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# Development of next-generation biopesticides as a control method for the small hive beetle *Aethina tumida* murray (coleoptera: nitidulidae), a serious pest of the European honey bee *Apis mellifera*

A thesis submitted by Michelle Emma Powell, BSc in accordance with the requirements of Durham University for the degree of Doctor of Philosophy

Department of Biosciences

Durham University

August 2018

**Development of next-generation biopesticides as a control method for the small hive beetle *Aethina tumida* murray (coleoptera: nitidulidae), a serious pest of the European honey bee *Apis mellifera***

**Michelle Emma Powell**

## Abstract

The small hive beetle (*Aethina tumida*) is a serious pest of the European honey bee (*Apis mellifera*), responsible for causing significant economic damage to the apiculture industry in North America and Australia. In 2014 *A. tumida* was detected in Italy, highlighting the potential for an outbreak within the UK. Current control measures rely on the use of organophosphate and permethrin, both are highly toxic to honeybees and continued use can give rise to resistance. Given these issues alternative control strategies are urgently required. The aims of this thesis were to explore potential for the development of next generation biopesticides, including RNA interference (RNAi) and fusion protein technology, as an alternative control method for *A. tumida*

The sequence specificity of RNAi makes it an ideal strategy to combat this parasite of honey bees. Here we report that microinjection of low (2-10 ng) doses of *V-ATPase subunit A* and *Laccase 2* dsRNAs resulted in 100 % mortality of *A. tumida* larvae. Quantitative PCR analysis confirmed that injections induced significant decreases in mRNA levels of the target genes with an enhancement of gene suppression over time providing evidence for systemic RNAi effects. Whilst oral delivery of *V-ATPase subunit A* dsRNA via “soaking” in dsRNA solutions resulted in 50 % mortality and malformed survivors, gene suppression could not be verified by qPCR analysis. Our results showed that dsRNAs are prone to degradation by extracellular nucleases following ingestion by feeding, but not wandering stage, larvae. We suggest that the lack of consistent RNAi effects in feeding experiments was a consequence of dsRNA degradation within the gut of *A. tumida*. Target specificity was confirmed by a lack of effect on survival or gene expression in honey bees injected with *A. tumida* dsRNAs. *A. tumida* show a robust response to injected dsRNA but further research is required to develop methods to induce RNAi effects via ingestion.

**Abstract**

The spider-venom peptide ω-hexatoxin-Hv1a (Hv1a) is highly potent by injection to a range of insects, but not vertebrates making it an ideal candidate for the development of bioinsecticides. Oral delivery of the toxin is largely ineffective due to failure to access its site of action in the central nervous system (CNS). Fusion protein technology allows oral delivery of Hv1a to the CNS via fusion to a "carrier" protein, snowdrop lectin *Galanthus nivalis* agglutinin (GNA), directing transport of the toxin across the insect gut to the circulatory system.

Constructs encoding Hv1a or modified Hv1a (K>Q modification to remove potential KEX2 cleavage site) linked to the N- or C-terminus of snowdrop lectin (GNA) were used to produce recombinant GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q. All four fusion proteins were toxic by injection to *A. tumida*. The $LD_{50}$'s for GNA/Hv1a and GNA/Hv1a(K>Q) were a similar 0.44 and 0.47 µg/µl, whilst Hv1a/GNA and Hv1a(K>Q)/GNA $LD_{50}$'s were slightly lower, at a respective 0.33 and 0.25 µg/µl. In contrast no effects on honeybee survival were observed when 20 fold higher doses were injected. When fed to *A. tumida* larvae, GNA/Hv1a was 2x more effective than Hv1a/GNA, GNA/Hv1a(K>Q) and Hv1a(K>Q)/GNA ($LC_{50}$s of 0.52, 1.14, 1.18 and 0.89 mg/ml, respectively). When fed to *A. tumida* adults no mortality was recorded for GNA/Hv1a(K>Q) or Hv1a(K>Q)/GNA treatments. However, both Hv1a/GNA and GNA/Hv1a were toxic to adults, with similar $LC_{50}$s of 2.52 and 2.02 mg/ml, respectively. Reduced efficacy of Hv1a/GNA and K>Q variants against larvae was shown to be attributable to differences in the stability of the fusion proteins in the presence of extracellular gut proteases. In laboratory assays *A. tumida* larval survival was significantly reduced when brood, inoculated with eggs, was treated with GNA/Hv1a. The dominant digestive protease in *A. tumida* larvae was identified as trypsin. Consequently, a trypsin inhibitor (Soybean Kunitz trypsin inhibitor: SKTI) was incubated together with *A. tumida* gut extracts and GNA/Hv1a and Hv1/GNA, with both fusion protein remaining fully intact after 24 hr. This contrasted with previous analysis that showed no intact GNA/Hv1a or Hv1a/GNA after incubation with gut extracts in the absence of the trypsin inhibitor under comparable conditions.

Consequently, SKTI was evaluated as an alternative carrier protein to GNA for the delivery of Hv1a to the circulatory system of *A tumida*. Preliminary studies indicated transport of SKTI into the haemolymph, suggesting SKTI could be used as an alternative carrier protein. An initial construct was designed based on GNA/Hv1a, however no biological activity was observed after injection into *A. tumida* larvae. It was

speculated that the lack of insecticidal activity was attributed to the misfolding of the toxin during expression in the yeast cells. As such two additional fusion proteins were designed incorporating either a flexible (Gly-Gly-Gly-Gly-Ser motif) or rigid linker (Proline rich motif region) to improve protein folding and function. Only inclusion of a rigid linker showed limited biological activity after injection into *A. tumida* larvae, again suggesting misfolding of the toxin.

Both RNAi and fusion protein technology hold enormous potential for the control of *A. tumida* in apiculture and to our knowledge this is the first study to demonstrate the use of a protein based biopesticide and RNAi as a possible control method for *A. tumida*.

