-operation and Development (OECD) guidelines (OECD, 1998a, 1998b). For contact toxicity assays, bees were cold anesthetized and individually treated by topical application of PBST (phosphate buffered saline - Tween; 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4.2H_2O$, 3 mM KH_2PO_4, pH 7.4, containing 0.05% Tween-20; negative control), GNA in PBST (20 μg/bee), Hv1a/GNA in PBST (20 μg/bee), or acetamiprid as the positive control (4, 8.09 or 16.18 μg/bee, in PBST), directly applied to the thorax using a micropipette. After application, insects were separated into storage boxes as described above. Ten bees were used per treatment, and each treatment replicated seven times.

For the acute oral toxicity assays, insects were starved for 2 h prior to testing, in order to encourage active feeding during the assay. Bees were collected, cold anesthetized and placed inside the storage containers, in replicates of ten individuals per container. After starvation bees were fed via a feeder with either 200 μl of sucrose (50% w/v) solution (negative control), or sucrose solution containing GNA (control; 100 μg/bee), Hv1a/GNA (100 μg/bee), or acetamiprid (positive control; 7.26, 14.52 or 29 μg/bee). Insects were allowed to feed, without restraint, on the treatments for up to 4 hours, after which these feeders were removed and replaced with sucrose solution (50% w/v) feeders to allow feeding *ad libitum*. Six replicates of 10 bees were used for the negative control, GNA, and Hv1a/GNA treatments, whereas four replicates of 10 bees were used for each concentration of the positive control.

Effects of the recombinant proteins were also evaluated by an injection bioassay. Adult honeybees (thirty per treatment) were cold anaesthetized and

injected into the thorax with either 1) 5 µl of PBS (phosphate buffered saline as described above); 2) 5 µl of a 4 µg/µl GNA solution in PBS buffer (20 µg of GNA/bee); or 3) 5 µl of a 4 µg/µl Hv1a/GNA solution in PBS buffer (20 µg of Hv1a/GNA/bee) using a Hamilton® syringe (Model 25F, needle gauge 25). After injection, bees were divided into groups of ten inside the storage containers.

4.3.4 Chronic toxicity tests of ω-ACTX-Hv1a/GNA

Bees were collected, anaesthetised, then transferred to storage containers with feeding tubes as described above. Bees were allowed to feed *ad libitum* for seven days on one of three treatment solutions: 1) 1 M sucrose, 2) 350 µg/ml Hv1a/GNA in 1 M sucrose, or 3) 10 ng/ml thiamethoxam in 1 M sucrose. Bees were maintained in an incubator at 34 °C for the duration of the treatment period, and mortality was recorded daily. Sample size was 40 bees per treatment group.

4.3.5 Testing of Hv1a/GNAfor acute toxicity towards honeybee larvae

Standard operating procedures established for the *in vitro* testing of pesticides were used to test for acute toxicity of Hv1a/GNA towards honeybee larvae (Aupinel et al., 2007). A single oral dose of 100 µg/larva of Hv1a/GNA was administered to four day-old larvae individually maintained in microtitre plate wells. Plates were incubated under controlled environmental conditions at 34 °C in the dark, 60% RH (relative humidity). A total of thirty larvae were treated alongside a control treatment, in which larvae were fed on a diet with no added protein. Fifteen larvae were sacrificed at 24 and 92 h after exposure to the fusion protein to obtain haemolymph, whole larval and diet samples for western blot analysis to assess the stability of the fusion protein. Haemolymph (at least 5 µl per insect) was obtained by piercing pre-chilled larvae with a fine needle and collecting into pre-chilled phenylthiocarbamide-phenol oxidase (PPO) inhibitor to prevent melanisation. The survival of the remaining 15 larvae was monitored for four days subsequent to the single acute Hv1a/GNA dose.

4.3.6 Acute Hv1a/GNA exposure for learning and memory experiments

Forager bees were collected from outside the hive in small plastic vials, cold anaesthetised, and restrained in harnesses (Bitterman et al., 1983). The bees

were fed 20 µl of 1 M sucrose solution, then left overnight to become sufficiently hungry and motivated to perform the olfactory learning task. One hour prior to the learning task, each bee was fed 5 µl of treatment solution. The treatment groups were: 1) a control group fed 5 µl of 1 M sucrose; 2) 100 µg of Hv1a/GNA in 5 µl of 1 M sucrose; 3) 100 µg of GNA in 5 µl of 1 M sucrose; and 4) 500 ng of benidipine HCl in 5 µl of 1 M sucrose. The experiment was repeated with 3 cohorts, and the total sample size of trained bees was $\geq$20 bees per treatment group.

4.3.7 Long-term Hv1a/GNA exposure for learning and memory experiments

Foraging worker bees were collected and cold anaesthetised. Ten bees were transferred to each feeding box (16.5 x 11 x 6.5 cm) fitted with 2 ml microcentrifuge tubes with evenly spaced holes for feeding the solutions. Bees were allowed to feed *ad libitum* for seven days on one of three treatment solutions: 1) 1 M sucrose, 2) 350 µg/ml Hv1a/GNA in 1 M sucrose, or 3) 10 ng/ml thiamethoxam (i.e. 10 ppb or 34 nM) in 1 M sucrose. Bees were maintained in an incubator at 34 °C for the duration of the treatment period, and mortality was recorded daily. After this, the bees were cold anaesthetised and restrained in harnesses, fed 20 µl of treatment solution, and left overnight to become sufficiently motivated to perform the olfactory learning task. The survival analysis was repeated four times (N = 40/treatment group). A subset of bees was selected from these cohorts for the olfactory conditioning assay.

4.3.8 Learning and memory experiments

An olfactory conditioning protocol based on the proboscis extension reflex (PER) was performed (Bitterman et al., 1983). The conditioned stimulus (CS, 1-hexanol) and unconditioned stimulus (0.2 µl of 1 M sucrose solution) were presented for six training trials, with a 10 min inter-trial interval. PER response to the CS was recorded. Two unreinforced recall tests (the CS and a novel odour) were administered at 10 min after conditioning and again at 24 h. The order of presentation of these two test stimuli was pseudorandomised across subjects.

4.3.9 Detection of Hv1a/GNA in honeybee tissues by western blotting

To test internalization of recombinant proteins, tissue samples were collected from bees following 24 h feeding on either GNA or Hv1a/GNA, as described above, using a modified version of the method described by Mayack and Naug (2010). For haemolymph from adults, insects were killed at -20 °C and immediately wrapped with Parafilm. The distal end of one of the antennae was cut and insects were placed individually in microcentrifuge tubes. Tubes were spun for 30 s at 5000 *g* and haemolymph collected and kept at -80 °C until use. Haemolymph was collected from larvae previously exposed to the recombinant proteins after either 24 h (five days-old larvae) or 92 h (eight days-old larvae), as detailed above. For brain samples from adults, insects were cold anesthetized, restrained in harnesses and fed with 20 μl of 1 M sucrose solution (negative control) or 100 μg Hv1a/GNA in 20 μl of 1 M sucrose solution. After 24 h, honeybees were freeze-killed and the brains removed. Six brains from each treatment were pooled and macerated in SDS sample buffer (100 mM Tris-HCl, pH 6.8, 4% SDS, 9% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue). Proteins from individual samples were separated in 15% SDS-PAGE, transferred to nitrocellulose membranes and screened for the presence of GNA or Hv1a/GNA by SDS-PAGE followed by western blotting using anti-GNA antibodies (Fitches et al., 2012).

4.3.10 Statistical analysis

Log-rank Kaplan-Meier (K-M) survival analyses with pairwise comparisons over strata were carried out using SPSS v. 19.0. The LD_{50} with 95% confidence intervals for positive controls on acute oral and contact bioassays were estimated by plotting log dose vs probit of corrected mortalities (Randhawa, 2009; Abbott, 1925; Miller and Tainter, 1944). PER response during the learning and memory tests was scored as a binary response, and data was analysed in SPSS using a binary logistic regression (lreg). Data from the first training trial was excluded from the analysis to facilitate model fit. Pairwise comparisons between different treatments, time points and odours were performed using least-squares *post-hoc* comparisons (lsc). PER data represent the mean probability of responding with a Wald chi-square 95% confidence interval.

4.4 Results

4.4.1 Testing the acute and chronic toxicity of Hv1a/GNA to honeybees

In order to assess the potential toxicity of Hv1a/GNA to pollinators, bioassays were carried out to measure the survival of honeybees after exposure to the fusion protein (Figure 4.1). The Hv1a/GNA treatment regimens included acute contact and oral exposure, acute injection, and a chronic 7-days oral exposure; the neonicotinoids acetamiprid and thiamethoxam, were used to compare mortality caused by a neonicotinoid to that of the fusion protein.

In the acute contact toxicity assays, the positive control acetamiprid induced bee mortality when compared to the negative control (PBST), GNA control, or Hv1a/GNA treatments (Figure 4.1a, K-M, PBST vs Ace, $\chi_1^2 = 57.1$, $P < 0.001$; Hv1a/GNA vs Ace, $\chi_1^2 = 49.9$, $P < 0.001$; GNA vs Ace, $\chi_1^2 = 49.9$, $P < 0.001$), with an estimated LD_{50} of 6.78±0.58 μg/bee, thus within the limits reported on the literature (Iwasa et al., 2004). When compared to the negative control, neither Hv1a/GNA nor GNA increased mortality after contact exposure (K-M, Hv1a/GNA, $\chi_1^2 = 1.34$, $P = 0.246$; GNA, $\chi_1^2 = 1.34$, $P = 0.246$) when applied at 20 μg/bee. It is unlikely that the fusion protein or the GNA are able to cross the insect cuticle, and thus a lack of toxicity in this assay is expected.

In the acute oral treatments with the compounds, bees fed the neonicotinoid, acetamiprid, were the least likely to survive of all treatments (Figure 4.1b, K-M, Suc vs Ace, $\chi_1^2 = 56.3$, $P < 0.001$). The estimated LD_{50} for this compound was 8.95±0.23 μg/bee, which is comparable to those reported for formulated products (European Comission, 2004). Survival of honeybees fed on Hv1a/GNA or GNA at the maximum recommended dose for oral toxicity assays (100 μg/bee) was reduced by 22% for the fusion protein (K-M, Suc vs Hv1a/GNA, $\chi_1^2 = 7.76$, $P = 0.005$) and 34% for the GNA (K-M, Suc vs GNA, $\chi_1^2 = 16.7$, $P < 0.001$). Survival of the bees fed either Hv1a/GNA or GNA was greater than those fed acetamiprid (K-M, Hv1a/GNA vs Ace, $\chi_1^2 = 35.5$, $P < 0.001$; GNA vs Ace, $\chi_1^2 = 31.5$, $P < 0.001$). We can therefore conclude that Hv1a/GNA and GNA are of relatively low toxicity to honeybees as the oral LD_{50}>100 μg/bee. An acute toxicity assay was also performed on larval honeybees: no mortality was

observed for either control or Hv1a/GNA treatments, with 100% survival recorded four days post treatment.

In order to exclude the possibility that low toxicity of Hv1a/GNA was due to inefficient transport of the Hv1a/GNA from the gut to the haemolymph, toxicity of Hv1a/GNA and GNA by injection was assessed to represent a 'worst case scenario'. In this test, injections were of 20 µg protein/bee. The mortality over 48 h was greatest for those injected with GNA (57% mortality; Figure 4.1c, K-M, PBS vs GNA, $\chi_1^2 = 23.4$, $P < 0.001$; GNA vs. Hv1a/GNA, $\chi_1^2 = 11.1$, $P = 0.001$). Whilst bees injected with Hv1a/GNA also had significantly greater mortality than the PBS control (K-M, PBS vs Hv1a/GNA, $\chi_1^2 = 5.35$, $P = 0.021$), mortality levels were relatively low (<17%). These low levels were similar to the acute oral treatment, confirming that only a very high dose of this compound could produce measurable mortality in honeybees. Most of this mortality occurred between the 24 and 48 h time points.
Previously, the Hv1a/GNA fusion protein has been shown to be an effective insecticide when used as a foliar spray; the protein is stable over timescales >2 weeks under these conditions, and provides continuing protection without the need for re-spraying (Elaine Fitches, unpublished data). The toxicity of chronic consumption of Hv1a/GNA at the effective concentration when delivered as a spray, 350 ppm (0.35 mg/ml), by adult forager honeybees was also investigated, and compared directly to the chronic toxic effects of the neonicotinoid, thiamethoxam, at the concentrations reported in the nectar and pollen of treated crops (Stoner and Eitzer, 2012; Pohorecka et al., 2013). Each bee consumed on average 63.8 ± 0.003 µl of the control solution, 62.1 ± 0.002 µl of the Hv1a/GNA solution, and 72.7 ± 0.004 µl of the thiamethoxam solution per day. Based on the average volume of solution consumed per day, the estimated dose of the Hv1a/GNA solution for each bee was 21.7 µg/bee/day, and the estimated dose of the thiamethoxam for each bee was 0.727 ng/bee/day. After seven days of treatment, thiamethoxam treatment significantly increased mortality compared to the other groups (Figure 4.1d, K-M, Suc vs TMX, $\chi_1^2 = 37.3$, $P < 0.001$). In contrast to this, there was no difference in survival between the control group and the Hv1a/GNA treatment group (K-M, Suc vs Hv1a/GNA, $\chi_1^2 = 1.16$, $P = 0.282$), again confirming low toxicity of Hv1a/GNA to honeybees.

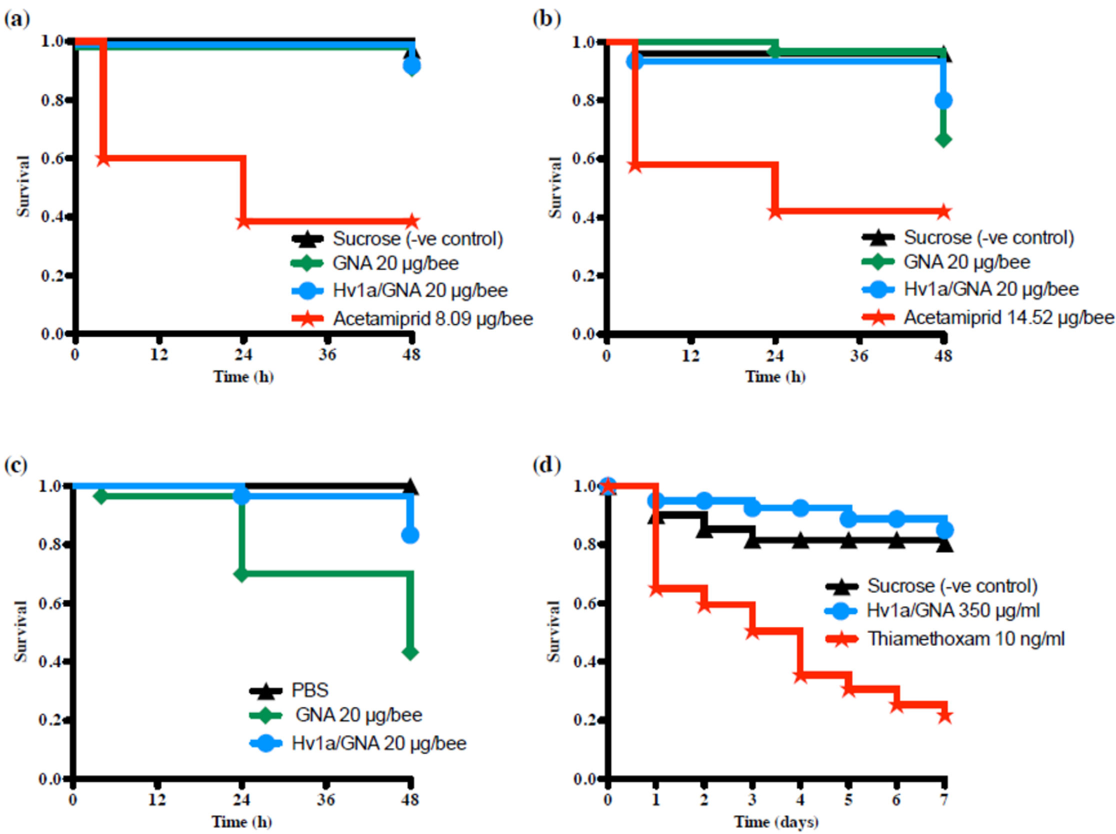

Figure 4.1: Survival analyses indicate Hv1a/GNA poses no substantial toxicity towards adult honeybees. (a) Acute contact toxicity assay of GNA and Hv1a/GNA with honeybees (20 µg of test protein/bee; N = 70 bees/treatment). Survival curve for the positive control acetamiprid (8.09 µg/bee) is shown. (b) Acute oral toxicity bioassays of GNA (N = 60) and Hv1a/GNA (N = 60) with honeybees (100 µg of test protein/bee). Survival curve for positive control acetamiprid (14.52 µg/bee, N = 40) is shown. (c) Effects of GNA and Hv1a/GNA on survival of honeybees following injection (20 µg of test protein/bee; N = 30 bees/treatment). (d) Honeybee survival was unaffected by chronic consumption of 21.7 µg/bee/day dose of Hv1a/GNA, but a 0.727 ng/bee/day dose of thiamethoxam increased mortality (N = 40 bees/treatment). Dose-response curves for both acute contact and acute oral bee toxicity assays for all acetamiprid concentrations are presented in ESM Figure 1a and Figure 1b, respectively.

4.4.2 Testing the effects of Hv1a/GNA on honeybee learning and memory

Experiments based on an olfactory conditioning protocol were performed to assess whether Hv1a/GNA affected olfactory learning and memory in the

honeybee following both acute and long-term oral exposure (Figure 4.2). Studies to investigate potential effects of acute exposure also included a positive control for testing the effects of a CaV channel blocker on this behavioural parameter (benidipine hydrochloride, BH), since a CaV channel is the target of the Hv1a toxin. As shown inFigure 4.2a, there was an overall difference in the rate of learning between the different acute treatment groups (lreg, $\chi_3^2 = 30.7$, $P < 0.001$). Benidipine hydrochloride (positive control) impaired the rate of olfactory learning by up to 50% over the course of six conditioning trials (lsc, $P = 0.026$). The rate of learning was unaffected when bees were treated with an acute dose of either Hv1a/GNA (lsc, $P = 0.957$) or GNA (lsc, $P = 0.702$) (Figure 4.2a). Treatment influenced the expression of short-term memory (Figure 4.2b); bees fed BH had lower responses than the control, GNA, or the Hv1a/GNA treated bees (lreg, STM, $\chi_3^2 = 7.82$, $P = 0.050$; lsc for the control vs. BH, $P = 0.025$). However, when tested for long-term memory 24 h later, there was no significant difference in the rate of response to the conditioned odour between the treatment groups (lreg, LTM, $\chi_3^2 = 4.67$, $P = 0.197$). For both tests, the rate of response was always greater towards the conditioned odour than a novel odour (data not shown, lreg, STM, $\chi_1^2 = 17.7$, $P < 0.001$; LTM, $\chi_1^2 = 10.3$, $P = 0.001$).

The effects of chronic oral exposure to Hv1a/GNA on olfactory learning ability and memory were also tested. The results showed that Hv1a/GNA did not influence the rate or asymptotic level of learning when compared to the control (lreg, $\chi_1^2 = 2.69$, $P = 0.107$) (Figure 4.2c). Similarly, bees fed Hv1a/GNA did not exhibit impaired short or long-term memory performance (lreg, STM, $\chi_1^2 = 3.30$, $P = 0.069$; LTM, $\chi_1^2 = 1.41$, $P = 0.235$) (Figure 4.2d). These results demonstrate that the fusion protein HV1a/GNA does not impair olfactory learning or memory formation, even though a positive control for the same target as the fusion protein (BH) significantly reduced the rate of learning and short-term memory.

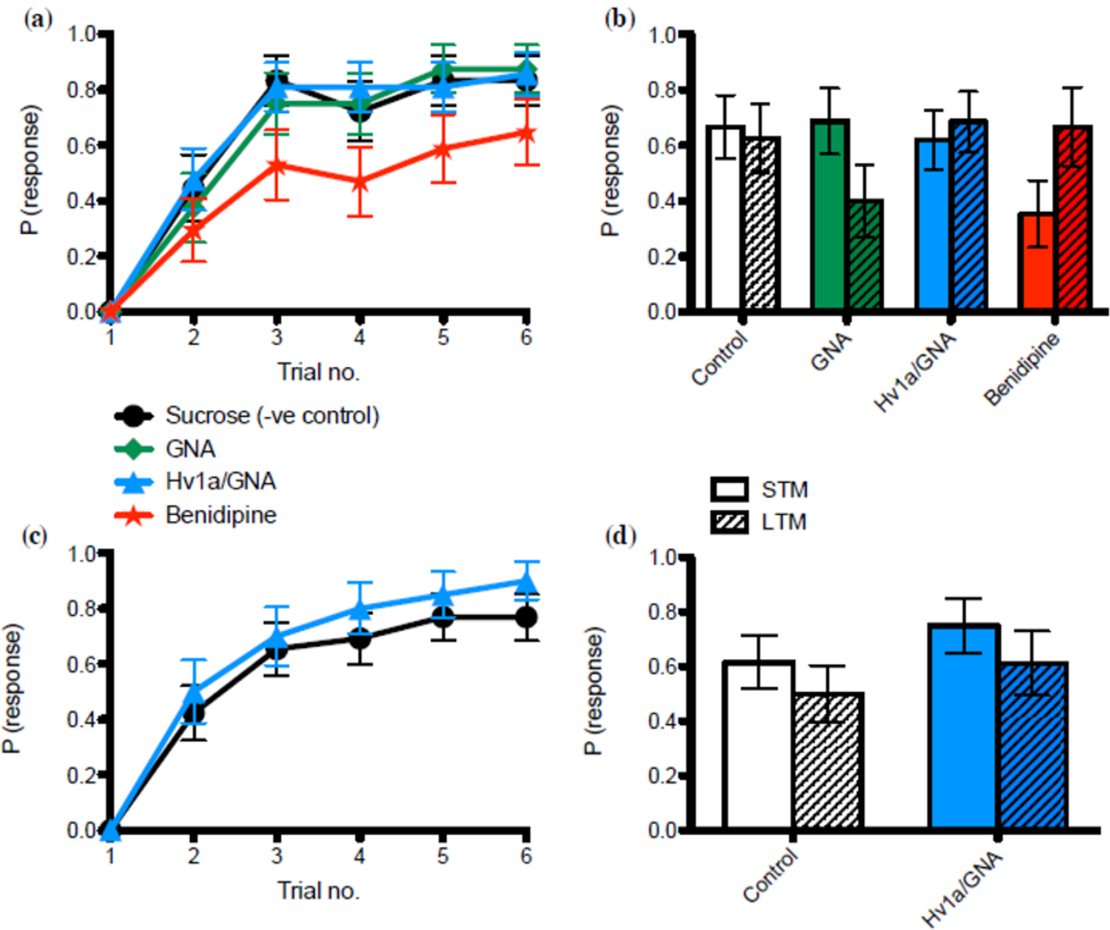

Figure 4.2: Hv1a/GNA consumption does not affect honeybee learning and memory. (a) The rate of learning is reduced in the positive control (the calcium channel blocker, benidipine HCl, Ben), whereas acute exposure to Hv1a/GNA (Hv1a), or GNA, does not significantly influence olfactory learning relative to the control (Con). $N_{control}$ = 20, N_{GNA} = 20, N_{BH} = 23, $N_{Hv1a/GNA}$ = 23 (b) Short-term memory was impaired for the Ben group, but not for the other treatments (lsc comparisons against the control: GNA, P = 0.740, Ben, P = 0.025, Hv1a/GNA, P = 0.661). (c) The rate of learning was not significantly different for bees fed Hv1a/GNA for 7 days. $N_{control}$ = 26, $N_{Hv1a/GNA}$ = 20 (d) Short term memory (STM, 10 min) and long term memory (LTM, 24 h) were not significantly different for bees fed Hv1a/GNA prior to conditioning; con = control, Hv1a = Hv1a/GNA. Data represents mean response probabilities ± 95% confidence intervals.

4.4.3 Detection of Hv1a/GNA in honeybee tissues by western blotting

To investigate potential internalization of HV1a/GNA in both adult and larval honeybees, tissue samples were collected from insects fed on diet containing either GNA or Hv1a/GNA 24 h after exposure and subsequently transferred to diet without treatment for varying times. In adult bees the Hv1a/GNA fusion protein was clearly visualised in haemolymph samples 24 h after feeding (Figure 4.3a), demonstrating that the GNA carrier component was able to direct transport of the toxin component across the gut epithelium, as has been observed in other insects (Fitches et al., 2012). Fusion protein was also detectable in brain tissue, showing that the toxin had been able to reach its site of action in the CNS, and that the lack of toxicity of Hv1a/GNA was not due to failure to transport or access its target. As in adult bees, the western blotting experiment for bee larvae showed evidence for transport of the GNA carrier across the gut epithelium, since GNA was present both in haemolymph and whole insect after feeding and chase (24 and 92 h). However, no evidence for toxin transport was seen, as all the fusion protein was degraded, and no intact Hv1a/GNA could be detected (Figure 4.3b). As expected, the levels of degraded protein, representing the GNA part of the fusion protein, were reduced by the longer chase period of 92 h compared to 24 h. The absence of toxicity of Hv1a/GNA to larval bees is thus primarily due to protein degradation in the gut preventing transport of the toxin to its sites of action, although on the basis of results from adult bees, it is likely that the toxin would not affect calcium channels if transported to the haemolymph.

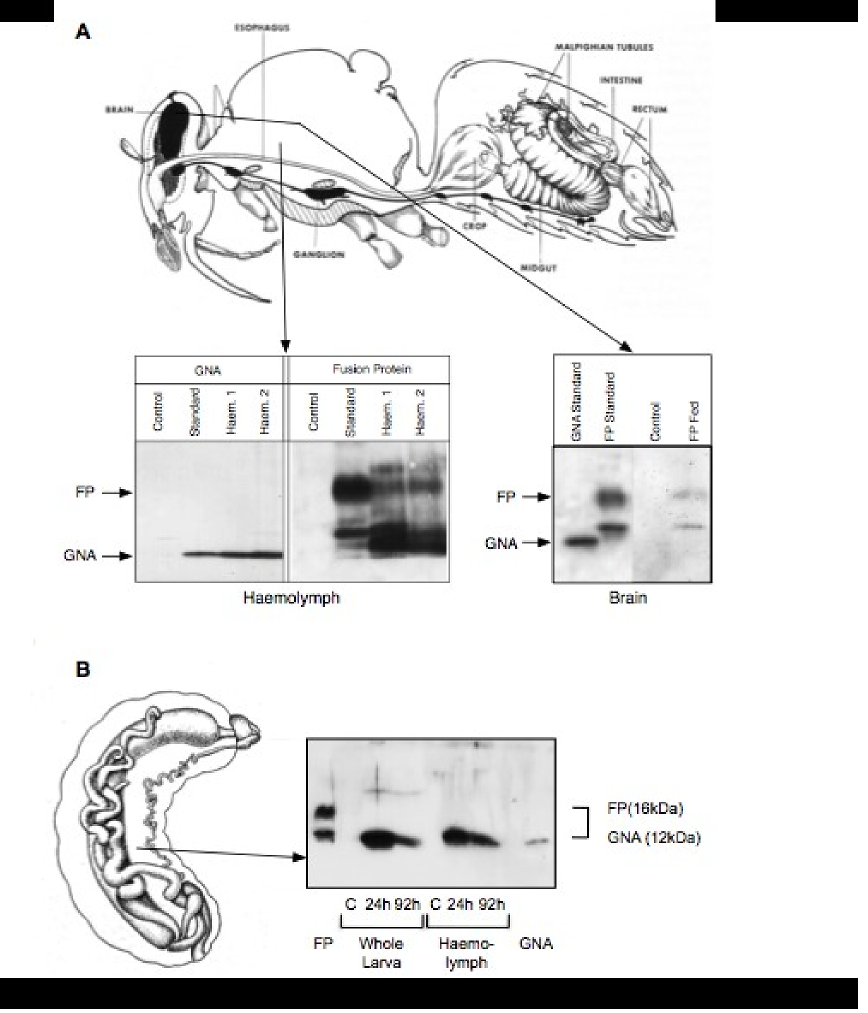

Figure 4.3: Immuno-assay by western blotting demonstrates internalization of Hv1a/GNA in adult honeybee tissues. Bands of GNA (12 kDa) and Hv1a/GNA (FP; 16 kDa) are indicated. (a) Diagram of adult honeybee showing the presence of GNA and fusion protein Hv1a/GNA (FP) in both the haemolymph and brain after feeding solutions containing proteins. Insects were fed 100 μg GNA or Hv1a/GNA and haemolymph or brain tissue was collected after 24 h for analysis. (b) Diagram of larval honeybee showing that Hv1a/GNA (FP) is degraded after ingestion; larvae were dosed with 100 μg Hv1a/GNA per larva and haemolymph was collected after 24 h for analysis.

4.5 Discussion

The fusion protein Hv1a/GNA complies with the current European and American risk assessments for pesticide toxicity to honeybees, as tests described in the OECD guidelines were fulfilled (OECD, 1998a, 1998b). Following those assays, acute oral and contact toxicity of Hv1a/GNA can be considered negligible (LD_{50}>100 μg/bee). Even when bees were injected with

Hv1a/GNA, only 17% of the bees died within 48 h. In comparison, lepidopteran larvae injected with comparable amounts of fusion protein typically show a 90-100% reduction in survival (Fitches et al., 2012). We assume this level of mortality can be considered low, as, according to the US Environmental Protection Agency (EPA), compounds with *contact* toxicity of LD_{50}>11 µg/bee are classified as "relatively nontoxic" (EPA, 2011). This suggests that the omega toxin does not reach or bind to the target site of action in the central nervous system (CNS) of bees as avidly as it does in lepidopteran larvae, or that there are critical differences in the ion channel binding sites in bees and lepidopteran larvae. Surprisingly, the survival of bees injected with GNA was significantly reduced (ca. 60%), as compared to the control treatment, whereas the injection of equivalent, high doses of GNA into lepidopteran larvae does not result in substantial mortality. In our experiments, GNA was only used as a control, in the event that the fusion protein had an influence on survival, learning and memory. Previous results of feeding bioassays have suggested that plant lectins have differing effects on insects, although the basis of this effect remains unclear. Hv1a/GNA did not have a measurable influence on survival or cognition in adult worker honeybees after acute or long-term oral exposure. The observed lack of Hv1a/GNA toxicity contrasts with lethal effects of neonicotinoids used as positive controls: acetamiprid was acutely toxic at similar concentrations to those previously reported (Iwasa et al., 2004), and chronic thiamethoxam ingestion at a field-relevant dose had significant lethal effects at the concentrations found in nectar and pollen (Stoner and Eitzer, 2012; Pohorecka et al., 2013).

No adverse effects of Hv1a/GNA on honeybee learning and memory were detected in the assays reported here, in spite of the fact that the doses we gave the bees prior to the assay were relatively high. In fact, the chronic exposure experiment is likely to have provided a dose to the bees far above what they would experience in the field; this is because the biopesticide is applied as a spray and not as a systemic pesticide and so would not be consumed in large amounts by bees in nectar and pollen. Previous studies have found that exposure to field-relevant doses of pesticides which target the central nervous system, such as neonicotinoids and organophosphates, impair the ability of honeybees to learn and remember the association between an olfactory cue and a sucrose reward (Decourtye et al., 2004a; Williamson and Wright, 2013).

The effect of Hv1a on insect calcium channels (Fletcher et al., 1997) suggests that it could have significant effects on learning and memory, especially if CaV channels are affected (Perisse et al., 2009). CaV channels are known to play a role in olfactory learning in mammals (Jerome et al., 2012), and are present in the areas of the honeybee brain where olfactory associations are processed (Schafer et al., 1994; Grunewald, 2003). This prediction of CaV involvement in honeybee learning was confirmed, as the positive control for CaV block, benidipine HCl (Yao et al., 2006), impaired olfactory learning and short-term memory. What was surprising, however, was that benidipine HCl (used as a positive control) did not influence long-term olfactory memory. A previous study of the influence of calcium on olfactory learning and memory in bees showed that blocking intracellular calcium release prior to conditioning impaired long-term memory formation (Perisse et al., 2009). Instead of blocking CaV channels as we did, however, this study used a chelator of calcium to prevent calcium binding to CaV channels. In contrast, Hv1a/GNA had no significant effect on olfactory learning or memory, indicating that at the doses we tested, it is an ineffective antagonist of the CaV channel in the honeybee brain.

This lack of observed adverse effects on either the survival or the learning ability of adult honeybees was not due to the fusion protein failing to reach the target site in the CNS. When orally administered to adult worker honeybees, Hv1a/GNA was capable of crossing the epithelial gut wall, as Hva1/GNA immunoreactivity was detected in the haemolymph and brain tissue one hour after ingestion. In contrast with adult honeybees, larvae were capable of cleaving the fusion protein within the digestive tract, preventing Hv1a/GNA from reaching the site of action. A decline in gut proteolytic activity is known to occur as bees develop into foragers (Moritz and Crailsheim, 1987; Free, 1979), reflecting the high protein content of the diet consumed by larval bees, in contrast to the low-protein nectar diet consumed by adults.

It would appear that despite reaching the CNS of adult bees, Hv1a/GNA does not block the CaV channels of *Apis mellifera*. Conversely, another peptide isolated from *H. versuta* venom, ω-ACTX-Hv2a, has been shown to block CaV channels in honeybee brain neurons (Wang et al., 2001). Although this protein has a similar disulphide connection pattern to Hv1a, it has only limited sequence similarity, which could account for differences in toxicity towards bees. Hv1a has insecticidal activity against Lepidoptera such as *Helicoverpa*

armigera (Atkinson et al., 1998), and has been shown to block CaV currents in CNS neurons from *D. melanogaster*, and the cockroach *Periplaneta americana* (Bloomquist, 2003; Fletcher et al., 1997). However, compared with other insecticide targets in the CNS such as acetylcholine receptors and NaV channels, CaV channels are less well conserved between different insect orders (King et al., 2008), thus conferring a certain degree of specificity. Functional expression of recombinant CaV channels from different insect orders would be necessary to fully elucidate the basis of this differential sensitivity to Hv1a.

The data we report here suggests that Hv1a/GNA is a potentially specific biopesticide, as it shows no adverse effects on the honeybee, *Apis mellifera*, an economically important pollinator, while being toxic to agronomically important insect pests. Another possible reason for this lack of toxicity towards honeybee is due to its degradation within the bee, preventing accumulation of the fusion protein even if exposure is repeated. The experiments we have performed exceed current European and American requirements for pesticide safety, and include an olfactory learning assay, which found no adverse effects of Hv1a/GNA on this behavioural parameter. These results show that Hv1a/GNA can be considered safer for honeybees than some currently used pesticides, such as neonicotinoids, although additional safety tests should be performed to confirm its safety against other beneficial hymenoptera, such as bumble bees and parasitoid wasps. This study also highlights the need to extend current guidelines for the safety testing of new pesticides to include behavioural studies, particularly for pollinating insects.

Chapter 5 Effects of Hv1a/GNA on *Varroa destructor* via its host, the Italian honeybee (*Apis mellifera ligustica*)

5.1 Abstract

The mite *Varroa destructor* is considered the major threat to the honeybee (*Apis mellifera*) worldwide, feeding on bee haemolymph and acting as vector for viruses. Control methods generally involve the use of chemical acaricides, resulting in the rapid development of resistance. Alternative/complementary approaches are therefore necessary to manage varroa populations. Previously, it was shown that *Galanthus nivalis* agglutinin (GNA) was transported to honeybee haemolymph following ingestion. In the present study, transport to the circulatory system was maintained even after GNA was fused to the miticidal spider venom peptide ω-ACTX-Hv1a. It was hypothesized that exposure of varroa to the fusion protein, Hv1a/GNA, via its host would cause a detrimental effect on mites. Immuno-assays by western blotting confirmed that Hv1a/GNA was transported to the haemolymph when newly emerged bees were fed *ad libitum* on 1 M sucrose solution containing the fusion protein (0.35 μg/μl). Similarly, GNA was also transported to the haemolymph. Irrespective of whether the bees were dosed orally (0.35 μg/μl) or directly injected with the fusion protein (20 μg/bee), no effects on mite survival were observed ($P>0.05$). Varroa degrades Hv1a/GNA following ingestion, which is a possible reason for its lack of toxicity.