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## List of Abbreviations

### Nucleic acid abbreviations:

A: Adenine

C: Cytosine

G: Guanine

T: Thymine

### Amino acid abbreviations:

| Amino acid | Single letter code | Three letter code |
|---|---|---|
| Alanine | A | Ala |
| Arginine | R | Arg |
| Asparagine | N | Asn |
| Aspartic acid | D | Asp |
| Cysteine | C | Cys |
| Glutamine | Q | Gln |
| Glutamic acid | E | Glu |
| Glycine | G | Gly |
| Histidine | H | His |
| Isoleucine | I | Ile |
| Leucine | L | Leu |
| Lysine | K | Lys |
| Methionine | M | Met |
| Phenylalanine | F | Phe |
| Proline | P | Pro |

| | | |
|---|---|---|
| Serine | S | Ser |
| Threonine | T | Thr |
| Tryptophan | W | Trp |
| Tyrosine | Y | Tyr |
| Valine | V | Val |

**Degenerate nucleic acid abbreviations:**

W: Weak (A/T) K: Keto (G/T)

S: Strong (G/C) R: purine (A/G)

Y: pYrimidine (C/T) M: aMino (A/C)

B: C/G/T

D: A/G/T

H: A/C/T

V: A/C/G

N: A/C/G/T

**Other abbreviations:**

| | |
|---|---|
| ºC | Degrees Celsius |
| % | Percentage |
| AOX1 | Alcohol oxidase 1 |
| AFB | American foulbrood |
| ANOVA | Analysis of Variance |
| Approx. | Approximately |
| BB | Binding buffer |
| bp | Base pair |
| BSA | Bovine serum albumine |
| Bt | *Bacillus thuringiensis* |

| | |
|---|---|
| ButaIT | Indian red scorpion (*Mesobuthus tamulus)* toxin |
| Cav | Voltage-gated calcium channels |
| CBB | Coomassie brilliant blue |
| cDNA | complementary DNA |
| CHS | chalcone synthase |
| CNS | central nervous system |
| Con A | Concanavalin A |
| CpTI | Cowpea trypsin inhibitor |
| dATP | Deoxyadenosine triphosphate |
| dCTP | Deoxycytidine triphosphate |
| dGTP | Deoxyguanosine triphosphate |
| dNTP | Dideoxynucleotides triphosphates |
| dsRNA | double-stranded RNA |
| dTTP | Thymidine triphosphate |
| DUM | dorsal unpaired median |
| DWV | Deformed wing virus |
| EC | European Commission |
| EDTA | Ethylenediaminetetraacetic acid |
| EPPO | European and Mediterranean Plant Protection Organization |
| ER | Endoplasmic reticulum |
| EU | European Union |
| FP | Fusion protein |
| GAPH | Glyceraldehyde-3-phosphate dehydrogenase |
| GFP | Green fluorescent protein |
| GNA | *Galanthus nivalis* agglutinin or Snowdrop lectin |

| | |
|---|---|
| GST | Glutathione S-transferase |
| hr | hour(s) |
| HEK | human embryonic kidney |
| Hv1a | ω-Hexatoxin-Hv1a |
| HVA | High-voltage activated |
| I-IV | Four repeat domains |
| ICK | Inhibitory cystine knot |
| IPM | Integrated Pest Management |
| K-M | Kaplan-Meyer analysis of survival |
| kDa | Kilodalton(s) |
| LB | Luria Bertani |
| $LC_{50}$ | Concentration of a toxin that causes the death of 50 % of test animals |
| $LD_{50}$ | Dose of a toxin that causes the death of 50 % of test animals |
| LSLB | Low salt Luria Bertani |
| LVA | Low-voltage-activated |
| Manse-AS | *Manduca sexta* allatostatin |
| Tm | Melting temperature |
| miRNA | microRNA |
| MGW | Molecular grade water |
| MS | Member State |
| *nptII* | bacterial nptII resistance gene |
| nt | Nucleotide |
| OD | Optical density |
| Or | Odorant receptor |

| | |
|---|---|
| PBS | Phosphate buffer saline |
| PCR | Polymerase chain reaction |
| piRNA | PIWI-associated RNA |
| PIs | Proteinase inhibitors |
| PHA | Phytohemagglutinin |
| PI1a | δ-amaurobitoxin-PI1a |
| qPCR | Quantitative polymerase chain reaction |
| RISC | RNA induced silencing complex |
| RNAi | RNA interference |
| S1-S6 | Six trans-membrane segments |
| SBBI | Soybean Bowman-Birk inhibitor |
| SF11 | *Segestria florentia* toxin 1 |
| siRNA | short interfering RNA |
| SDS-PAGE | Sodium dodecyl sulphate Polyacrylamide gel electrophoresis |
| SKTI | Soybean Kunitz trypsin inhibitor |
| SMD | Protease deficient *Pichia pastoris* strain |
| SP | Sodium phosphate buffer |
| SPIs | Serine proteases inhibitors |
| ssRNA | single-stranded RNA |
| TBE | Tris-borate EDTA buffer |
| TEMED | N,N,N',N'-tetramethylethane |
| TEs | Transposable elements |
| UK | United Kingdom |
| USA | United Sates of America |
| v/v | volume/volume |

| | |
|---|---|
| w/w | weight/weight |
| WGA | Wheat germ agglutinin |
| X33 | Wild-type *Pichia pastoris* strain |
| YPG | Yeast peptone glucose media |

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### Declaration

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**Date:** August 2018

**Signed:** 

### Publication

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I would like to dedicate my thesis to the memory of my Pa, Oswald Peter Powell.

Michelle Powell

# CHAPTER 1 | INTRODUCTION

## 1.1 Small hive beetle (*Aethina tumida*)

The small hive beetle (*Aethina tumida Murray*, Coleoptera: Nitidulidae) (Figure 1.1 A) belongs to the order Coleoptera, consisting of beetles and weevils. This order contains over 370,000 described species, representing approx. 40 % of the known insects, and is the largest order in the animal kingdom. *Aethina tumida* a small dark brown to black beetle is a member of the family Nitidulidae, consisting of >2500 described species worldwide (Habeck, 2002). Most Nitidulid species feed on a large variety of different food sources, mostly of plant origin after fungal invasion such as decaying fruit, fermenting plant juice and trees with fungi, however some Nitidulids frequent flowers, carrion or crops (Neumann and Elzen, 2004). The ability to feed and reproduce on a range of diets, has enabled *A. tumida* to opportunistically switch hosts to honey bee colonies (Neumann and Elzen, 2004; Stedman, 2006).