Keywords: Honeybee, *Varroa destructor*, GNA, Hv1a/GNA, fusion protein

5.2 Introduction

Pollinators provide a valuable ecological service in food crops, estimated at more than €150 billion worldwide (Gallai et al., 2009). One of the most important pollinator species, the honeybee *Apis mellifera*, is being threatened by an unexpected bee decline, characterized by a loss of adult workers that invariably leads to the death of colonies (Oldroyd, 2007). In the United States alone, it has caused from ~20 to 56% colony losses (van Engelsdorp et al., 2008). Although no single cause (pesticides parasitism, pathogens, adult physiology) can be held responsible, bees in collapsing colonies tend to have higher pathogen loads and lower levels of miticides than non-affected counterparts (van Engelsdorp et al., 2009).

The ectoparasite mite *Varroa destructor* is currently the most damaging pest to apiculture worldwide, playing a major role in bee decline (Boecking and Genersch, 2008; USDA, 2012). Female mites parasitize adult worker bees and drones, which transport varroa to the hive. The parasite then invades a brood cell just prior to capping and, approximately three days later, oviposits a male egg, followed by up to four female eggs. Mating occurs within the sealed brood cell, until the bee emerges and the adult varroa mite can detach from its host, subsequently parasitizing another bee or invading a brood cell (Rosenkranz et al., 2010). Apart from the damage caused by feeding on the bee's haemolymph, varroa not only suppresses the bee immune system (Yang and Cox-Foster, 2005), but also vectors viruses, such as the Deformed wing virus – DWV (Yue and Genersch, 2005). Typically, hives collapse within one or two years of infestation (Boecking and Genersch, 2008).

Currently, varroa control involves the use of chemical acaricides, such as the alpha-adrenoreceptor agonist amitraz, the organophosphate coumaphos and the pyrethroid τ-fluvalinate. However, constant application of those compounds poses serious threats to apiculture, such as selection of resistant mites (Sammataro et al., 2000; González-Cabrera et al., 2013), contamination of bee products (Wallner, 1995) and impairment of bee learning and memory caused by commonly used miticides such as coumaphos and thymol (Williamson et al., 2013; Bonnafé et al., 2014). Additionally, co-application of the antibiotic oxytetracycline for disease control and the miticides coumaphos/τ-fluvalinate

inhibits bee molecular xenobiotic transporters, increasing adverse effects caused by those compounds and possibly by other pesticides (Hawthorne and Dively, 2011). Consequently, the development of alternative control tools/strategies is important for the sustainability of beekeeping.

The spider venom peptide ω-ACTX-Hv1a (Hv1a) from *Hadronyche versuta* is a calcium channel blocker that has high insecticidal (Fletcher et al., 1997) and miticidal activity (Mukherjee et al., 2006). Previously, it has been linked to the molecular carrier *Galanthus nivalis* agglutinin (GNA), which delivers the peptide to its target sites in the central nervous system following ingestion by insects (Fitches et al., 2012). Additionally, both GNA alone and Hv1a/GNA are able to cross the gut barrier in the honeybee (*A. mellifera*), being detected as intact proteins in the haemolymph from adults after oral exposure (Nakasu et al., 2014). Although the fusion protein presents high toxicity towards insects from different orders (Hemiptera, Coleoptera and Lepidoptera), it had virtually no effects against *A. mellifera*, with LD_{50} >100 μg/bee.

In this work, it was hypothesized that bees feeding on GNA or Hv1a/GNA would internalize proteins and make them available to varroa mites, which would potentially serve as a concept for horizontal delivery of miticidal molecules via host. The aim of the present study was therefore to investigate the potential of this fusion protein to control the varroa mite.

5.3 Material and Methods

5.3.1 Insects

Italian honeybees (*A. mellifera ligustica*) were obtained from untreated brood frames from hives kept on the experimental apiary of Università degli Studi di Napoli Federico II, Naples, Italy. Frames were collected, kept at 34 °C, 80% relative humidity (RH) and, after up to three days, newly emerged bees were collected for the experiments. Varroa mites were collected by scraping a sample drawer placed underneath untreated hives. Mites were taken to the laboratory, separated from colony debris using a fine brush and immediately used as described below.

5.3.2 Bioassays

Newly emerged honeybees were taken from brood frames and two varroa mites were placed on each bee. Parasitized insects were separated in groups of ten inside 500 ml plastic cups with a lateral hole covered with muslin for aeration, one hole on top to add the bees and another small one for adding 2 ml centrifuge tubes used as feeders (Figure 5.1). The bottom of the cup was covered with white tissue paper, secured with a Petri dish, to allow easy counting of unattached mites. Feeders containing one of the three protein treatments (Bovine Serum Albumin – BSA as negative control, GNA or Hv1a/GNA) at 0.35 μg per μl of 1 M sucrose solution were then offered *ad libitum* for the bees. For each treatment, three replicates of 10 bees and 20 mites were carried out (as detailed on Table 5.1). Cups were kept at 34 °C, 80% RH in the dark. Bee mortality and the number of mites at the bottom of the cups (unattached from bees) were assessed regularly for a period of 96 h and analysed using Kaplan-Meyer survival.

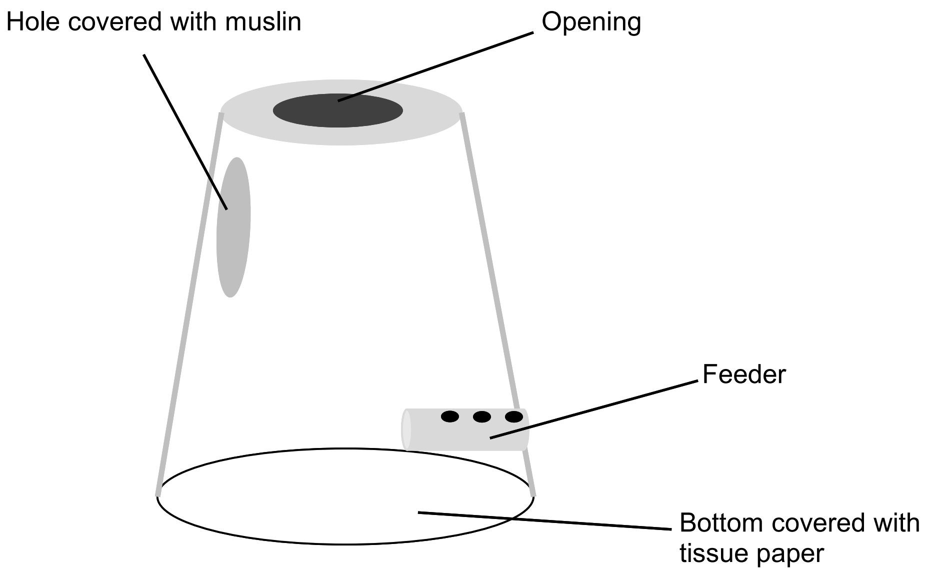

Figure 5.1: Cages used for the bioassays were made with 500 ml plastic cups. The bottom was covered with white paper tissue to allow easy varroa counting. Parasitized bees were added via the opening on the top of the cage. A hole covered with muslin provided aeration while a feeder contained one of the test proteins suspended in sucrose solution.

Table 5.1: Diet regimes used for the bioassays in order to test the effects of GNA and Hv1a/GNA on varroa mites.

Treatment	μg/μl	Replicates	bees/rep	total bees	mites/bee	total mites
BSA	0.35	3	10	30	2	60
GNA	0.35	3	10	30	2	60
Hv1a/GNA	0.35	3	10	30	2	60

5.3.3 Detection of fusion protein in haemolymph and varroa samples

Haemolymph samples from honeybees were collected as described in Chapter 4 (Nakasu et al., 2014). In order to assess the fate of the fusion protein following ingestion by varroa mites, honeybee hosts (30 per treatment) were first injected with 20 μg of BSA, GNA or Hv1a/GNA. Mites (two per bee) were then placed on injected bees, allowed to feed for 48 h and collected. Proteins were extracted from whole mites using Tissue Extraction Reagent I (Invitrogen®; 50 mM Tris, pH 7.4, 250 mM NaCl, 5 mM EDTA, 2 mM Na_3VO_4, 1mM NaF, 20 mM $Na_4P_2O_7$, 0.02% NaN_3) with Protease Inhibitor Cocktail diluted to 1x (Sigma-Aldrich; 2 mM AEBSF, 0.3 μM Aprotinin, 130 μM Bestatin, 1 mM EDTA, 14 μM E-64, 1 μM Leupeptin), at the rate of 10 μl of buffer per mite. After macerating varroa mites on ice, samples were centrifuged for 2 min at 10000 *g*, the supernatant collected and protein content quantified using the method of Bradford (1976). Thirty micrograms of mite or honeybee samples were ran on a 4-12% SDS-PAGE gel on an XCell SureLock™ (life technologies™) system. After electrophoresis, proteins were transferred to PVDF membranes by using iBlot (Invitrogen®). Membrane blocking and GNA and fusion protein detection were carried out as previously described (Fitches et al., 2012), using anti-GNA as primary antibodies at 1:5000 dilutions and enhanced luminol-based chemiluminescent (ECL) detection.

5.4 Results

5.4.1 Effects of recombinant proteins on varroa mites

In order to test whether GNA or Hv1a/GNA would affect the varroa mites, a simple bioassay method was set. Honeybees parasitized with varroa (two mites per bee) were given sucrose solution containing one of three different proteins,

BSA (negative control), GNA or Hv1a/GNA. Consumption of solutions containing proteins was similar in all groups and did not differ significantly (data not shown; ANOVA, P=0.474). Honeybee survival was not affected by treatment (K-M Survival, χ^2 = 1.200, 2 d.f., P=0.549, Figure 5.2).

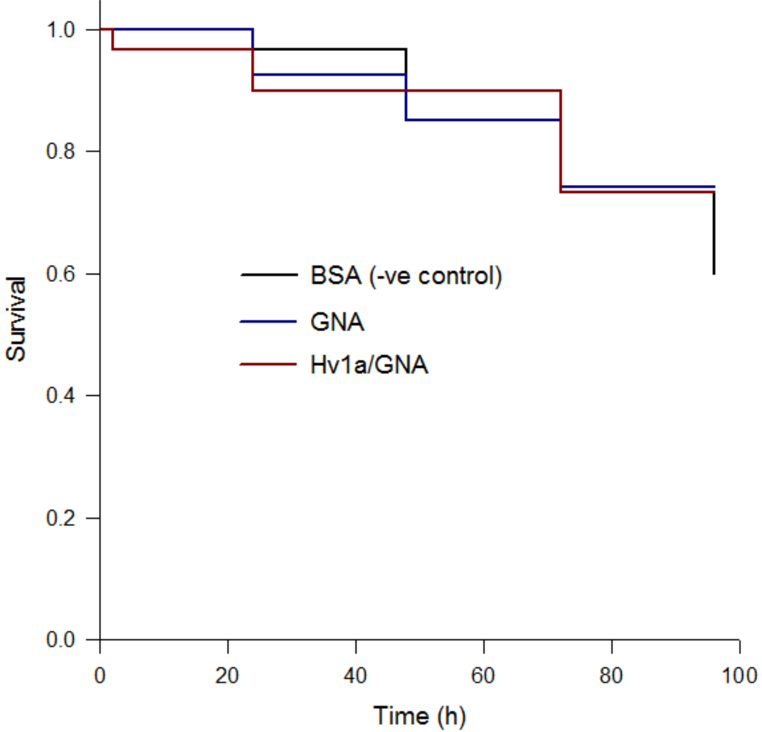

Figure 5.2: Honeybee survival was not affected by treatment. Parasitized insects were constantly fed with sucrose solution containing BSA (negative control), GNA or Hv1a/GNA for up to 96 h (n=30 insects/treatment).

Similarly, mite survival was unaffected by treatment (K-M Survival, χ^2 = 1.748, 2 d.f., P=0.417, n= 60 mites per treatment, Figure 5.3), with most of the mites detaching from bees after only 2 h from the beginning of the experiments (60% in control, 65% in GNA and 76% in Hv1a/GNA treatment). Thereafter, there was little change in survival rates for the remainder of the bioassay.

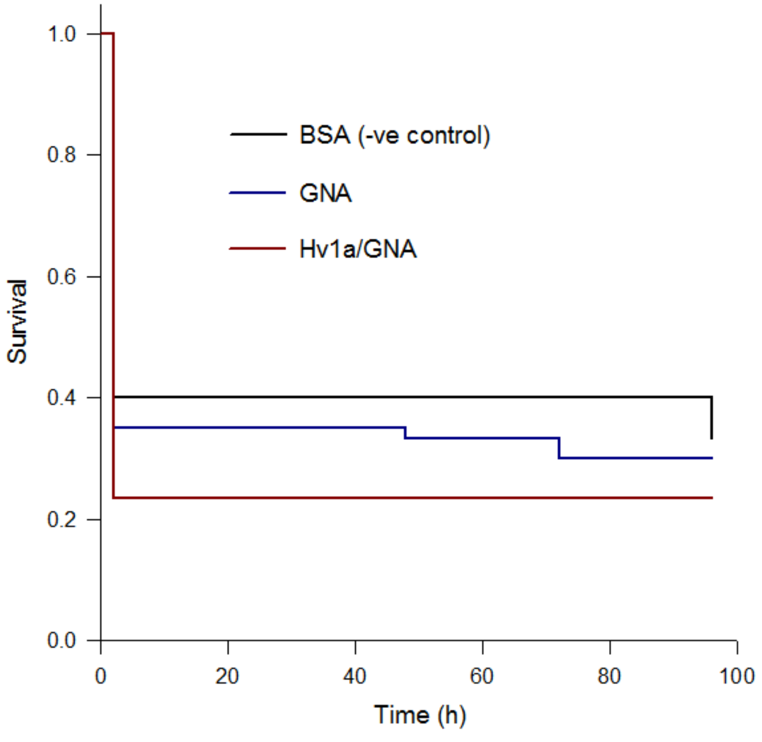

Figure 5.3: Survival of varroa mites feeding on bees exposed to different proteins. Most of the mortality occurred soon after the beginning of the assays.

5.4.2 Fate of Hv1a/GNA following ingestion by varroa mites

Both GNA and Hv1a/GNA were detected as intact proteins in honeybee haemolymph following oral exposure (Figure 5.4). However, it was not possible to detect GNA or the fusion protein in mites feeding on bees orally exposed to the test proteins. Therefore, in order to facilitate immunological detection and evaluate the fate of GNA and Hv1a/GNA after ingestion by the mite, honeybees were first injected with high doses of GNA or the fusion protein (20 μg/bee). Mites were then introduced and allowed to feed on the bees for up to 48 h, when they were collected and subjected to western blot analysis. GNA remained intact after ingested by varroa mites. However, Hv1a/GNA did not retain its integrity, and no signs of the whole fusion protein were detected (Figure 5.4).

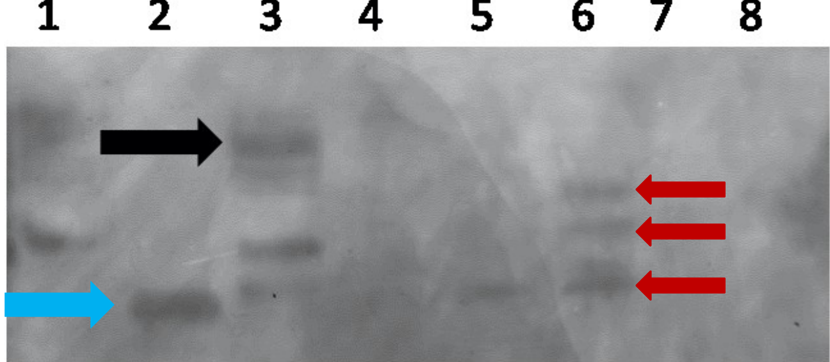

Figure 5.4: The fusion protein is digested by varroa mites, while GNA remains intact. Loading as follows: 1) Hv1a/GNA (+ve control); 2) Haemolypmh from bees fed on GNA; 3) Haemolypmh from bees fed on Hv1a/GNA; 4) varroa collected from bees injected with BSA (-ve control); 5 and 6) varroa collected from bees injected with GNA and Hv1a/GNA, respectively; 7 and 8) varroa collected from bees feeding on GNA and Hv1a/GNA, respectively. Black arrow shows intact fusion protein, blue arrow indicates GNA, and red arrows indicate digestion products of Hv1a/GNA after ingestion by varroa mites.

5.5 Discussion

In this study, tri-trophic effects of the fusion protein Hv1a/GNA on varroa mites were evaluated. Firstly, honeybees were fed with sucrose solution containing fusion protein at levels known to be innocuous to bees, but toxic to target species (0.35 µg/µl). BSA and GNA were used as negative controls. Although bee mortality was to some extent expected on GNA treatment (Nakasu et al., 2014), its effects on mites were unpredictable, and the hypothesis that the lectin portion of the fusion protein might exert more toxic effects on varroa than the neurotoxic peptide Hv1a could not be excluded. In fact, plants genetically engineered to express GNA have shown detrimental effects on at least two different mite species. For example, spider mites (*Tetranychus cinnabarinus*) feeding on papaya plants expressing GNA lay fewer eggs than control lines (McCafferty et al., 2008), and GNA-expressing potato plants have a deterrent effect on egg laying behaviour of *Tetranychus urticae* mites (Rovenská and Zemek, 2006). However, GNA did not influence bee or varroa mortality at the levels tested, although other non-measured biological parameters, such as fecundity, might have been affected. Similarly, there was no link between varroa mortality and exposure to Hv1a/GNA, as no differences between the control BSA, GNA and fusion protein treatments were detected. A previous study by

Mukherjee et al. (2006) has shown that the peptide Hv1a is orally toxic at picomolar (LD_{50}=716±23 pmol/g) levels to the lone star tick (*Amblyomma americanum*), which belongs to the same subclass as varroa mites (Acari). For that reason, it was expected that the Hv1a/GNA would be able to block varroa calcium channels, causing a toxic effect.

In order to test if the lack of observable effects was due to mites not being exposed to test proteins, haemolymph was collected from honeybees feeding on the treatments and subjected to immunoassays. Even though it was demonstrated that they internalized GNA and Hv1/GNA following feeding, these proteins were not detected in varroa mites parasitizing those bees. It is likely that mites ingested the protein, but the immunoassay was not sensitive enough to detect them. To circumvent this limitation and examine the fate of GNA and Hv1a/GNA after ingestion by mites, bee hosts were injected with 20 µg of either protein. Varroa mites were allowed to feed on those bees for 48 h, after which time they were collected and used in western blots. GNA, as demonstrated before in insects (e.g., Fitches and Gatehouse, 1998; Hogervorst et al., 2006), has remained intact after ingestion, whereas Hv1a/GNA was proteolytically cleaved in three visible bands. Cleavage of the fusion protein by varroa proteases probably prevented GNA from carrying the Hv1a peptide to the haemolymph, thus preventing it from reaching its targets in the CNS. Similarly, Fitches et al. (2004b) have fused a chitinase, toxic to *Lacanobia oleracea*, to the GNA carrier. Whereas the fusion between the chitinase and lectin induced mortality in *L. oleracea* via injection, it was ineffective via an oral route, as insects were able to digest the fusion protein, preventing GNA from carrying the chitinase to the haemolymph. However, even if the fusion protein is cleaved, preventing GNA from acting as a carrier molecule, this does not explain why the atracotoxin itself was not toxic to varroa, but toxic to a closely related mite even in the absence of a carrier molecule. It is possible that varroa mites can also cleave the Hv1a toxin, preventing it from acting as a calcium channel blocker. Directly injecting mites with Hv1a/GNA or Hv1a alone might elucidate as to whether the spider venom peptide can be toxic or not.

The bioassay had several limitations, such as high mite mortality during the first hours, short observation periods (>5 days) due to high bee mortality in all

treatments (including negative control) and incapability of evaluating biological parameters other than mite mortality. Nevertheless, it was possible to measure the effects of GNA and Hv1a/GNA on mites and test the concept of using GNA as a carrier of miticidal molecules via hosts. Bees consumed sucrose solutions containing GNA or Hv1a/GNA at similar rates to the controls, indicating that they do not avoid the lectin. Furthermore, GNA is internalized by bees and detected in their haemolymph (Nakasu et al., 2014), which in turn is ingested by parasitic mites. A similar approach using a tri-trophic system was reported by Garbian et al. (2012), in which bees were fed dsRNA targeting housekeeping genes in varroa, reporting up to 60% decrease in mite populations in small colony assays. In the present study, it was expected that, after ingestion by the parasite, Hv1a/GNA would cross the mite’s gut, reach the haemolymph and be transported to its targets in the CNS. In the future, it might be more feasible to fuse GNA to miticidal peptides that target proteins from the digestive system rather than the CNS. Such an approach might circumvent the issue of having to cross the mite gut, and possibly sustaining a toxic effect despite of protease activity.

Chapter 6 Silencing of Voltage-Gated Calcium channels in aphids and in the beetle *Tribolium castaneum*

6.1 Abstract

Insect voltage-gated calcium channels are potential targets for novel pest control molecules. For example, the spider venom peptide ω-ACTX-Hv1a (Hv1a) from *Hadronyche versuta*, one of the most potent insecticidal peptides discovered to date, is an insect calcium channel blocker. In *Drosophila melanogaster*, the Hv1a target is likely to be coded by *Dmca1D*, a calcium channel gene. In this work, RNAi techniques were used against the beetle *Tribolium castaneum* and the aphid *Myzus persicae*, two insect species susceptible to Hv1a, in order to silence *Dmca1D* homologues. Species-specific double-stranded RNA (dsRNA) against *Dmca1D* homologues and against a positive control, the proton pump V-ATPase, were used. In *M. persicae*, no effects on gene expression were detected when insects were fed or injected with dsRNA targeted to any of the selected genes. Conversely, *T. castaneum* larvae presented ca. 6-fold down-regulation of the V-ATPase, but not of the calcium channel gene, when orally exposed to dsRNAs. On the other hand, injection of dsRNAs produced 2- and 3-fold down-regulation of V-ATPase and calcium channel gene, respectively. However, this effect did not induce mortality or any other visible phenotypical changes. These results indicate that *Dmca1D* homologues are not viable targets for inducing mortality in insects via RNAi, even when the species is amenable to the technique.

Keywords: aphids, calcium channels, *Tribolium castaneum*, RNAi, dsRNA, Dmca1D

6.2 Introduction

RNA interference (RNAi) by double-stranded RNA (dsRNA), described in animals for the first time by Fire et al. (1998), is nowadays a powerful tool in functional genetics. The mechanism is initiated by processing of long dsRNA molecules into ~21 nucleotides small interfering RNAs (siRNAs) through Dicer enzymes (Bernstein et al., 2001). Resulting siRNAs are then taken up by the RNA-induced silencing complex (RISC), which recognizes and degrades target mRNA, leading to inhibition of translation (Hanon, 2002). The potential of RNAi technology to suppress essential genes for insect survival has opened the possibility to generate pest-resistant transgenic plants via expression of insect-specific dsRNA (Baum et al., 2007).

Aphids are major crop pests, directly damaging plants by feeding on phloem sap, or indirectly, by serving as vectors for viruses. The deployment of transgenic plants expressing dsRNA could be effective at controlling aphids (Price and Gatehouse, 2008), and RNAi components have been characterized in at least two representative species, *Acyrthosiphon pisum* and *Aphis glycines* (Jaubert-Possamai et al., 2010; Bansal and Michel, 2013). In fact, RNAi effects have been reported in *A. pisum* (e.g., Shakesby et al., 2009, Whyard et al., 2009), although expression of dsRNA in transgenic plants has only resulted in low to moderate levels of aphid control (Pitino et al., 2011, Xu et al., 2013). On the other hand, the beetle *Tribolium castaneum* presents a strong and well-characterized RNAi response (Tomoyasu et al., 2008), serving as a model organism for reverse genetics experiments. Furthermore, as opposed to aphids in general, detailed information for designing experiments and effectively testing gene knockdown in this species are available in the literature (e.g., Posnien et al., 2009; Miller et al., 2012).

Several different synthetic and natural insecticidal compounds have ion channels as their primary targets (Bloomquist, 1996). For example, the venom peptide ω-ACTX-Hv1a (Hv1a) from the spider *Hadronyche versuta* is a potent calcium channel blocker (Fletcher et al., 1997), presenting oral toxicity against insects when fused to the molecular carrier *Galanthus nivalis* agglutinin (GNA) (Fitches et al., 2012). The high-voltage-activated calcium channel α_1 subunit Dmca1D from *Drosophila melanogaster* is likely to be the molecular target for

Hv1a, as the phenotype of flies expressing this peptide resembles that of those carrying a hypomorphic allele for this gene (Tedford et al., 2007). Furthermore, Dmca1D plays a vital, non-redundant role in fly larvae and adults (Eberl et al., 1998), being then sensible to assume that silencing of this gene would induce mortality in insects.

In this study, RNAi techniques were used in order to silence *Dmca1D* homologues in two insect species susceptible to Hv1a, the coleopteran *T. castaneum* (Back, 2011) and the aphid *Myzus persicae* (Pal et al., 2013), in an attempt to induce insect mortality. If effective silencing of this putative Hv1a receptor is achieved, the usage of dsRNA might act synergistically with administration of Hv1a/GNA fusion protein to susceptible insects.

6.3 Material and Methods

6.3.1 Insects

M. persicae were kept on Chinese cabbage plants (*Brassica rapa*) at 25 °C, 16:8 h (L:D). Before the bioassays, adults were transferred from plants to Petri dishes containing artificial diet (Febvay et al., 1988) in Parafilm sachets as described by Down et al. (1996), and allowed to reproduce for 24 h. Neonate nymphs were used for feeding assays, whereas 5-days old aphids were used for injection assays with dsRNA against V-ATPase E and the calcium channel gene.

T. castaneum were reared on whole flour containing 5% brewer's yeast, at 30 °C, 16:8 h (L:D). For the feeding assays, flour was sieved in order to separate the eggs from larvae and adults. Newly ecloded larvae (less than two weeks old) were used for those assays, whereas late instars were used for injection bioassays.

6.3.2 RNA extraction and complementary DNA (cDNA) synthesis

For RNA extractions, insects were collected, freeze-killed in liquid nitrogen and stored at -80 °C until further use. Total RNA was extracted from whole insects

using PureLink® RNA kit (Ambion®), following the manufacturer's instructions. DNA contamination was removed by using on-column PureLink® DNAse Set (invitrogen™). RNA was quantified on a NanoDrop spectrophotometer (model ND-1000, Thermo Scientific) and complementary DNA (cDNA) synthesized with poli-T primer oligo d(T)18V (Sigma) using SuperScript® II Reverse Transcriptase (invitrogen™).

6.3.3 Templates for Double-stranded RNA (dsRNA)

Except when targeting V-type ATPase E from aphids, all dsRNAs were produced via PCR products containing T7 promoters (Table 6.1). A sequence from a kanamycin-resistance gene from the plasmid pSC-A-amp/kan vector (Agilent Technologies) was amplified using a pair of primers containing opposing T7 promoters. The dsRNA derived from this sequence was used as a negative control in all experiments. Mixed instars of *T. castaneum* larvae were used to synthesize the cDNA that served as template to amplify sequences coding for a calcium channel and for a V-type ATPase dsRNAs. cDNA from a mixed population of *M. persicae* was used as template to amplify two different regions of a calcium channel gene. PCR conditions were as follows: 94 °C for 3 min, followed by 30 cycles of 45 s 94 °C, 30 s at 55 °C and 45 s at 72 °C, and a final extension step of 7 min at 72 °C. Dr. Michelle Powell (FERA – Sand Hutton, York) kindly provided a construct containing *M. persicae* V-type ATPase sequence in a pLITMUS 28i vector (New England BioLabs®). Plasmids were extracted from cells harbouring the construct and digested with either *Xho*I (10 min at 37 °C in 1x Tango buffer, Thermo Scientific) or *Xba*I (1 h at 37 °C in 1x Tango buffer). Digested plasmids and PCR products were precipitated with 3 M NaOAc (Sodium acetate, 1/10 of reaction volume) plus three volumes of ethanol and re-suspended in nuclease-free water prior to RNA synthesis.

Table 6.1: Primer pairs used for RNA syntheses prior to dsRNA production. T7 promoter is shown in **bold**. Insect cDNAs were used as templates for RNAi against *M. persicae* (*M.p.*) and *T. castaneum* (*T.c.*).

Gene	Access code	Template	Primer sequence	size	Reference
voltage-gated calcium channel alpha 1 subunit	LOC659557 (*T.c.*)	*T.c.* cDNA (larva)	TCGTCGAGTGGAAACCTTTT	369bp	This work
			TAATACGACTCACTATAGGGGACTGGGAACACCAGAAACG		
			TAATACGACTCACTATAGGGTCGTCGAGTGGAAACCTTTT		
			GACTGGGAACACCAGAAACG		
V-ATPase E subunit	XM_965528 (*T.c.*)	*T.c.* cDNA (larva)	AGGGACGCCACTGGTAAAGACGTT	185bp	Whyard et al., 2009
			TAATACGACTCACTATAGGGCCAAACAAGGCCGTACGAATTTC		
			TAATACGACTCACTATAGGGAGGGACGCCACTGGTAAAGACGTT		
			CCAAACAAGGCCGTACGAATTTC		
voltage-dependent calcium channel type A subunit alpha-1-like	EC388785 (*M.p.*)	*M.p.* cDNA	ACAGCGGCTCATATCTTCGT	536bp	This work
			TAATACGACTCACTATAGGGGATGGTAGTCCACCCTTCCA		
voltage-dependent calcium channel type A subunit alpha-1-like	EC388785 (*M.p.*)	*M.p.* cDNA	AAGCCGTACAACGCGTACTT	265bp	This work
			TAATACGACTCACTATAGGGCGCTAAGCACACCGAGAACT		
			TAATACGACTCACTATAGGGAAGCCGTACAACGCGTACTT		
			CGCTAAGCACACCGAGAACT		
			TAATACGACTCACTATAGGGACAGCGGCTCATATCTTCGT		
			GATGGTAGTCCACCCTTCCA		
V-ATPase catalytic subunit A-like	similar to XP_001950890 (*A. pisum*)	*M.p.* cDNA	GGCCCTGGCATTTTGGG	279bp	Powell, unpubl.
			GTTTCCCGGTGGAGCTTGG		
neomycin/kanamycin resistance	JN638547 (synthetic construct)	pSC-A-amp/kan	**TAATACGACTCACTATAGGG**CATTCGCCGCCAAGTTCTTC	468bp	This work
			TAATACGACTCACTATAGGGTGCTCGACGTTGTCACTGAA		

6.3.4 dsRNA production

Single-stranded RNAs (ssRNA) were synthesized using MEGAscript T7 kit (Ambion®) following the manufacturer's instructions. For each strand, 1 μg of digested product from pLITMUS 28i vector or PCR products were used as templates. Transcription was carried out for 16 h at 37 °C. Complementary RNAs were then combined, incubated at 75 °C for 5 min and allowed to cool down to room temperature. Resulting dsRNAs were bound to filter cartridges provided in the kit and ssRNA and template DNA were removed by digestion. Eluted dsRNA was quantified on a spectrophotometer at A_{260}.

6.3.5 Feeding assays

Double-stranded RNAs were mixed into aphid artificial diet at a final concentration of 100 ng/μl. Neonate *M. persicae* were placed in small Petri dishes (35 x 10 mm) in groups of ten per dish. Diets containing dsRNA against Km (negative control), Ca1 region 1 or 2, or V-ATPase genes were then offered to aphids in Parafilm sachets, as described above (section 6.3.1), and changed every two days in order to avoid contamination or oxidation of the diet. For *T. castaneum* assays, dsRNAs were delivered via flour disks prepared as described by Xie et al. (1996). Briefly, 5000 ng of dsRNA (Km, Ca1 or V-ATPase) in a volume of 500 μl were added to 100 mg of sieved whole-wheat flour supplemented with 5% brewer's yeast. Ten microliters of the suspensions were directly poured into flat bottom wells of a 96-wells microtiter plate and allowed to dry out at room temperature for 16 h. After this period, one small larva (less than two weeks-old) was added to each well (Table 6.2).

Table 6.2: Oral delivery of dsRNA to *M. persicae* (*M.p.*) and *T. castaneum* (*T.c.*).

Bioassay #	**Insect**	**Treatment**	**insect age**	**Number of insects**	**dsRNA concentration**
1	*M.p.*	Km	neonate	30	100 ng/µl
	M.p.	Ca1 region 1	neonate	30	100 ng/µl
	M.p.	Ca1 region 2	neonate	30	100 ng/µl
2	*M.p.*	Km	neonate	30	100 ng/µl
	M.p.	V-ATPase	neonate	30	100 ng/µl
3	*T.c.*	Km	<2 weeks	26	50 ng/mg
	T.c.	Ca1	<2 weeks	25	50 ng/mg
	T.c.	V-ATPase	<2 weeks	25	50 ng/mg

For aphid bioassays, insect mortalities were compared using Kaplan-Meyer survival analysis, and number of nymphs produced/adult recorded throughout the bioassay. For *T. castaneum*, mortalities were analysed using Abbott's corrected mortality formula (1925).

6.3.6 Injection assays

For the injection bioassays, glass capillaries (3.5'' Drummond #3-000-203-G/X) were pulled with a needle puller (INTRACEL P-1000, Sutter Instruments), programmed as follows: Pull 70, Velocity 80, Delay 150, Press 500. Tips were broken with a forceps in order to create a sharp end. The needles were then filled with mineral oil and mounted on a Nanoject II™ injector (Drummond Scientific Company). The dsRNAs were loaded into the needles and injected as described on Table 6.3.

Table 6.3: Injection bioassays with *M. persicae* (*M.p.*) and *T. castaneum* (*T.c.*).

Bioassay	Insect	Treatment	Insect age	Number of insects	[dsRNA]	Volume injected
1	*M.p.*	Km	5 days	20	600 ng/µl	9.2 nl
	M.p.	Ca1 region 1	5 days	20	600 ng/µl	9.2 nl
	M.p.	Ca1 region 2	5 days	20	600 ng/µl	9.2 nl
2	*M.p.*	Km	5 days	70	1200 ng/µl	13.8 nl
	M.p.	Ca1 region 2	5 days	70	1200 ng/µl	13.8 nl
3	*M.p.*	Km	5 days	30	1000 ng/µl	13.8 nl
	M.p.	V-ATPase	5 days	30	1000 ng/µl	13.8 nl
4	*T.c.*	Km	late stage	27	1000 ng/µl	138 nl
	T.c.	Ca1	late stage	62	1000 ng/µl	138 nl
	T.c.	V-ATPase	late stage	23	1000 ng/µl	138 nl
5	*T.c.*	Km	late stage	17	1000 ng/µl	69 nl
	T.c.	Ca1	late stage	17	1000 ng/µl	69 nl
	T.c.	V-ATPase	late stage	19	1000 ng/µl	69 nl

6.3.7 Quantitative real-time PCR (qPCR)

Gene expression was evaluated via qPCR using SYBR® GreenER™ (invitrogen™), following the manufacturer's instructions. The regions to which primer pairs for qPCR (Table 6.4) were designed diverged from those targeted by dsRNA. Quantitative real-time PCR conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 15 sec at 95 °C and 60 sec at 60 °C. Fluorescence was recorded by a Chromo4™ detector (Bio-Rad).

Table 6.4: Primers used for qPCR analyses in *M. persicae* (*M.p.*) and *T. castaneum* (*T.c.*).

Gene	Access code	Insect	Primer sequence	size	reference
voltage-gated Ca^{2+} channel α1	LOC659557 (*T.c.)*	*T.c.*	AGCATCTGAATGCAACGGGA AGGGTCAACATGGCCTTAGC	129bp	This work
Ribosomal protein S6 (RpS6)	NM_001172390 (*T.c.)*	*T.c.*	GAAGCAGGGTGTTCTCACGA GTTTCCTTTCACCGTCACGC	92bp	This work
V-ATPase E	XM_965528 (*T.c.*)	*T.c.*	GAGAACAATATAGTGGTGAGAGTC TATTTCGTCGCAACAACTGG	80bp	Whyard et al., 2009
voltage-dependent Ca^{2+} channel type A α1-like	XM_001943894.2 (*A. pisum*)	*M.p.*	GTTAGCCGCCGAAGATCCGA TCTGAGGTACGAACCGGGAT	151bp	This work
V-ATPase subunit A-like	Similar to XP_001950890 (*A. pisum*)	*M.p.*	CGTTCACTATGTTGCAAGTTTGG AAGTGGATAGTTGGCAGGCAAT	288bp	Powell, unpub.
Ribosomal protein L27 (Rpl27)	NM_001126221.2 (*A. pisum*)	*M.p.*	CCGAAAAGCTGTCATAATGAAGAC GGTGAAACCTTGTCTACTGTTACATCTTG	231bp	Mutti et al., 2006

6.4 Results

6.4.1 Oral bioassays targeting a calcium channel gene in aphids

When compared to the negative control, aphid survival was unaffected on insects feeding on 100 ng/μl dsRNA targeted against any of two regions of the calcium channel gene (K-M Survival, 0.190, 2 d.f., P=0.909, Figure 6.1). Additionally, no differences in the number of nymphs produced/adult after 10 days were recorded (ANOVA, F=1.008, P=0.419). As expected from these results, neither of the constructs induced changes to calcium channel expression following qPCR analysis (F=0.426, P=0.671, Figure 6.2).