Since 1996 *A. tumida* has become a major pest to beekeepers, through extension of its geographical range, having a significant economic impact on managed, wild and feral bee populations (Elzen et al., 1999). The spread of *A. tumida* into new regions can be attributed to the increase in international trade of packaged bees, queen cages, whole honey bee colonies and their products over the last few decades (Cuthbertson and Brown, 2009). In its native range of sub-Saharan Africa it is considered an occasional parasite and scavenger of colonies of African honey bee, *Apis mellifera scutellata* (Lundie, 1940; Roberts, 1971; Smith 1953) and the Cape honey bee, *Apis mellifera capensis* (Elzen et al., 2000). Through co-evolution African honey bees have been able to develop traits that limit the damage *A. tumida* can cause to their colonies. Africanised bees in general exhibit a more aggressive behaviour, with the guard bees acting as the first line of defence in limiting *A. tumida* invasions. Additionally, the guard bees will modify the hive entrance by using propolis or bee resin, which is produced by bees by collecting resin from trees and other sources and mixing it with a small amount of honey, this in turn reduces the size of the entrance and chances of colony invasion. Within the hive African bees patrol particularly the brood area and are able to remove free roaming beetles, exposed eggs and larvae, when present in low numbers (Lundie, 1940; Elzen et al., 2001; Neumann et al., 2001). Cape honey bees are reported to hold *A. tumida* captive and this confinement behaviour is likely to play an important role in preventing *A. tumida* from mating. When large numbers of *A.*

*tumida* are present within a hive, African bees show a tendency to abscond limiting the available resources for *A. tumida* population growth (Neumann et al., 2001; Spiewok et al., 2007).

As an invasive species *A. tumida* has caused severe economic damage in colonies of the European honey bees (*Apis mellifera*), as they have not developed the necessary behavioural resistance mechanisms required to keep *A. tumida* population growth under control (Ellis et al., 2003). In North America *A. tumida* have been observed to readily take over strong *A. mellifera* colonies with little resistance from worker bees, allowing adults to readily lay eggs within the cracks and crevices of the hive or directly on the comb. Worker bees allow *A. tumida* adults and larvae to feed unhindered on pollen, honey and brood, causing honey to ferment, leaving it unusable and 'slimed' (Figures 1.1 B & C). Additionally, this unrestricted feeding results in comb destruction which often lead to full structural collapse of the nest and absconding of the colony within as little as 2 weeks (Neumann et al., 2001; Spiewok et al., 2007).

Honey bees are threatened by numerous pathogens and viruses that pose a key threat to the health of their host (Chen and Siede, 2007). Deformed Wing Virus (DWV) is a single-strand positive sense RNA virus, known to cause overt wing deformities, such as shrunken and crumpled wings in developing honey bees, which results in emerging honey bees that are unable to fly or survive (Chen and Siede, 2007). Replication of DWV proceeds via the production of a negative-strand intermediate and its presence is indicative of active viral replication (Boncristiani et al., 2009). Eyer et al. (2009) provided evidence that adult *A. tumida* are carriers of DWV following ingestion of dead workers, brood, and wax contaminated with the virus and become increasingly infected with DWV following continual ingestion of contaminated food. More importantly *A. tumida* was implicated as a biological vector of DWV, as the results indicated that 40 % of infected *A. tumida* carried negative stranded RNA of DWV, confirming active viral replication (Eyer et al., 2009). Additionally, it has been demonstrated that *A. tumida* can transmit *Paenibacillus larvae* the bacterial causative agent of American foulbrood (AFB), consider to be the most widespread and destructive disease of bee brood. According to Schäfer et al. (2010) both larvae and adults become infected with spores when exposed to honey bee brood combs with clinical symptoms of AFB in the laboratory, demonstrating the potential of *A. tumida* to act as a vector of *P. larvae*.