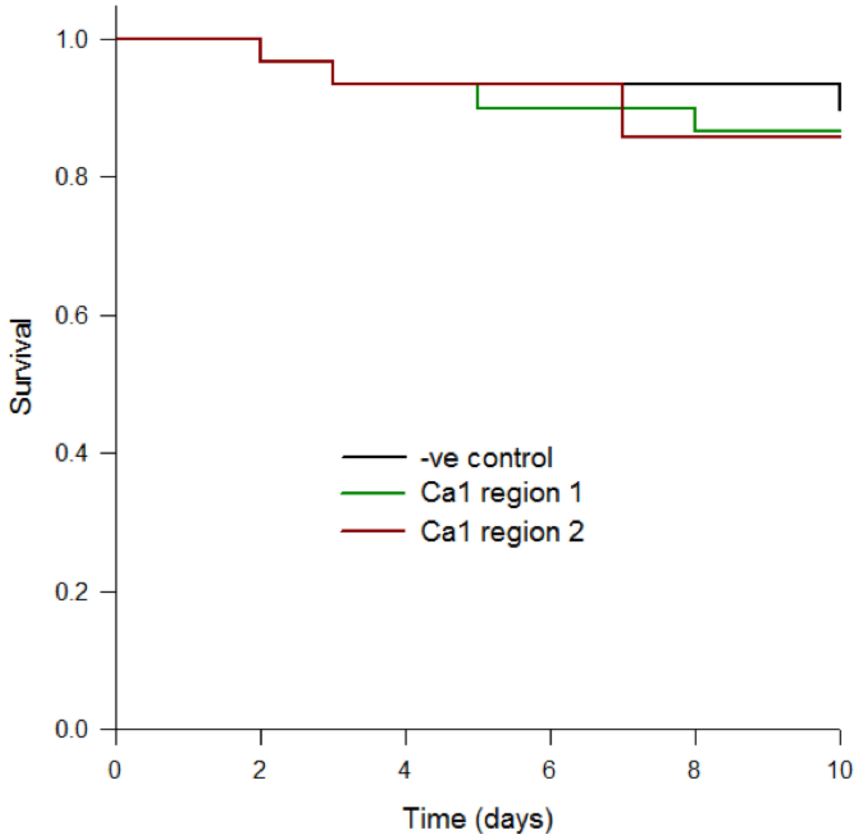

Figure 6.1: Aphid survival after feeding on artificial diets containing dsRNA against two different regions of a calcium channel or a kanamycin-resistance gene (negative control). No significant differences were found between treatments (K-M Survival P=0.909, n= 30 aphids/treatment).

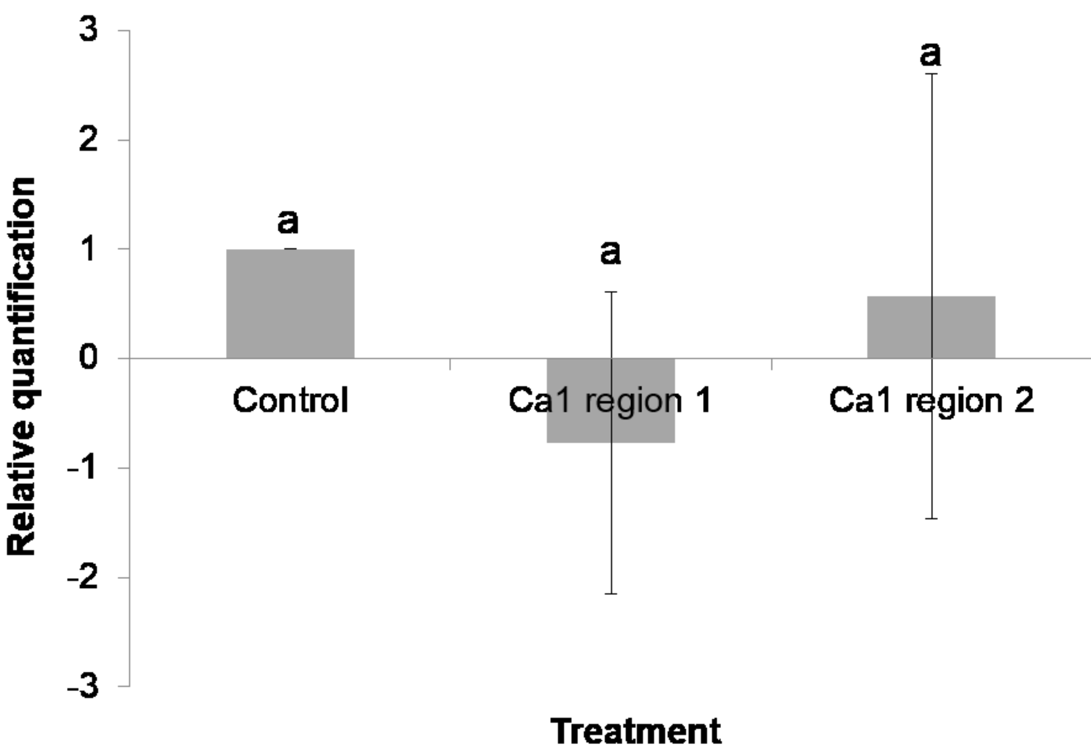

Figure 6.2: Feeding on diets containing dsRNA did not induce gene down regulation, according to qPCR analyses. Means±SEM are shown; same letters indicate no significant differences between treatments (P<0.05).

6.4.2 Injection bioassays targeting a calcium channel gene on aphids

An initial screening was carried out in order to evaluate which of the two calcium channel regions targeted by dsRNA would be more effective following injection into *M. persicae*. Although no significant differences on aphid survival were found between treatments (K-M Survival, χ^2= 3.238, P=0.198, Figure 6.3), the dsRNA targeting Ca1 region 2 has caused higher mortality than dsRNA against region 1 or the negative control. For that reason, dsRNA targeting the Ca1 region 2 was selected for further analysis.

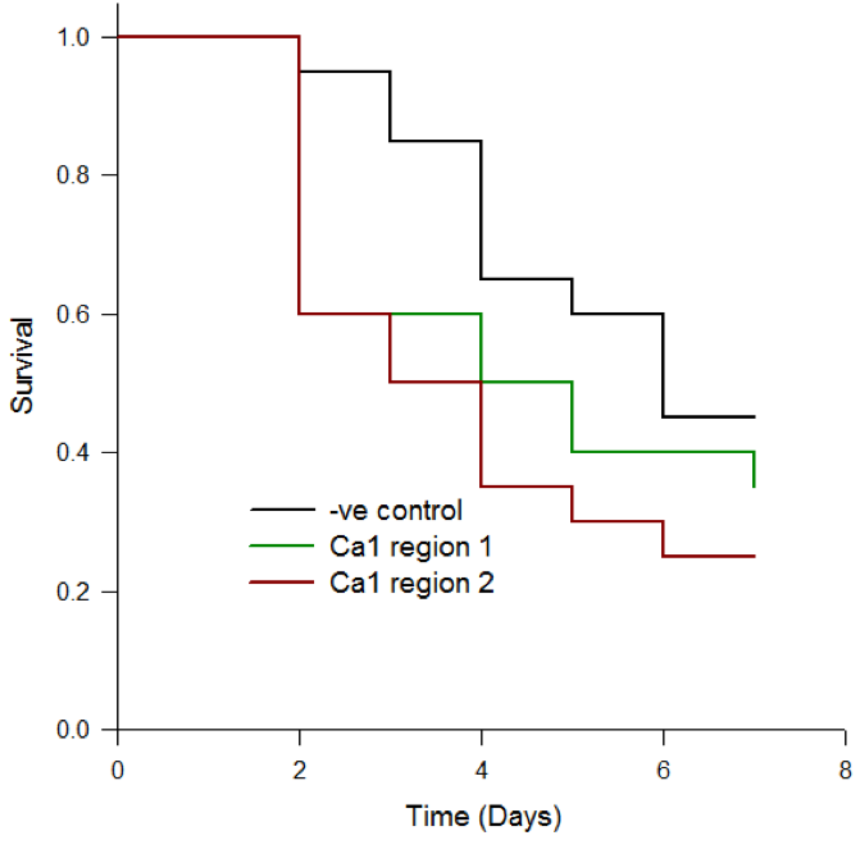

Figure 6.3: Injection of of dsRNA against calcium channel genes did not induce a significant mortality on aphids (K-M Survival P=0.198, n=20 aphids/treatment).

On a second bioassay, the concentration of dsRNA was increased from 600 ng/μl to 1.2 μg/μl, in order to induce a strong RNAi response. Insects were injected with either a negative control (dsRNA Km) or dsRNA targeting Ca1 region 2. Aphid survival, however, was not affected by treatment (K-M Survival, χ^2= 2.088, P=0.148, Figure 6.4), nor was nymph production/treatment (Figure 6.5, P=0.224).

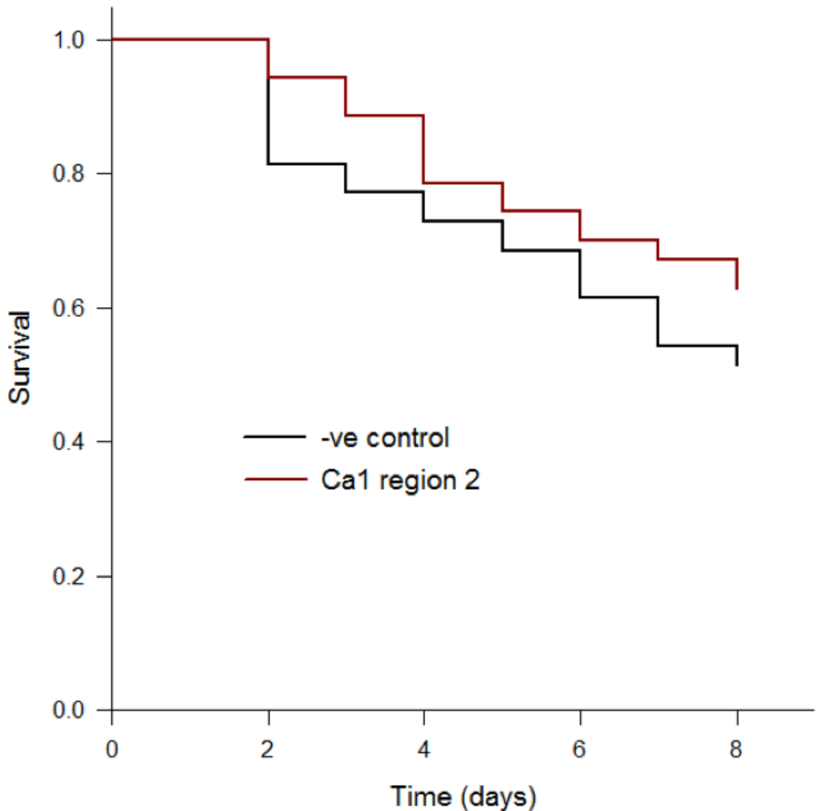

Figure 6.4: Injection of highly concentrated dsRNA against a calcium channel gene did not increase aphid mortality when compared against a negative control (K-M Survival, P=0.148).

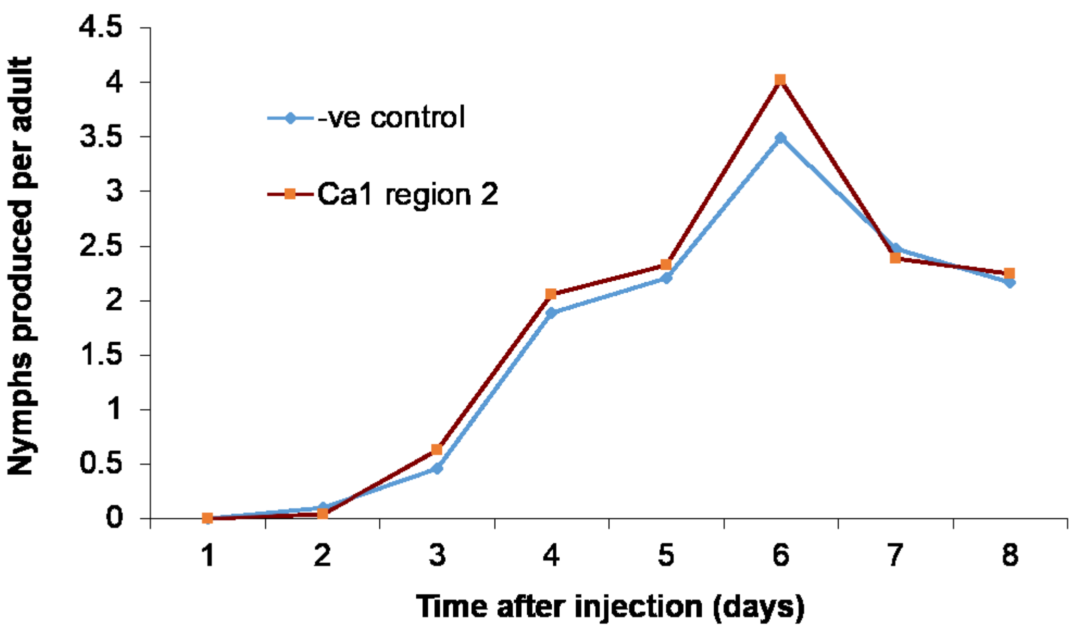

Figure 6.5: Aphid fecundity was unnafected by injection of dsRNA against a calcium channel gene (P=0.224).

Analyses of calcium channel gene expression were carried out with aphids collected one and three days after injection (DAI) and with the offspring nymphs from injected aphids. Although no biological replicates have been carried out, it is possible to conclude that injection of dsRNA against Ca1 gene did not cause its down-regulation (Figure 6.6).

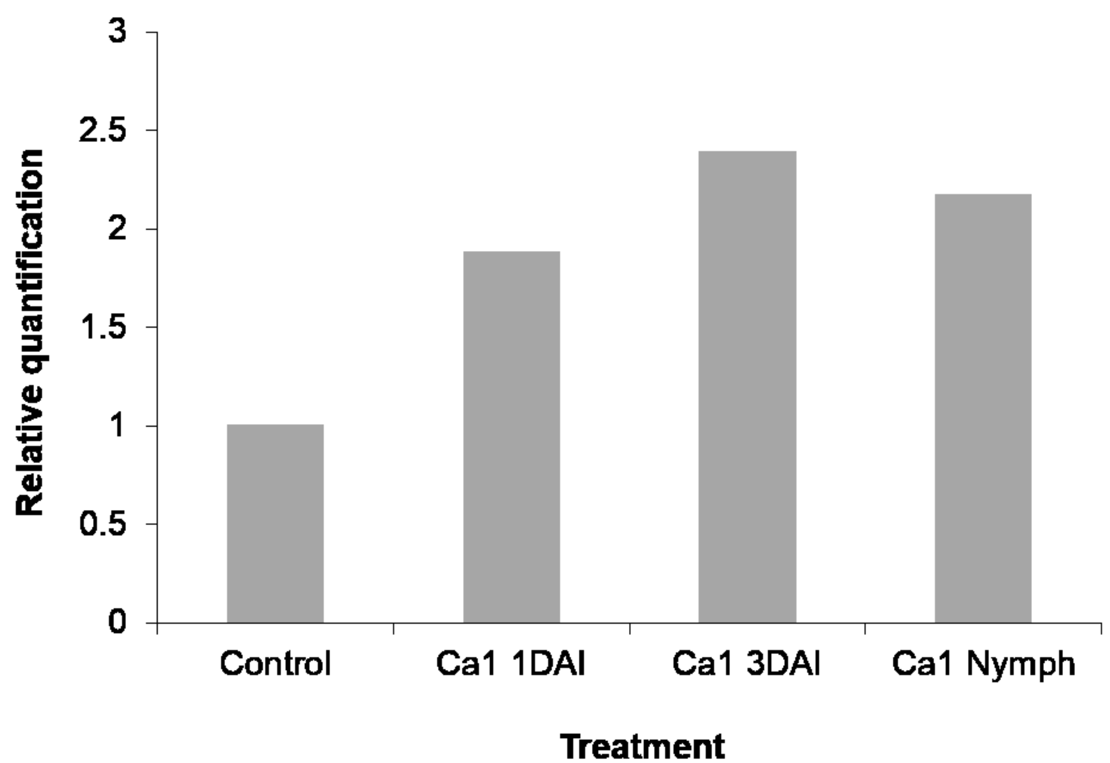

Figure 6.6: Injection of highly concentrated dsRNA against Ca1 region 2 did not induce down regulation in aphids one or three days after injection (DAI) or in their offspring (Ca1 Nymph).

6.4.3 Bioassays targeting V-ATPase E gene in aphids

Aphids feeding on diets containing 100 ng/µl dsRNA against a V-ATPase E gene did not induce mortality in neonate aphids when compared to a control (K-M Survival, χ^2=2.160, P=0.142, Figure 6.7).