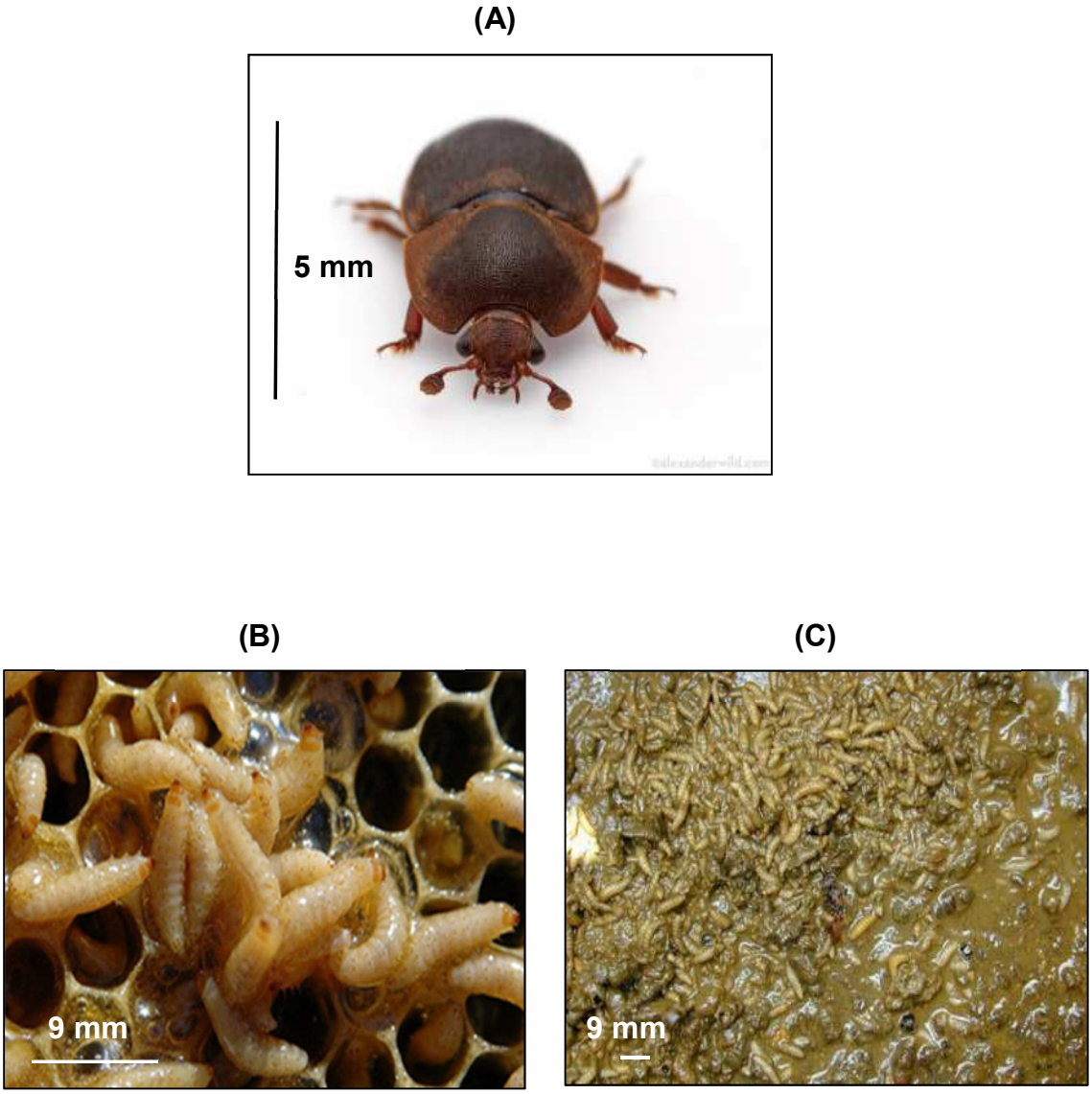



**Figure 1.1. (A)** *Aethina tumida* adult and (B) larvae. (C) Damage caused by larvae resulting from their feeding activity. Scale bars are indicated for the larvae and adult beetle.

### 1.1.1 Morphology and life cycle

Nitidulid beetles can be distinguished from other similar beetles by their characteristic club-shaped antennae, shield-shaped thorax and broad flattened legs (Habeck, 2002). Adult *A. tumida* are oval in shape, average 5.7 mm in length and 3.2 mm in width, however this varies according to the availability of food and climatic conditions (Ellis et al. 2004; Ellis, 2004). Directly after emergence they are reddish-brown in colour, becoming dark brown or black when fully mature. Adult *A. tumida* are sexually mature approximately one week after emergence from the soil and it has been estimated that females can lay between 1000 to 2000 eggs in their lifetime (Ellis, 2004; Schmolke, 1974; Somerville, 2003). Female beetles lay eggs in irregular masses, which are pearly white in appearance and are approximately 1.4 mm long and 0.26 mm wide. Females will oviposit in hive crevices, directly onto pollen or brood if not hindered by worker bees (Ellis, 2004). On average eggs will hatch after 3 days, however egg hatching viability is directly affected by the relative humidity. Humidity >65 % leading to rapid egg hatching in 24 hr, with relative humidity of 34 % preventing egg survival (Lundie, 1940; Mostafa and Williams, 2002). The larvae emerge from the egg through longitudinal slits made at the anterior end of the egg and the larvae begin to feed on whatever food source is available (honey, wax, eggs and bee brood). Larvae tend to give preference to bee eggs and brood, as the protein content is higher which positively effects their growth rate. Larvae are creamy white in colour and have characteristic rows of spines on the back and 3 pairs of tiny legs near the head (Lundie, 1940). The larvae are about 1 cm in length when fully mature, with the larval period lasting on average 13.3 days inside the bee colony. Mature larvae enter a wandering stage whereby they exit the hive *en masse* in search of a suitable pupation site in the surrounding soil as close to the hive entrance as possible, however larvae have been observed migrating >200 m to locate a pupation site (Stedman, 2006). Wandering larvae burrow 10-20 cm into the soil creating smooth walled cells, where they pupate over a period of three to four weeks. Pupae are initially white in colour and darken as metamorphosis takes place. Soil moisture, temperature and type directly affect the pupation period which varies from 15-60 days; temperatures of over 10°C are required to complete their development. Sandy, moist soils and warm temperatures are ideal for *A. tumida* development, with only 23 days being required for complete metamorphism (Neumann et al., 2001; De Guzman et al., 2009). Female beetles

pupate slightly faster than males and in population development *A. tumida* display a female biased sex-ratio. Adults beetles can survive on average up to 188 days and under moderate United States of America (USA) and South African climatic conditions there may be six generations a year. However, development and reproduction heavily relies on an adequate source of nutrition and the availability of honey has a key role in adult beetle longevity (Neumann et al., 2001; Ellis et al., 2002; Neumann and Elzen, 2004).

### 1.1.2 Origin and Spread as a Pest of Honey Bee Hives

*Aethina tumida* was first described by Murray (1867) and is native to sub-Saharan Africa, with published records verifying its distributed throughout tropical and sub-tropical Africa (Lundie, 1940; Roberts, 1971; Smith 1953). In November 1996 *A. tumida* samples were collected in Charleston, South Carolina, but it was not until June 1998 that it was detected and formally identified in St Lucie, Florida USA (Elzen et al., 1999; Hood, 2000; Ellis, 2003). After this inception, apiary inspections were carried out during June and July in Florida, Georgia and South Caroline and by autumn *A. tumida* was found in all three states including North Carolina (Elzen et al., 1999; Hood, 2000). The failure to identify *A. tumida* in 1996 led to the loss of 30 000 colonies in Florida in 1998 with an estimated cost to the industry of $3 million (Neumann and Elzen, 2004). By December 1999 *A. tumida* had been found in 12 states, increasing to 26 states by 2003 and to date has been recorded in 48 states (Hood, 2000; Ellis, 2003; Neumann et.al, 2016). The rate at which *A. tumida* spread throughout USA was mainly a consequence of the movement of infested bee colonies, packaged bees and/or beekeeping equipment (Hood, 2004).