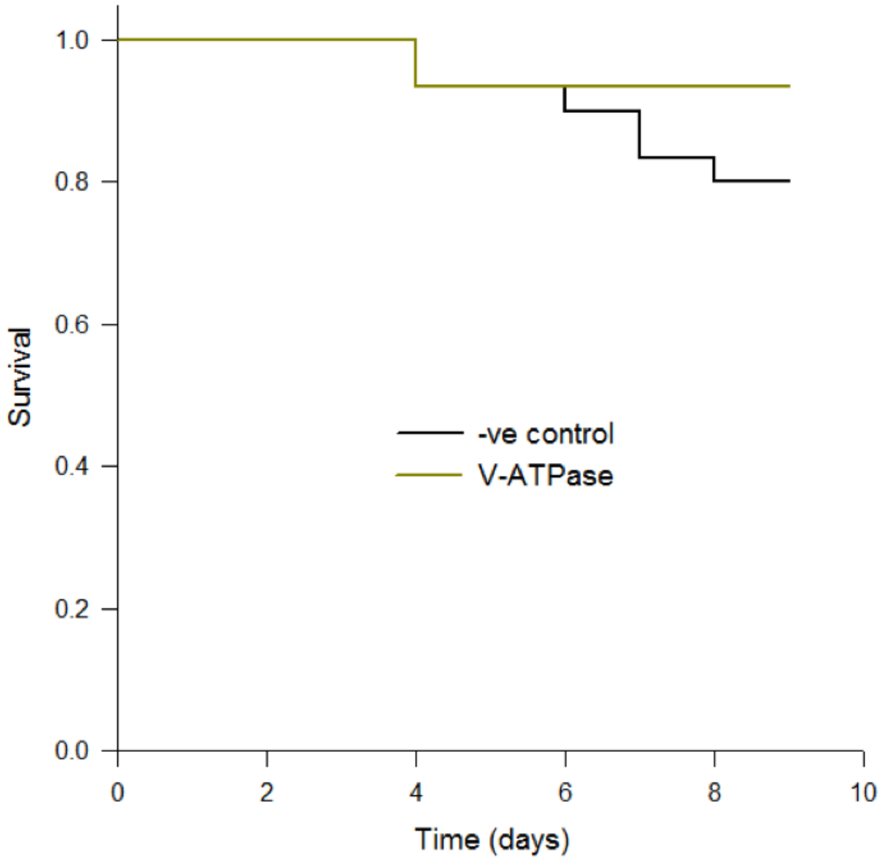

Figure 6.7: Aphid survival was unaffected when insects were exposed to artificial diet containing 100 ng/μl dsRNA against a V-ATPase E (K-M Survival, P=0.142).

Insect feeding or injection of the same dsRNA into *M. persicae* did not cause gene down-regulation following a period of 48 h (Figure 6.8).
Therefore, dsRNA targeted to Ca1 regions 1 or 2, or targeted to V-ATPase E, neither caused a significant phenotypic effect (measured in terms of survival), nor caused down-regulation of gene expression of the targeted genes in *M. persicae*.

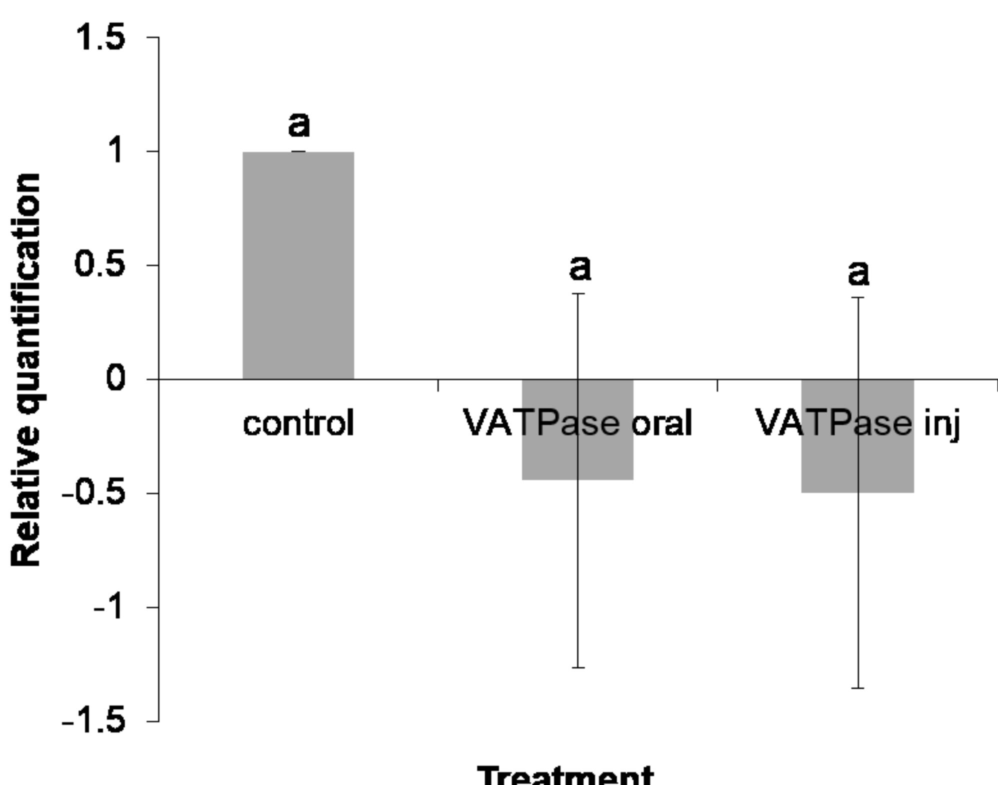

Figure 6.8: Aphid exposure to dsRNA against a V-ATPase E via oral feeding or injection did not cause gene down regulation. Means±SEM are shown; same letters indicate no significant differences between treatments.

6.4.4 Feeding of dsRNA to **T. castaneum**

Oral delivery of dsRNA targeted against a V-ATPase or Calcium channel genes to *T. castaneum* at 50 μg/g of diet did not cause significant mortality when compared to a control (K-M Survival, χ^2=2.502, P= 0.286, Figure 6.9). Furthermore, corrected mortality (Abbott, 1925) for each treatment after eight days was only 0.33% for V-ATPase A and 7.43% for dsCa1 ($n_{-ve\ control}$=98; $n_{dsV-ATPase}$=93; n_{Ca1}=103 insects.). Expression was, however, down-regulated by more than 6-fold in insects feeding on dsRNA targeting V-ATPase, although no changes on gene expression were detected on those insects exposed to dsRNA against Ca1 (Figure 6.10).

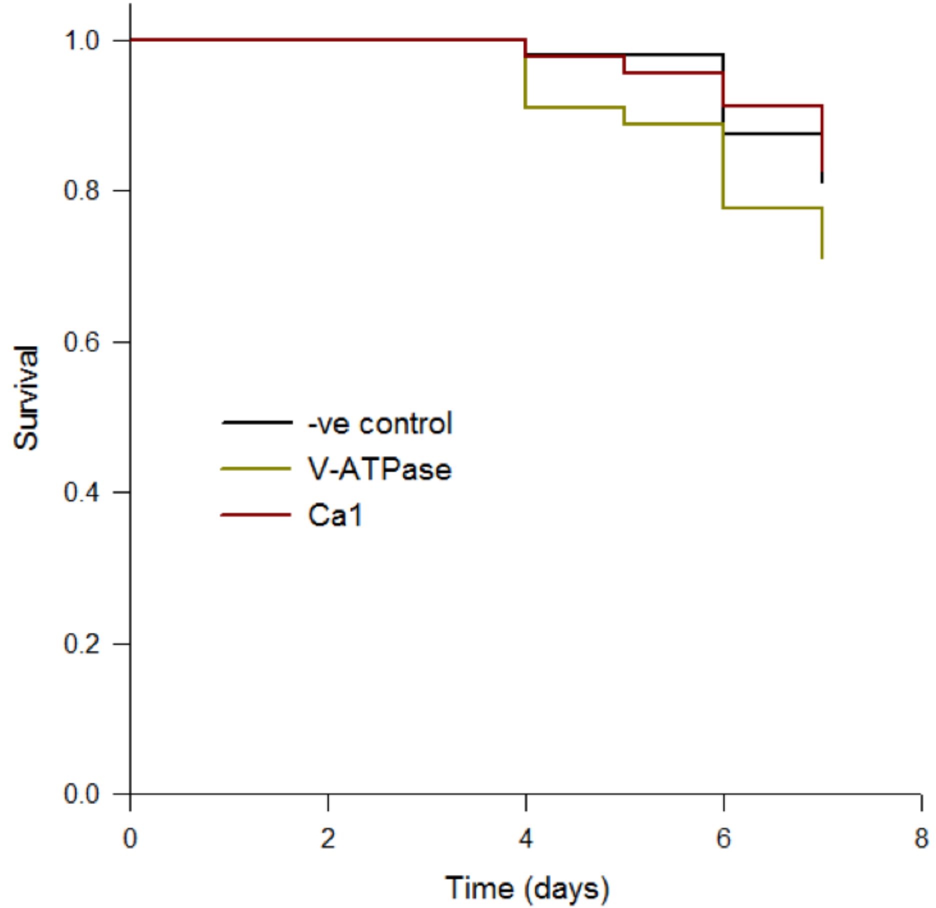

Figure 6.9: Beetle survival was unaffected by feeding on flour disks containing dsRNA (K-M Survival, P= 0.286).

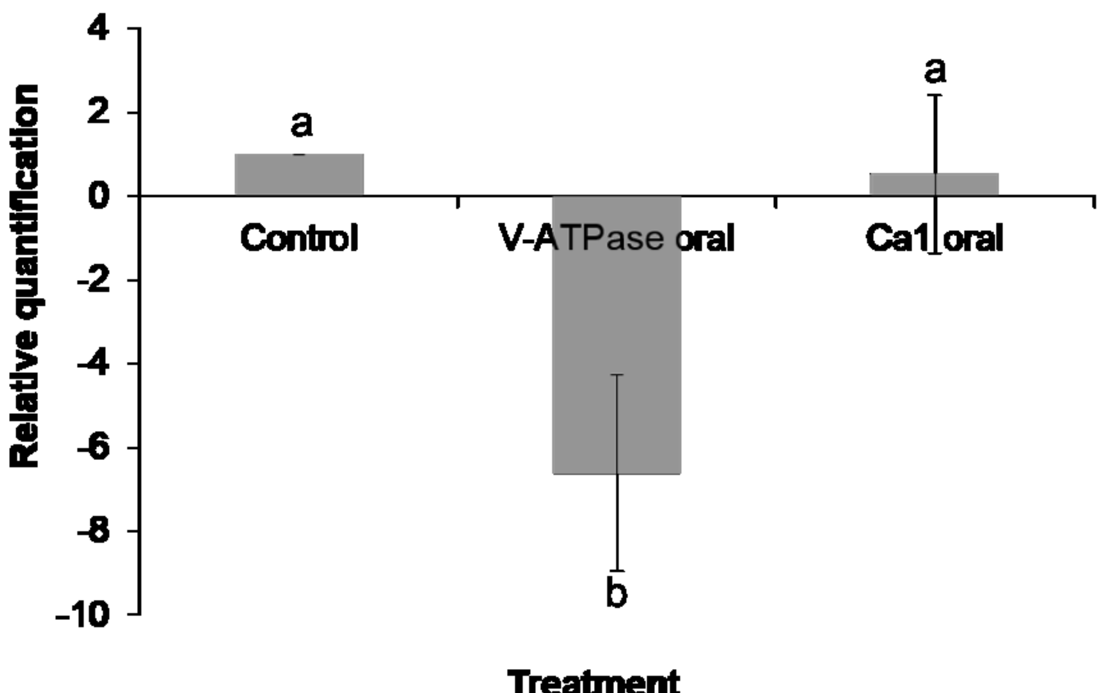

Figure 6.10: Feeding of *T. castaneum* larvae with dsRNA against a V-ATPase gene, but not against a Calcium channel gene, induced gene down regulation by 6.6±2.3-fold. Data are shown as means±SEM; different letters represent differences between treatments ($P<0.05$).

In contrast to oral delivery of dsRNA, injection of *T. castaneum* larvae induced high mortality in all treatments, although no differences in this parameter were detected between treatments (data not shown, $P>0.05$). However, expressions of both genes, V-ATPase A and Ca1, were significantly down-regulated by -2.2±0.6- and -3.1±1.2-fold, respectively ($P<0.05$, Figure 6.11).

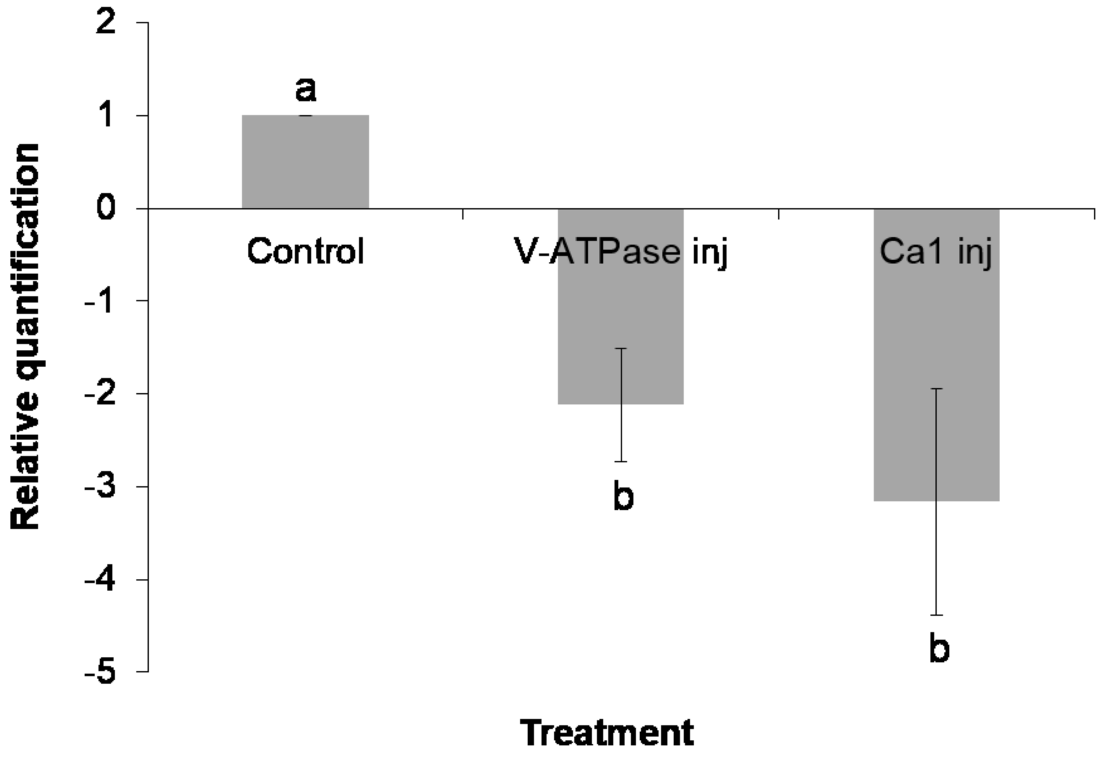

Figure 6.11: Expression of V-ATPase and Ca1 genes are down regulated following dsRNA injection into *T. castaneum* larvae (means±SE). Different letters mean statistically significant differences (P<0.05).

6.5 Discussion

In this work, the effects of injected and orally delivered dsRNA were compared in two insect species, the coleopteran *T. castaneum*, which presents a robust RNAi response (Tomoyasu et al., 2008), and the aphid *M. persicae*. In contrast with *T. castaneum*, aphids generally present a low and variable response to dsRNA, with the success depending on several different parameters, such as dsRNA sequence and concentration, tissues and genes targeted, and methods of uptake (Li et al., 2012; Christiaens et al., 2014). For both species, two silencing targets were chosen, DmCa1D homologues and the proton pump V-ATPase. Dmca1D homologues were chosen because they are likely to be the receptors for ω-ACTX-HV1a (Tedford et al., 2007) in *D. melanogaster* and could be targets for insect control, as loss of function by mutations on this gene are lethal for fly embryos (Eberl et al., 1998). The latter was used as positive control due to reports in the literature demonstrating ds-V-ATPase has caused insect

mortality when used against *D. melanogaster*, *T. castaneum*, *A. pisum*, *M. sexta* (Whyard et al., 2009) and *N. lugens* (Li et al., 2011).

Two different regions of the DmCa1D homologue in *M. persicae* were chosen and targeted, arbitrarily named Ca1 region 1 and Ca1 region 2, and neither yielded any silencing effect by oral delivery or injection. Injection of dsRNA for Ca1 region 2 at 600 ng/µl appeared to have a detrimental effect on adult survival, almost reaching significance when compared to control following survival analysis (P=0.055). However, when the concentration was increased to 1200 ng/µl in a subsequent experiment, no differences in survival were found between Ca1 region 2 and the control treatment, for which actually a slightly higher mortality was recorded. Parental RNAi was evaluated in the offspring of insects injected with dsRNA, but no down-regulation of DmCa1D homologues was observed.

In order to test whether *M. persicae* would be amenable to RNAi, insects were also injected with dsRNA against a V-ATPase subunit gene, which commonly induces mortality when silenced in other insects. For example, Baum et al (2007) showed that ingestion of a V-ATPase dsRNA caused gene down-regulation and mortality when fed to coleopterans *Diabrotica virgifera* and *Leptinotarsa decemlineata* via artificial diet. In addition, the authors also showed that transgenic maize expressing a V-ATPase dsRNA protected plants against *D. virgifera*. Double-stranded RNA-mediated gene silencing in *M. persicae* has been reported when delivered via plants (Pitino et al., 2011; Bhatia et al., 2012; Mao and Zeng, 2014), but not via artificial diet or injection. Surprisingly, although the three papers targeted different genes (Pitino et al. targeted *Rack1*, expressed in the gut and *MpC002*, expressed in salivary glands; Bhatia et al. targeted a gut serine protease and Mao and Zeng silenced *hunchback*, a zinc-finger-containing transcription factor), they all affected aphid reproduction. In the present work, however, aphid survival, fecundity and gene expression were unaffected whether dsRNAs were delivered via injection or artificial diet.

It is possible that, similarly to the aphid *A. pisum*, *M. persicae* is able to degrade dsRNA by nucleases present in both the saliva and haemolymph (Christiaens et

al., 2014). If this is the case, then the use of this technology for aphid control would not be viable, and would account for the observed lack of effect. Despite the lack of a phenotypic effect in *M. persicae* in the present study with either dsRNA directed to V-ATPase or voltage-gated calcium channels, there are examples where this technology has produced promising results for reverse genetics studies in aphids. For instance, Sapountzis et al. (2014) showed that silencing a gene for cathepsin-L in different body parts of *A. pisum* can be induced by injection or feeding. However, knock-down of this gene in the carcass and head was more effective by injection, whereas administration of dsRNA by feeding was more effective for inducing RNAi response in the gut.

Primer sequences used to produce dsRNA and perform qPCR against *T. castaneum* in this work were the same as reported by Whyard et al. (2009). The authors showed increased mortality when beetles were feeding on flour containing dsRNA, with an estimated LC_{50} of 0.0025 mg/g of diet, or 2.5 ng/mg of diet. In the present study, beetles were exposed to 20 times that concentration, 50 ng/mg of diet. Although a six-fold, significant ($P<0.05$) down-regulation was observed for *T. castaneum* feeding on dsRNA against V-ATPase, no effects on mortality were detected. A likely explanation is that the beetle strain presently used was not as amenable to RNAi as the one used by Whyard et al. (2009), or the dsRNA suffered degradation in the diet. Furthermore, in that study the authors used neonate larvae, whereas those used in this study were more than a week old. Conversely to V-ATPase dsRNA, ingestion of dsRNA against a CaV channel did not cause suppression of gene transcription, possibly due to poor delivery of the dsRNA to target cells in the insect CNS. However, when injected into the haemolymph, beetles presented gene down-regulation for both targets, although no effects on survival were observed. While expression of the DmCa1D-homologue after injection was around 3-fold lower than for controls, the effect was probably not sufficiently great to eliminate transcription or to fully deprive the cells of mRNA needed for translation of new calcium channels. The lack of effects in survival might also be related to a low calcium channel protein turnover, explaining the reason why no phenotypical responses were seen. As far as I am aware, no other studies have targeted insect voltage-gated calcium channels with RNAi. Nevertheless, further advancements in the design and delivery of dsRNA molecules need to

be made for their deployment as insecticidal molecules for crop protection, and also for its use in reverse genetics studies in aphids.

Chapter 7 General Discussion

There is a pressing need for the development of effective crop protection agents that are able to control insect pests whilst posing little to no hazard to non-target organisms. This results not only from the loss of efficacy of available pesticides due to the rise of pest resistance and indiscriminate poisoning of pests, biological control and pollinator agents, but also from EU policies, such as Directives 2009/128/EC and 91/414/EEC, bans on chemical control agents (e.g. neonicotinoids), and demands made by NGOs and the general public.

In this context, a novel biopesticide comprising a voltage-gated calcium channel blocker, the spider venom peptide ω-ACTX-Hv1a from *Hadronyche versuta* and snowdrop lectin (GNA) was developed by Fitches et al. (2012). Contrary to its counterparts alone, the fusion protein, Hv1a/GNA, was orally toxic to the lepidopteran *Mamestra brassicae*. Moreover, Back (2011) showed that this fusion protein is also orally toxic to the beetle *Tribolium castaneum*, causing reduction in beetle survival and larval weight. When sprayed on potato plants as a 350 ppm (0.35 µg/µl) suspension, it also induces 100% mortality on another coleopteran larva, the Colorado potato beetle (CPB, *Leptinotarsa decemlineata*) in glasshouse trials (EC Fitches, personal communication), which is comparable to levels attained by commercial *Bacillus thuringiensis* biopesticide formulations. This is particularly relevant because the neonicoitinoid imidacloprid, banned in the EU for being linked with pollinator declines, was an important tool in controlling this particular insect pest (Horowitz and Ishaaya, 2004). Additionally, the efficacy of imidacloprid against CPB has been declining, as field-evolved resistance to this insecticide and other neonicotinoids alike has been reported (Mota-Sanchez et al., 2006; Szendrei et al., 2012). The effectiveness of Hv1a/GNA against representatives of coleopteran and lepidopteran pests and its potential as a biopesticide have been therefore demonstrated in previous work. In this thesis, the efficacy of Hv1a/GNA and RNAi techniques targeting voltage-gated calcium channels, a distinctive insecticidal target from those commonly exploited by conventional insecticides, were assessed for their efficacy and specificity (as summarized in Table 7.1).

Table 7.1: A summary of the effects of Hv1a/GNA towards different arthropods, as evaluated in the present work.

Organism	Exposure route	Parameters analyzed	Effects
M. persicae	artificial diet	mortality	LC_{50}=0.58 µg/µl after 4 days
		fecundity	significant reduction
		size	significant reduction
	transgenic plants	mortality	significantly higher
		fecundity	no effects observed
S. avenae	artificial diet	mortality	LC_{50}=0.73 µg/µl after 2 days
L. oleracea	droplet	mortality	no effects observed
		weight	no effects observed
	injection	mortality	no effects observed
		weight	significant reduction
E. pennicornis	tri-trophic	oviposition	no effects observed
		number of pupae/host	no effects observed
		emergences	no effects observed
	injected hosts	number of pupae/host	hosts not viable
		emergences	hosts not viable
A. mellifera	topical	mortality	LD_{50}>20 µg/bee after 2 days
	acute oral	mortality	LD_{50}>100 µg/bee after 2 days
		learning and memory	no effects observed
	Chronic oral	mortality	no effects observed
		learning and memory	no effects observed
	oral single dose (larva)	mortality	no effects observed
V. destructor	tri-trophic	mortality	no effects observed

Firstly, the effects of the fusion protein towards aphids, a group of insects not commonly targeted by biopesticides, was assessed (**Chapter 2)**. Bioassays conducted against two important aphid pests, *Myzus persicae* and *Sitobion avenae*, demonstrated that insects were susceptible to the fusion protein, even though *M. persicae* was able to partially digest it. Contrary to CPB and other chewing insects that feed on plant tissues, aphids feed on the phloem sap. As the fusion protein needs to be ingested in order to exert its toxic effect, the most feasible means of delivering it to aphids is by expressing the fusion protein in plants, specifically in the phloem. To achieve this goal, Arabidopsis plants were transformed with a construct coding for the fusion protein under the constitutive

promoter CaMV35S, as a model for testing Hv1a/GNA efficacy against *M. persicae*. Western blot analyses have shown Hv1a/GNA expression in leaves (ca. 25 ng/mg FW) and that the protein was functional, with levels of toxicity to *M. persicae* comparable to those seen in artificial diet bioassays. It was not possible to test whether transgenic plants would be effective against CPB, as this insect does not feed on Arabidopsis. In the future, it would be desirable to also transform plants that are compatible with CPB (such as potato plants) or *S. avenae* (such as wheat), as Hv1a/GNA is more effective against those insect pest species than to *M. persicae*. The approach of using transgenic plants expressing this fusion protein for insect control has been, however, proven to be viable. As for any other insect control compound, evolution of resistance to the fusion protein would be expected to occur if pests are continuously exposed to it. Although speculative, mechanisms might include mutations in the points of interaction between CaV and Hv1a; decline in transport efficiency of GNA; and increase in proteolytic activity in the insect gut, digesting the fusion protein and preventing GNA from carrying the spider venom peptide to the haemolymph. In the future, deploying the ‘high dose’/refuge strategy (Gould, 1998), effective and already used for Bt-expressing plants (Gryspeirt and Grégoire, 2012), might prevent the evolution of insect pest field-resistance to transgenic plants expressing Hv1a/GNA.

Even though Hv1a/GNA has been shown to be toxic to a number of economically important insect pests, the safety of the fusion protein to non-target organisms has not been assessed. Therefore, the effects of Hv1a/GNA on two non-target beneficial insects, representatives of biological control agents (*Eulophus pennicornis*) and pollinators (*Apis mellifera*) were evaluated. Should the fusion protein be sprayed on crops and consumed by phytophagous insects, it will be made available to higher trophic levels, i.e., arthropods that prey on whole insects, such as ladybirds, lacewings and spiders. If the herbivorous insect internalizes the fusion protein, which then reaches the haemolymph, Hv1a/GNA will also be taken up by parasitoids that feed on haemolymph, such as *E. pennicornis*. Therefore, tri-trophic toxicity of Hv1a/GNA against *E. pennicornis* via its host *Lacanobia oleracea* was assessed (**Chapter 3**).

Fitches et al. (2012) reported that Hv1a/GNA was orally toxic to noctuid larvae of the moth *Mamestra brassicae*. In the present work, the fusion protein did not induce mortality in larvae of another noctuid species, *L. oleracea*, even though it was internalized following feeding. This finding presented an advantage when testing the effects of Hv1a/GNA consumption by the parasitoid *E. pennicornis* in tri-trophic assays, as indirect effects on parasitoids arising from poor host quality could be discounted. As no effects were detected in these assays where the fusion protein was delivered to the parasitoid via its host, it is expected that this novel biopesticide will be compatible with biological control methods, with no direct effects on this parasitoid.

In honeybees (**Chapter 4**), the fusion protein induced a slight increase in mortality (20%) when insects were fed high doses (100 μg/bee). However, in chronic assays, in which insects were continuously feeding on a 350 ppm Hv1a/GNA solution for seven days, survival was unchanged. Furthermore, those bees were able to perform learning and memory tasks at rates similar to control bees (Nakasu et al., 2014). The amounts of Hv1a/GNA used in those assays were, however, much higher than what bees would encounter in the field. This is because Hv1a/GNA is sprayed on plants as a 350 ppm suspension, and as bees consume only pollen and nectar from those plants (or any other plant), exposure would be even lower. Accidental bee poisoning by insecticides has been recorded from the 1870's, and now timing of application generally takes into account the bee's foraging times (Johansen, 1977; Johansen et al., 2014). Additionally, colonies have to be kept at least four miles away from crops that are sprayed with chemicals that present high toxicity to bees as an exposure mitigation measure (Johansen et al., 2014). As opposed to commonly used chemical pesticides, the fusion protein is not insecticidal via contact and requires ingestion to trigger its toxic effect. In practical terms it means that, even if Hv1a/GNA did have toxic effects when ingested, it could still be sprayed at times in which bees would be foraging, and in the absence of any bee-toxic compound being concomitantly applied, colonies could be kept closer to crops.

With the finding that GNA was able to transport the Hv1a peptide through the bee's midgut, it was hypothesized that the fusion protein would also be

delivered to the parasite *Varroa destructor*, which feeds on the bee haemolymph (**Chapter 5**). The rationale is that since bees are not susceptible to Hv1a/GNA, this fusion protein could be incorporated into food supplements fed to bees. The bees would internalize the fusion protein that would then be consumed by and exert a toxic effect on varroa mites. There are several occasions when bees need food supplements such as bee candy, syrup or water in which the fusion protein could be incorporated, particularly during removal of 'honey crops' or nectar shortages (Food and Environment Research Agency – Fera, 2011). It is likely that varroa did ingest GNA and the fusion protein after consumption by bee hosts, although those proteins could not be detected in mites via immunoassays. The use of this strategy for mite control therefore needs to be improved, starting by designing more effective bioassays to test miticidal activity of proteins against varroa.

The other approach employed for targeting voltage-gated calcium channels, RNAi (**Chapter 6**), did not give promising results for insect control, although a decrease in CaV expression was seen in the model insect *T. castaneum*. Furthermore, dsRNA against *T. castaneum* V-ATPase, previously reported by Whyard et al. (2009) to produce an estimated LC_{50} of 0.0025 mg/g of diet, did not result in significant mortality even when the same dsRNA was administered at a concentration 20 times higher. Cases of contrasting results from similar experiments have also been reported for Lepidoptera, with low reproducibility of RNAi experiments between research groups, and variation in efficiency of the technique related to species, tissues and genes targeted (as reviewed by Terenius et al., 2011). For the aphid *M. persicae*, no changes in expression were detected following dsRNA feeding or injection of dsRNA against V-ATPase, consistent with results from research groups at Durham University (Min Cao, pers. comm.) and Fera (Michelle Powell, pers. comm.), or against CaV genes. Double-stranded RNA-based insect control has potential for selectively killing pests whilst being innocuous to non-target organisms, as sequences specific to an order, species or genotype can be targeted (Scott et al., 2013). However, the negative results obtained from RNAi experiments indicate that further advances are needed in order to use dsRNAs against insect pests. Those include an appropriate selection of molecular targets that

are decidedly effective and a thorough assessment of the amenability of different species to RNAi techniques.

The results obtained in this thesis showed that using voltage-gated calcium channels as targets for insecticides has the potential to selectively kill pests whilst being innocuous or posing negligible risks to non-target organisms, in particular to hymenopterans. The use of biopesticides, such as Hv1a/GNA, might reduce the deployment of broad-spectrum chemical insecticides (Wilson et al., 2013), benefitting crop yields by not only reducing damage by pests, but also by not disturbing important ecological services such as pollination and biological control. Expression of the fusion protein in genetically engineered plants might be more effective than spray applications, although this strategy needs first to gain a wider public acceptance (Godfray et al., 2010). While stricter safety requirements are being imposed for conventional insecticides (Chandler et al., 2011), development and production of biopesticides come forward as viable alternative/complementary tools for IPM strategies. Efforts are currently being made in order to produce Hv1a/GNA on a commercial scale (Pyati et al., 2014) before it can be produced at economically viable costs.

7.1 Conclusions

The work carried out in this thesis demonstrated that the fusion protein Hv1a/GNA is orally toxic to two aphid species, *M. persicae* and *S. avenae*, when tested in artificial diets. Additionally, Hv1a/GNA was shown to be functionally active when expressed in Arabidopsis as a model plant system, conferring enhanced levels of toxicity to *M. persicae*. Whilst toxic to aphids, the fusion protein presented no detectable effects against two hymenopteran beneficial species, the parasitoid *E. pennicornis* and the honeybee *A. mellifera*. Similarly, it did not have any effects on the bee parasite *V. destructor*. Silencing of the putative Hv1a receptor in *T. castaneum* did not result in reduced insect survival.

7.2 Future work

- Modifications in the triple-alanine linker region between Hv1a and GNA should increase protein stability, thus preventing the fusion protein from

being cleaved following expression in plants or ingestion by target pests. This may result in increased insecticidal activity and the fusion protein becoming a more active biopesticide.

- Expression of Hv1a/GNA in wheat, potato and tomato plants should render plants with increased resistance to *S. avenae*, *L. decemlineata* and *M. brassicae*, respectively, as the fusion protein is toxic to these insects via artificial diet and, in the case o *L. decemlineata* and *M. brassicae*, also as a biopesticide.
- It is not expected that this fusion protein will cause major hazards to honeybees and at least one parasitoid species. However, as both insects are representatives of hymenopterans, it would be desirable to test Hv1a/GNA for potential effects against beneficial species from other Orders, such as Coleoptera (e.g. ladybirds) and Hemiptera (e.g. predatory Heteroptera), that would also be exposed to the fusion protein via their hosts or preys.
- No effects on *V. destructor* were detected when mites fed on bees previously exposed to Hv1a/GNA. However, GNA might still be used as a means for the delivery of other miticidal molecules to varroa via honeybee hosts.

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Appendices

Appendix A

Tests of recombinant ω-ACTX-Hv1a/GNA for potential allergenicity

The potential allergenicity of the recombinant fusion protein, ω-ACTX-Hv1a/GNA, was carried out *in silico* and by pepsin digestion according to FAO/WHO guidelines (2001).

For database searches, the Modified ω-ACTX-Hv1a/GNA sequence was first aligned using the BLAST algorithm against nr, RefSeq, Swissprot, pat and pdb databases, with an 'allergen' filter. No major significant matches were found using this method. Using the NetNGlyc software, a potential glycosilation site was found in the GNA portion of the fusion protein. The Hv1a/GNA sequence was then aligned with allergens from four different databases: Evaller, Allermatch, Allergenonline and SDAP. Again no significant matches were found, suggesting that the fusion protein is not allergenic.

The fusion protein was shown to be resistant to proteolytic cleavage by pepsin, although the intensity of its band decreased as incubation time increased (Fig 1C). The positive control (potato acid phosphatase, Fig 1A) was degraded within 30 s, whereas the negative control (soybean trypsin inhibitor) remained stable for the duration of the assay (60 min) (Fig 1B).

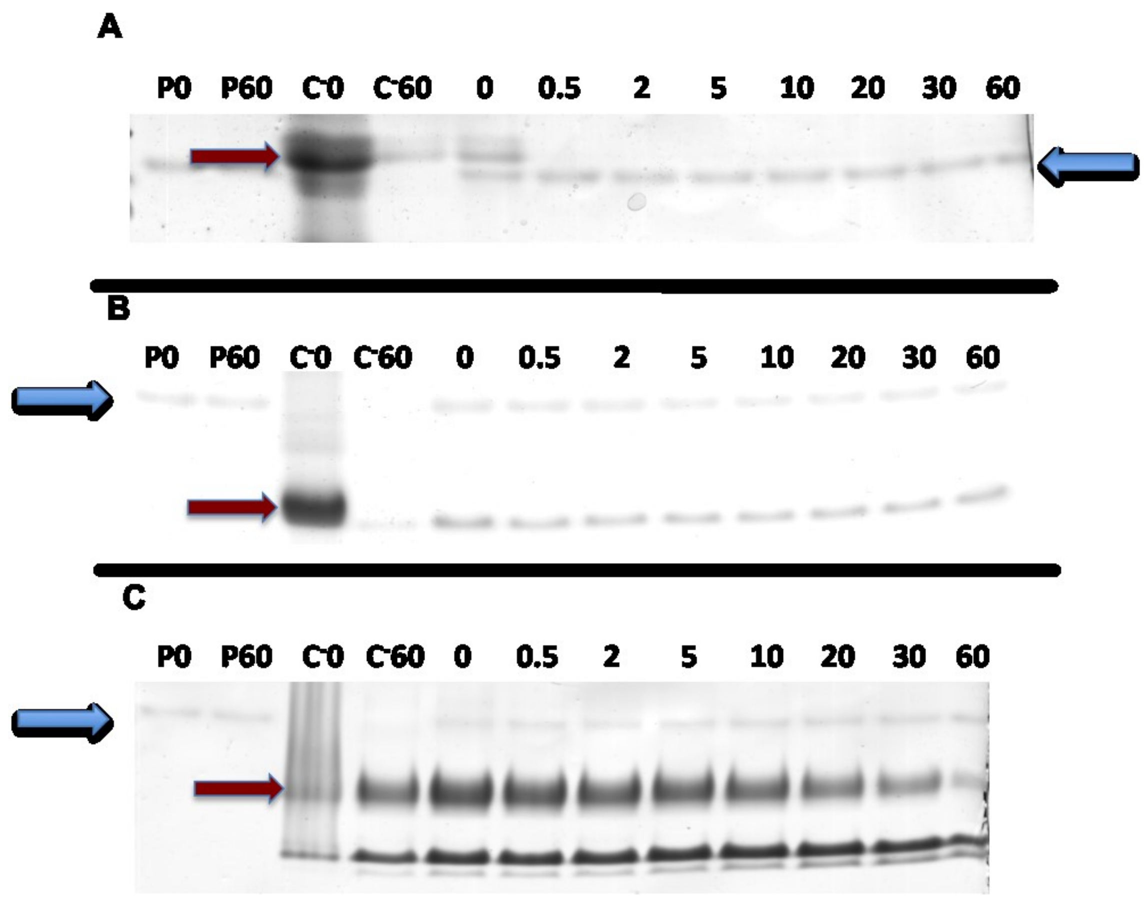

Figure 1: Pepsin digestibility assay. A) positive control (potato acid phosphatase); B) Negative control (soybean trypsin inhibitor) and C) ω-ACTX-Hv1a/GNA. Numbers represent incubation time at 37 ºC, in minutes. Hv1a/GNA appears to be stable and resistant to proteolytic cleavage by pepsin. Blue arrows show pepsin band, whereas red arrows demonstrate test protein.