In July 2002 *A. tumida* was detected in Richmond, New South Wales, Australia and was formally identified in October of that year (Minister for Agriculture 2002; Gillespie et al., 2003). In April 2003 *A. tumida* was detected in Queensland and hive inspections revealed that they had started spreading throughout the state (Somerville, 2003). Thereafter they were detected in Victoria on several occasions (2003, 2005 and 2007) and in 2007 *A. tumida* was detected and has now become established in honey bee colonies at Kununurra, Western Australia (Manning, 2008). Further incursions of *A. tumida* were recorded in Perth, Western Australia, in 2008, Naracoorte in Eastern

South Australia in 2012 and Renmark, South Australia, 2014 (Neuman et. al., 2016). *Aethina tumida* populations have become rapidly established, particularly along the warmer coastal strip where climatic conditions are better suited to *A. tumida* life-cycle. Establishment in South Australia has been limited by the hot dry summer, as these climatic conditions do not seem to suit their development. In Australia, heavy damage to strong colonies took approx. 4 years (2002-2006), whereas in USA it took 2 years and this time delay was thought to be a consequence of a drought Australia was experiencing at the time (Neuman et. al., 2016). Regardless, a survey in Queensland documented over 3000 colonies that had been lost to *A. tumida* amounting to economic damage of $1, 200, 000 (Mulherin, 2009).

In 2004 the larvae and eggs of *A. tumida* were identified in cages of queens imported from the USA to Portugal, but all hives were immediately destroyed (Murilhas, 2005). In 2005, *A. tumida* were introduced into Jamaica (FERA, 2010) and has subsequently spread across the island (Neuman et. al., 2016). Since the first report in 2007, *A. tumida* has become well established in at least eight states of Mexico. In 2012 *A. tumida* was confirmed in Cuba, with no effect on local bee population to date, owing to low infestation rates. In 2013 they were detected in El Salvador, but it is unclear whether *A. tumida* is established in the country or localised (Del Valle Molina, 2007; Arias, 2014; Milian, 2012). In Canada there has been several outbreaks: 2002 (Manitoba), 2006 (Alberta and Manitoba), 2008 (Quebec and Ontario), 2009 (Quebec) and 2013 (Ontario) (Clay 2006; Neumann and Ellis 2008; Giovenazzo and Boucher 2010; Kozak 2010; Dubuc 2013), with the only established population (since 2010) being reported in Ontario (Neuman et. al., 2016). In September 2014 *A. tumida* was detected in South West Italy (Mutinelli et al., 2014) and by November 2016, the infestation of 40 apiaries in the region of Calabria was confirmed. In June 2014, an outbreak was documented in Lupon, Philippines, causing the collapse of managed colonies of European honey bees (Brion ,2015). In 2015 adult *A. tumida* was detected in an apiary in Piracicaba, São Paulo State, Brazil and urgent measures are underway to determine the extent of the outbreak (Hasan et al., 2017). Detections not establishments have been reported in Egypt (2002); Sudan (2007) and Hawaii (2010) (Ellis et al., 2004; Hassan and Neumann, 2008; Stephen, 2013). Figure 1.2 shows the current global distribution of *A. tumida*.

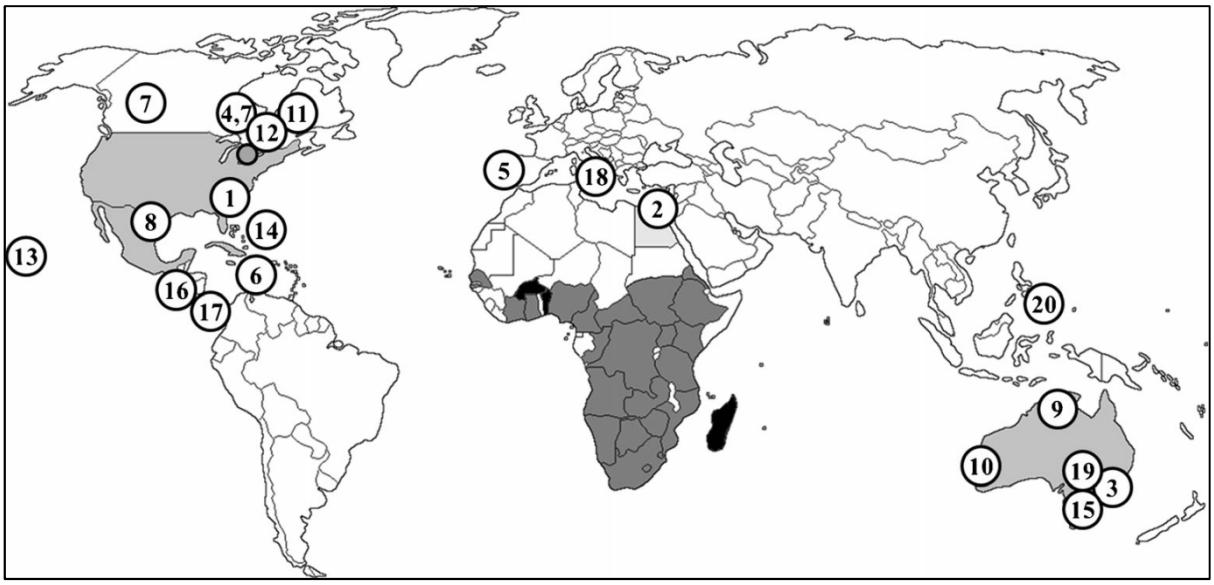



**Figure 1.2.** Current global distribution and reported introductions of *Aethina tumida* up to November 2015. Dark grey area depicts endemic distribution range in sub-Saharan Africa, medium grey areas shows countries with well-established invasive populations, light grey area indicates the not well-established population, black area shows new records in endemic range and introductions are circled in white (Source: Neumann et.al, 2016).

## 1.2 Legislation within EU and UK to mitigate the introduction of *Aethina tumida*

*Aethina tumida* was exotic to Europe until 2014 where it is considered a notifiable pest under European Union (EU) and United Kingdom (UK) legislation (Council Directive 92/65/EEC; Commission Decision 2004/216/EC) and a World Organisation for Animal Health (OIE) listed infestation (OIE, 2014). This dictates that any identification of *A. tumida* must be reported to national competent authorities, to the European Commission and to the OIE. Upon identification of *A. tumida*, Member States (MS) of the EU must implement passive surveillance programmes and in the case of detection, contaminated apiaries should be destroyed. The confirmation of *A. tumida* presence in Italy led to the implementation of formal protective measures by the EU (16 December 2014; Commission implementing decision 2014/909/EU). This document outlined that Italy must immediately prevent the dispatch of shipments of honey bees; bumble bees; unprocessed apiculture by-products; beekeeping equipment; and comb honey intended for human consumption from the entire territory of Calabria and some regions of Sicily to other areas of the EU. This document also stipulated that inspections and epidemiological investigations needed to be conducted immediately in the infested area (Mutinelli et al., 2014).