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Appendix B

Detailed method for producing and purifying Hv1a/GNA and GNA via fermentation.

Proteins were produced by heterologous expression in *Pichia pastoris* (SMD1168H strain) carrying sequences encoding GNA or Hv1a/GNA. Initially, *P. pastoris* harboring GNA or Hv1a/GNA genes were grown in baffled flasks containing YPG broth (1% yeast extract, 2% peptone, 4% glycerol) with 100 µg Zeocin/ml for 36 h at 180 rpm, 30 °C. Cultures (100ml) were then inoculated in Bio Console ADI 1025 (Applikon) fermentors containing 1 l of basal media (Table 1) supplemented with 4.6 ml/l of *Pichia* Trace Metals 1 (PTM_1, table 2). Fermentors were settled for 30% dO2, pH 5.0, 30 °C under agitation and continued 50% glycerol feeding with added PTM_1 (12ml/l). Glycerol feed was initiated 6 h after inoculation at a rate of 5 ml/h for 8h. After this period, glycerol feed was increased to 10 ml/h for 24 h and finally to 20 ml/h for another 24 h.

Table 1: basal media used in fermentations.

Reagent	Quantity
85% Phosphoric acid (H_3PO4)	26.7 ml/l
Calcium sulphate ($CaSO_4$)	0.93 g/l
Potassium sulphate (K_2SO_4)	18.2 g/l
Magnesium sulphate 7-hydrate ($MgSO_4.7H_2O$)	14.9 g/l
Potassium hydroxide (KOH)	4.13 g/l
Glycerol	40 ml/l
Water	to 1 l

Table 2: PTM_1 salts added to the basal media and glycerol feed.

Reagent	**Quantity**
Cupric sulphate ($CuSO_4.5H_2O$)	3 g
Sodium iodide (NaI)	0.04 g
Manganese (II) sulphate ($MnSO_4.H_2O$)	1.5 g
Sodium molybdate ($Na_2MoO_4.2H_2O$)	0.1 g
Boric acid (H_3BO_3)	0.01 g
Cobalt chloride ($CoCl_2$)	0.25 g
Zinc chloride (ZnCl)	10 g
Ferrous sulphate ($FeSO_4.7H_2O$)	32.5 g
Biotin	0.1 g
Sulphuric acid (H_2SO_4)	2.5 ml
Water	to 500ml

After expression, cultures were centrifuged at 7500 *g*, 4 °C for 35 min and supernatants collected. Samples were then sequentially filtered to remove remaining particles and cells (pore sizes of 2.7 μm, 1.2 μm and 0.7 μm).

For GNA recombinant protein, NaCl was added to the supernatant to a final concentration of 4M. Proteins were then purified by hydrophobic interaction chromatography on a phenyl-sepharose resin packed onto a Pharmacia XK16 column. After loading, the column was washed with solution A (4M NaCl), then with 30% solution B (deionized water) and finally eluted with a linear gradient from 30% to 100% of solution B. Fractions containing GNA were pooled, dialyzed, freeze-dried, re-suspended in dH2O and reloaded onto a size-exclusion column (HiPrep™ 16/60 Sephacryl S-100, GE-Healthcare). After purification, proteins were dialyzed, freeze-dried and stored at -20 °C.

For His-tagged Hv1a/GNA purification, supernatants were diluted in 4X binding buffer (BB; final concentration of 0.02 Sodium phosphate, 0.4M NaCl, pH 7.4). Samples were loaded onto a HisTrap™ (GE Healthcare) column and then eluted with BB containing 0.2 M imidazole.

After purification, samples were extensively dialyzed in water and freeze-dried.

The concentration of Hv1a/GNA was estimated by comparing band intensities with known amounts of GNA on SDS-PAGE.

Appendix C

Aphid diet contents, as describe by Febvay et al. 1988 (See References).

Amino acids	mg per 100 ml	Concentration (mM)
Alanine	178.71	20.06
β-alanine	6.22	0.7
Arginine	244.9	14.06
asparagine(H_2O)	295.55	19.88
aspartic acid	88.25	6.63
Cysteine	29.59	2.44
glutamic acid	149.36	10.15
Glutamine	445.61	30.49
Glycine	166.56	22.19
histidine (HCl)	136.02	6.49
isoleucine (allo free)	164.75	12.56
Leucine	231.56	17.65
Lysine (HCl)	351.09	19.22
Methionine	72.35	4.85
ornithine (Hcl)	9.41	0.56
Phenylalanine	293.03	17.73
Proline	129.33	11.23
Serine	124.28	11.83
threonine (allo free)	127.16	10.67
Tryptophan	42.75	2.09
Tyrosine	38.63	2.13
Valine	190.85	16.29
Sucrose	20000	580
Vitamins:	**mg/l**	
amino benzoic acid	100	
ascorbic acid	1000	
Biotin	1	
Calcium pantothenate	50	
Choline chloride	500	
folic acid	10	
i-inositol	420	
nicotinic acid	100	
pyridoxine (HCl)	25	
riboflavin	5	
Thiamine (HCl)	25	
Trace metals		
$CuSO_4.5H_2O$	4.7	
$FeCl_3.6H_2O$	44.5	
$MnCl_2.4H_2O$	6.5	
NaCl	25.4	
$ZnCl_2$	8.3	
Calcium citrate	100	
cholesterol benzoate	25	
$MgSO_4.7H_2O$	2420	

KH_2PO_4	2500

Appendix D

Sequences used for plant transformation on chapter 2.

Hv1a/GNA sequence with codons optimized by ShineGene Molecular Biotech,Inc. for expression in *Arabidopsis thaliana*, inserted into plants (Chapter 2).

ATGGCTAAGGCAAGTCTCCTCATTTTGGCCGCCATCTTCCTTGGTGTCATC
ACACCATCTTGCCTGAGTTCTCCTACTTGTATTCCTTCTGGACAACCTTGTC
CTTATAATGAAAATTGTTGTTCTCAATCTTGTACTTTTAAGGAAAATGAAAAT
GGAAATACTGTTCAAAGATGTGATGCTGCTGCTGACAATATTTTGTACTCC
GGTGAGACTCTCTCTACAGGGGAATTTCTCAACTACGGAAGTTTCGTTTTT
ATCATGCAAGAGGACTGCAATCTGGTCTTGTACGACGTGGACAAGCCAAT
CTGGGCAACAAACACAGGTGGTCTCTCCCGTAGCTGCTTCCTCAGCATGC
AGACTGATGGGAACCTCGTGGTGTACAACCCATCGAACAAACCGATTTGG
GCAAGCAACACTGGAGGCCAAAATGGGAATTACGTGTGCATCCTACAGAA
GGATAGGAATGTTGTGATCTACGGAACTGATCGTTGGGCTACTGGAACTC
ACACCGGACTTGTTGGAATTCCCGCATCGCCACCCTCAGAGAAATATCCTA
CTGCTGGAAAGATAAAGCTTGTGACGGCAAAGTAA

Sequence translation: GNA precursor in black; ω-ACTX-Hv1a portion in red, the AAA linker in bold and GNA in blue.

MAKASLLILAAIFLGVITPSCLSSPTCIPSGQPCPYNENCCSQSCTFKENENGN
TVQRCD**AAA**DNILYSGETLSTGEFLNYGSFVFIMQEDCNLVLYDVDKPIWATN
TGGLSRSCFLSMQ TDGNLVVYNPSNKPIWASNTGGQNGNYVCILQKDRNV
VIYGTDRWATGTHTGLVGIPASP PSEKYPTAGKIKLVTAK

Appendix E

dsRNA design against *Myzus persicae* Calcium channel:
EC388785.1
CGTGGTGTTGGCTCTAGAGGAACATCTGCCCAGGGGAGACAAGACTGTAC
TTGCTAAGACTTTGGAGGCAACCGAACTCGTTTTCCTAGCAATTTTTTGCG
TCGAAGCGACTCTCAAAATATTAGCTTTAGGTTTTTTACTGCACAGCGGCT
CATATCTTCGTAATATATGGAACATTATGGACTTTTTTGTAGTTGTAACAGG
GTCAATGACTGAATTCATGGAGTCCAACATGCTGGACATGAGGATGTTGAG
GTCCTTTCGGGTGCTCAGGCCTTTGAAACTGGTTTCGAGAATCCCAAGTCT
GCAAGTCGTGCTGAAGTCCATCATCAAGGCGATGGCTCCGCTACTCCAGA
TTGGTTTATTGGTGTTATTTGCTATCATCATATTCGCAATCATCGGACTGGA
GTTTTACTCCGGAGCGTTGCACAAGACTTGCTACAAGTTGGATAATCTGAT
TGCGATGGAAATCGAGGGAGCGAACCCGGCGCCTTGTAACTCGGACTCG
GACTCAGACGAGGGAACGAAGCCGTACAACGCGTACTTCTGTGATAACAC
GACGTCCACGTGCATAGAAAACTGGGTAGGTCCCAACTACGGGATCACGT
CGTTCGATAACATCGGACTGGCTATGCTCACCGTTTTCCAATGCATCACTA
TGGAAGGGTGGACTACCATCTTGTACTGGATGAACGATGCGTACGGCGTG
CTATTCAATTGGATATATTTTGTACCACTTATTATTCTAGGTTCATTTTTTAT
GCTCAATTTAGTTCTCGGTGTGCTTAGCGGAGAATTCGCTAAAGAA

Red highlight: pair of primers for RNAi (calcium channel region 1).
Green highlight: pair of primers for RNAi 2 (calcium channel region 2).

Appendix F

Effects of Hv1/GNA on honeybees (*Apis mellifera ligustica*) – acute oral assays

Material and Methods

Unprocessed, non-purified Hv1a/GNA (3% active ingredient in powder), or BSA were resuspended in 1M sucrose. Italian honeybees (*Apis mellisfera ligustica*, see chapter 5) were allowed to feed on one of three test solutions (sucrose alone, BSA or Hv1a/GNA at 100 μg/bee) for up to four hours, when feeders containing test solutions were removed. Insects were then allowed to feed *ad libitum* on 1 M sucrose solution. Three groups of ten forager bees were used per treatment. Mortality was assessed four, 24, 48, 72 and 96 h after administration of the treatments.

Results:

Survival was poor for all treatments, with more than 40% mortality in negative control (sucrose) after 96 h. However, no significant differences between treatments were found at any time point (figure 1; P>0.05).

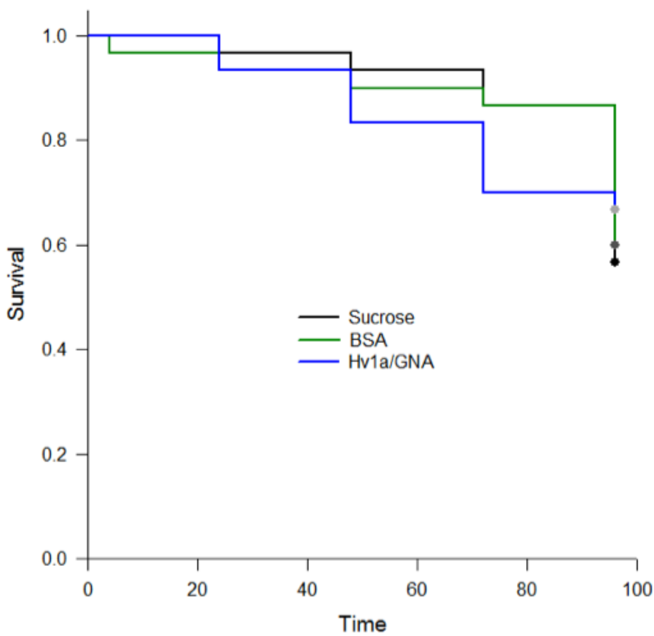
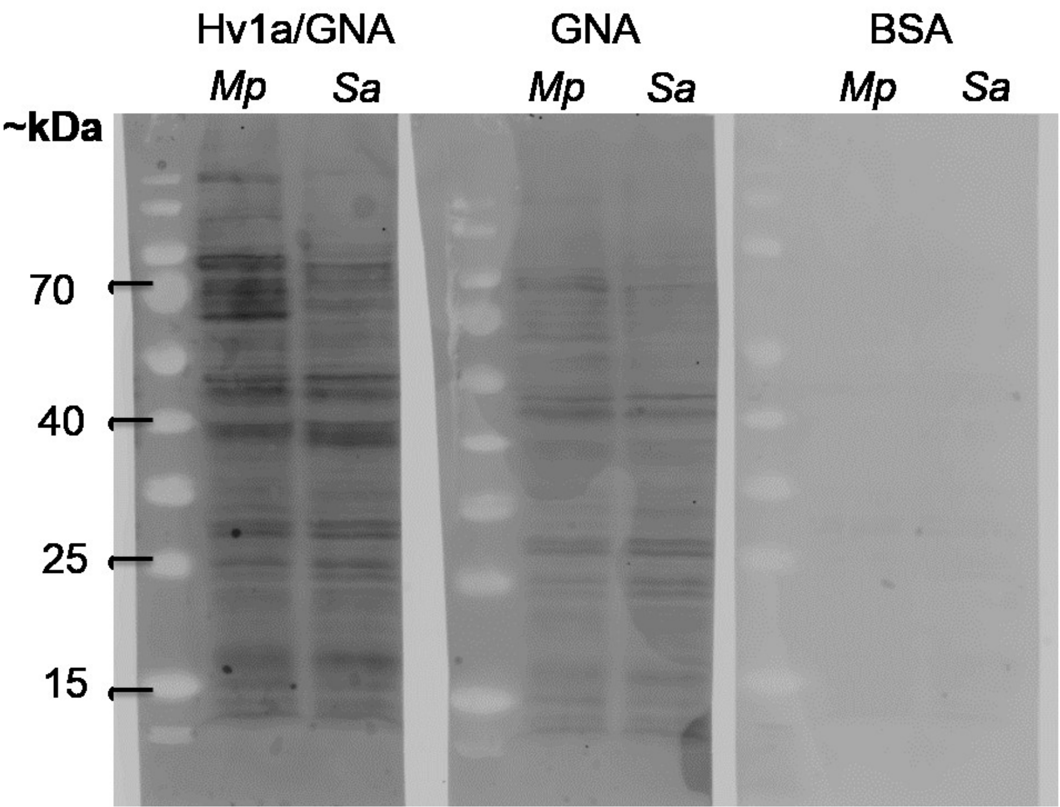


Figure 1: Bee mortality after 96 h on an acute toxicity assay. A sharp increase in mortality is seen for BSA and sucrose treatments, but no differences were found (P=0.945)

Appendix G



Whole aphids wore macerated in 1.5x Sample Buffer (20 µl/mg of aphids). 20 µl of extracts were loaded onto 12% SDS-PAGE gels. They were transferred to nitrocellulose (130 mA, 40'), blocked and incubated O.N. at 4 degrees with proteins labeled with FITC. Order is always Myzus and then Sitobion

Appendix H

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Novel biopesticide based on a spider venom peptide shows no adverse effects on honeybees

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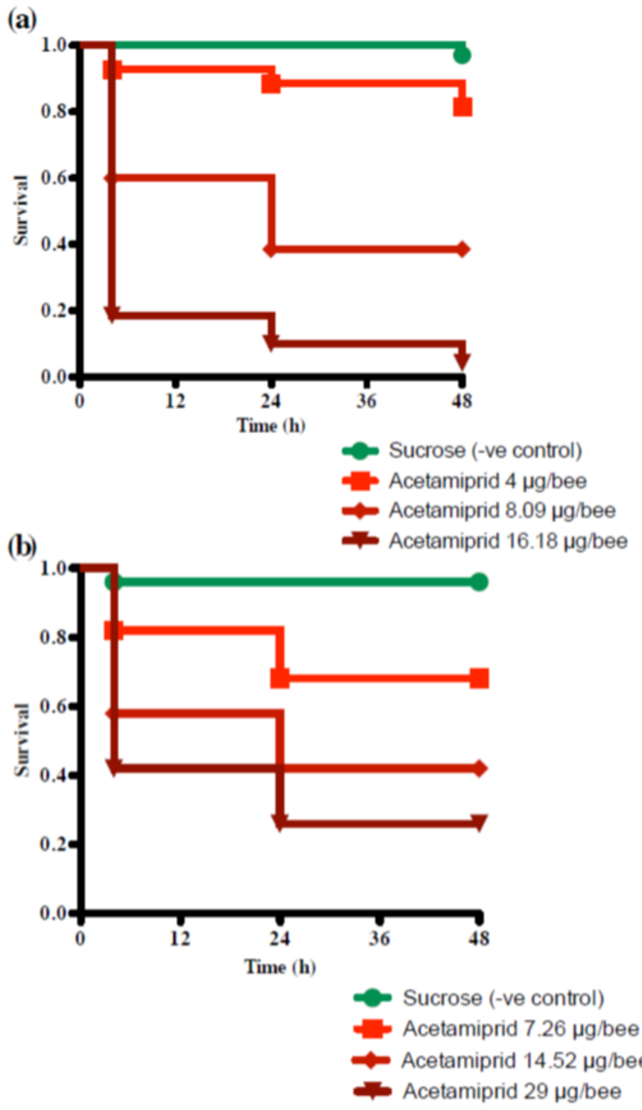
Appendix I



ESM Figure 1:

Dose response for the insecticide acetamiprid for a) acute contact toxicity (4, 8.09 and 16.18 µg/bee) and, b) acute oral toxicity (7.26, 14.52 and 29 µg/bee). The intermediate dose tested for both assays represents the published LD50 value for the pesticides for bees (EU, SANCO/1392/2001). These LD50 valuses were confirmed in the present study.

Appendix J

Transgenic plants expressing ?-ACTX-Hv1a and snowdrop lectin (GNA) fusion protein show enhanced resistance to aphids

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