Import of queen bees and particularly package bees offer the greatest potential for the spread of *A. tumida* (Brown et al., 2002). Import of honey bees into the UK are only permitted if stringent health certification is met and imports from countries where *A. tumida* is present, except for Australia, is prohibited. In the case of Australia, imports are only permitted from a certified limited area that is at least 100 km from any location where *A. tumida* are known to be present (Marris et al., 2012). Imports of honey bees from countries outside the EU are restricted to queen bees and no more than 20 attending workers. Packaged bees from any other country outside the EU are also prohibited, except for New Zealand under a derogation of the Commission Decision 2006/855/EC. All honey bees imported directly into UK from outside of the EU must enter through one of two designated Border Inspection Posts, whereby they are inspected by Veterinary Officers (Marris et al., 2012; Defra 2015). In contrast there are no border inspection points for the checks of EU imports and the requirement is that 50 % of the paperwork and 10 % of consignments are physically checked, however most of the inspections are conducted when the honey bees/bumble bees have reached their final destinations (Brown, 2006; Bee Health Policy, 2009; NBU, 2010).

Another pathway for the movement of *A. tumida* into the UK is through the import of bumble bees for pollination purposes. *Bombus terrestris* is the species imported for this purpose (not know to be a host for *A. tumida*), however *Bombus impatiens* have been identified as an alternative host. Given the opportunist nature of *A. tumida*, the import of bumble bees is legislated in the same manner as the import of honey bees. Soil imports have been implicated as the primary mode of introduction of *A. tumida* into Australia (White, 2004). As such soil and growing media containing soil is prohibited from countries outside of the EU other than from Egypt, Israel, Libya, Morocco and Tunisia. The exception to this is if soil is required to maintain a plant and then documentation is required stating that the necessary phytosanitary measures have been met and that the soil is free of harmful organisms (Plant Health Directive 2000/29/EC, 2009). *Aethina tumida* is able to successfully live and reproduce on rotten and fresh Kei apple (*Dovyalis caffra*), avocado, banana, melon, grapefruit, pineapple, mango, grape and orange (Eischen et al., 1999; Ellis, 2002; Buchholz et al., 2008). Thus, fruit imports present another avenue for the introduction of *A. tumida* and it has been speculated that this beetle arrived in Italy on ripe fruit (Mutinelli et al., 2014). Fruit imports are subject to plant health inspections but as *A. tumida* is not a plant pest they are not specifically looked for. No limitations have been put on the movement of fruit from countries where *A. tumida* is present (Plant Health Directive 2000/29/EC, 2009).

## 1.3 Climatic condition in UK suitable for *Aethina tumida* survival?

Environmental temperatures are a crucial factor in governing the successful establishment of *A. tumida* in a new geographical area. It has been reported that development of any life stage is arrested when temperatures are below 10 ºC or above 35 ºC and exposure to temperatures below -1 ºC for an hour results in mortality (Meikle and Patt 2011; Bernier et al., 2014). Ideal temperatures for completion of *A. tumida* life cycle ranges from 17 ºC to 25 ºC. Hence climatic conditions within much of the UK meet the developmental needs of *A. tumida* (Brown et al., 2002). Survival and establishment of *A. tumida* is also influenced by soil moisture, soil temperature and soil type. Somerville (2003) and Stedman (2006) have reported that soil moisture needs to be above 5 % to allow for successful pupation, and for much of the year most European countries, including the UK, have soils that meet this saturation requirement (EFSA, 2015). Soil temperatures below -1 ºC at a depth of 20 cm would result in the death of *A. tumida* pupae, preventing completion of the life cycle (Pettis and Shimanuki, 2000). With reference to the UK and shown in Figure 1.3, the average soil temperature at 30 cm is approximately 5 ºC, which is adequate for *A. tumida* to overwinter (Brown et al., 2002). Pupation can occur in any soil type, however for course textured soils moisture levels can drop below 5 %, within the top 10 cm. Pupation success tends to be higher in sandy loam soils and many areas within the UK meet these requirements (Brown et al., 2002). Furthermore, Hood (2000) and Neumann and Elzen (2004) have documented *A. tumida* ability to overwinter within a hive, utilising the warmth and food within the honey bee colony cluster. This has been further demonstrated by *A. tumida* ability to survive colder climates in areas of North America.

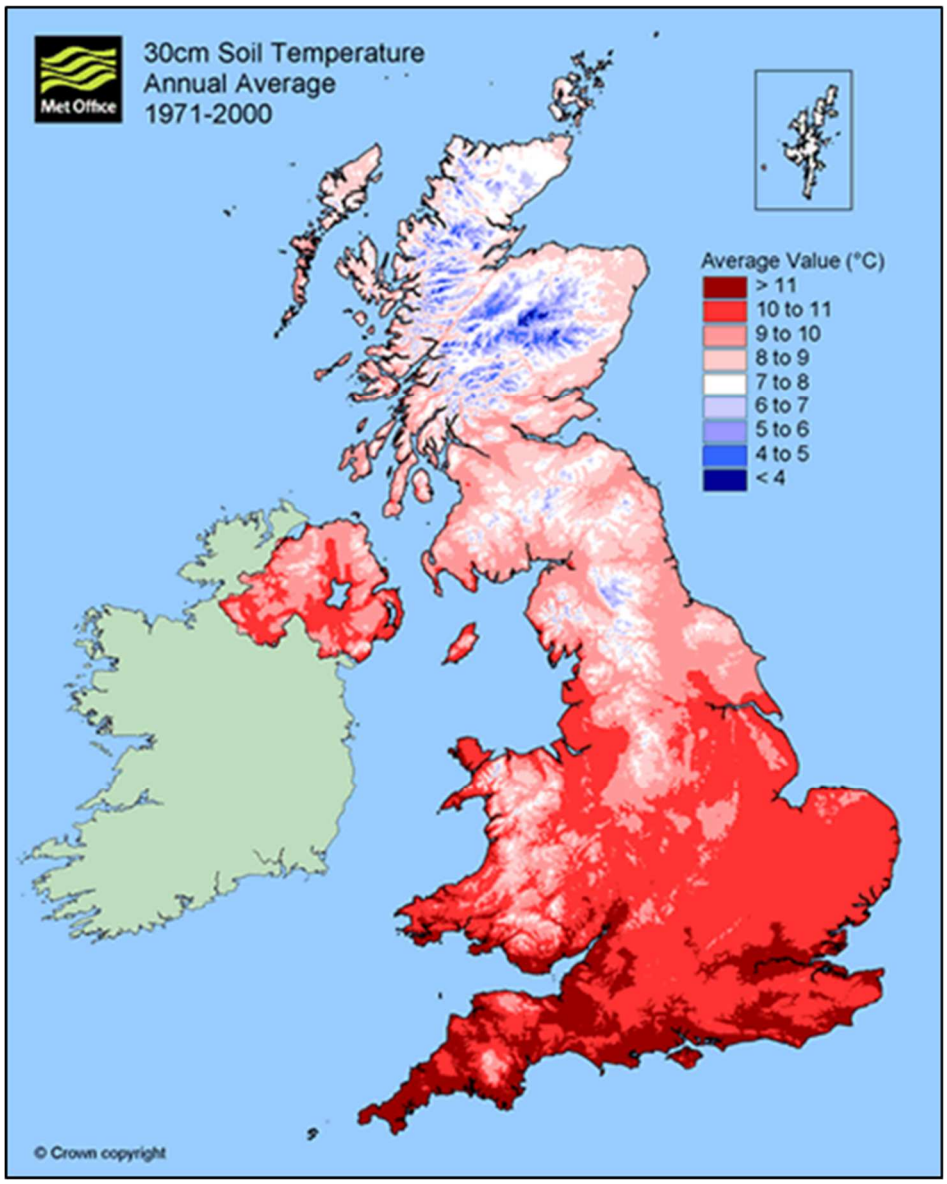



**Figure 1.3.** The annual average 30 cm soil temperature in the UK from 1971-2000 (Source: metoffice.gov.uk).

## 1.4 Value of honey bees as pollinators

Honey bees have been managed in Europe for several hundred years, contributing to human diets through the production of honey (Crane, 1999). Both wild and managed honey bees play a vital role in pollinating numerous crops, contributing to both food security and the economy (Potts et al., 2010). Over the last 46 years, the total global land area under cultivation has increased and during this time there has been a decrease in the land used for non-pollinator crops compared to an increase in the land dedicated to pollinator dependant crops (Aizen et al., 2008). In the developing world agricultural reliance on pollinator dependent crops has increased by >300 % since 1961, which has surpassed the global increase (approx. 45 %) in honey bee colonies, placing pressure on pollinator services worldwide (Aizen et al., 2009; Van Engelsdorp and Meixner, 2010). It has been estimated that 52 of the 115 leading global food crops rely on honey bee pollination for either fruit or seed set. This shift towards pollinator dependant crops is thought to be attributed to the higher economic value of these crops compared to non-pollinator dependant crops (Gallai et al., 2009).

The domesticated honey bee *A. mellifera* is considered the most economically valuable crop pollinator worldwide and it is predicted that the yields of some fruits, seeds and nut crops would decrease by more than 90 % without this pollinator (Klein et al., 2007). Managed honey bees are an ideal source of pollinators for agricultural crops as they have a relatively large work force ranging from approx. 10 000-40 000 individuals. Furthermore, they are generalist pollinators visiting a range of flower types, travelling on average 4.5 km to forage pollinating crops over a 6360 ha area and have the ability to communicate location of floral resources (Seeley, 1985). Additionally, the population within a colony can be increased via dietary supplements, which increases the overall foraging capacity of the colony thereby enhancing crop pollination in a given area. (Van Engelsdorp and Meixner, 2010).

In the UK approx. 70 % of crops are pollinated by bees. In 2009 the value of bees in the UK as pollinators of commercially grown crops was estimated to be £200 million per annum, with the value of honey production fluctuating between £10 and £35 million per annum (Cuthbertson and Brown, 2009). The stability of UK pollination services has come into question as a decline in honey bee colonies has been documented; with a 54 % decrease being recorded between 1985 to 2005 in England alone (Potts et al., 2010). Given their important role in ecosystem services, the decline in honey

bee numbers has received much attention, and this decline is thought to be attributed to several factors. The main threats to honey bee colonies within the UK are pathogens and parasites such as *Mellisococcus pluton* (European Foulbrood), AFB and *Ascosphaera apis* (Chalkbrood), microsporidian *Nosema ceranae* and *N. apis*, viral infections and pests such as the ectoparasite *Varroa destructor* and the tracheal mite *Acarapis woodi* (Klein et al., 2007). Other factors potentially responsible for the decline in honey bee numbers include the misuse of pesticides and herbicides and fragmentation and degradation of natural habitats (Kluser and Peduzzi, 2007).

The damage that *A. tumida* can cause to the apiculture industry has been documented in the USA where it has caused losses of approx. $3 million to the apiculture industry alone, just two years after the introduction of *A. tumida* (Ellis et al., 2002). In 2008 a survey conducted in Queensland showed a loss of 3000 colonies, with clean up, control and restoration costs amounting to approx. $ 1. 2 million (Mulherin, 2009). This highlights the significant risk *A. tumida* poses to UK apiculture, agriculture and horticulture through the further disruption of already fragile and declining pollinator services.

## 1.5 Current control methods-existing practises and recent research

### 1.5.1 Hive treatments

The structure of a typical honey bee hive, depicted in Figure 1.4, comprises of a screened bottom board, deep supers for the brood chamber, medium supers for the honey chamber, which is separated by a queen excluded, and outer cover placed on top of the honey super. CheckMite +$^{TM}$ strips (10% w/w coumaphos) are typically employed as a method to control the parasitic mite *V. destructor* but have been shown to provide some level of control against feeding larvae and adults of *A. tumida* (Elzen *et al*., 1999; Elzen and Westervelt, 2002; Ellis and Delaplane, 2007; Neumann and Hoffmann, 2008) (Figure 15 A). Typically, CheckMite +$^{TM}$ strips are placed underneath corrugated cardboard or plastic sheets on the bottom board. This treatment has limited efficacy against wandering larvae as even after exposure to CheckMite +$^{TM}$ for 24 hr they are still capable of burrowing into the soil, thus reducing the control of *A. tumida* outside of the hive (Ellis and Delaplane, 2007). CheckMite +$^{TM}$ together with a bottom board trap (Beetle Barn $^{TM}$), has shown to have high efficacy against adults (Bernier

et al., 2015) (Figure 15 B). The openings in the trap are large enough to allow access to adults, excluding honey bees, and once they are inside the trap contract is made with the pesticide. Coumaphos is the main pesticide used against *A. tumida* in honey bee colonies. Coumaphos belongs to the group of highly toxic insecticides referred to as organophosphates, as such no allowance for any residues in wax or honey products is allowed. Consequently, application of CheckMite +™ is labour intensive as all honey supers must be removed prior to treatment. Application rate is restricted to four times a year and upon application the CheckMite +™ is placed face down in the centre of the bottom board and is left in the colony for at least three days and a maximum of 45 days. Once the strip has been removed from the hive the supers can only be replaced after 14 days (Hood, 2000; Ellis, 2004).

In Australia APITHOR™ hive beetle insecticide was granted a temporary permit after a short-term trial demonstrating its effectiveness as a control method against adult *A. tumida* and its safety towards honey bees (Figure 15 C). APITHOR™ is composed of fipronil (0.48g $kg^{-1}$) treated corrugated card permanently enclosed in a plastic shell to prevent access or contact by honey bees (Levot and Somerville, 2012). Fipronil is a broad spectrum neurotoxic insecticide that is highly toxic towards bees and has been implicated in colony losses in France (Chuzat et al., 1999) and sublethal doses have been reported to reduce foraging and olfactory learning in honey bees (Colin et al., 2004). As such there was a great deal of controversy over the use of APITHOR™, which led to a six month trial being conducted. The results of the study showed that no fipronil or metabolite residues were found in in honey or bees wax extracts from treated colonies and no deleterious effects on honey bee health were observed. In December 2013 APITHOR™ was granted full product registration by the Australian Pesticides and Veterinary Medicines Authority, as the safety and the effectiveness of the product had been demonstrated (Levot et al., 2016).


**Figure 1.4.** Structure of a honey bee hive comprising of a screened bottom board, deep supers for the brood chamber, medium supers for the honey chamber, a queen excluded, and outer cover. (Source: https://www.beverlybees.com/wp-content/uploads/2005/09/Langstroth-Hive-Parts.jpg)

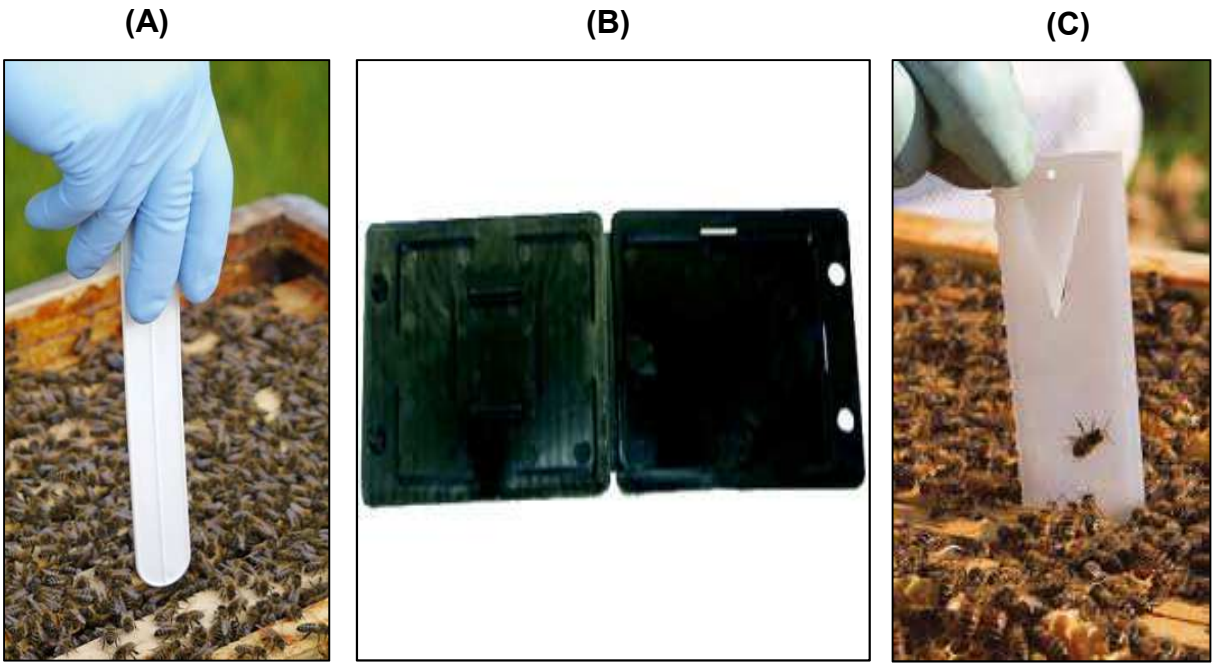



**Figure 1.5.** (A) CheckMite +™ strips (10% w/w coumaphos), (B) Beetle Barn ™ and (C) APITHOR™. (Source: https://www.animalhealth.bayer.ca/en/bees/checkmite/ ;https://gabees.com/product/m90d-beetle-barn/ and https://www.dadant.com/wpcontent/ uploads/2012/04/2011/09/Apivar-Brochure-USA.pdf.

### 1.5.2 Soil drench

Pyrethroid permethrin (sold as GardStar® in USA) applied as a ground drench, has shown to have some success at controlling *A. tumida* wandering larvae and pupae. Permethrin ground drench is widely used for *A. tumida* control as direct contact with honey bees is reduced (Delaplane, 1998; Hood and Miller, 2003). To maximise the effects of the soil drench the ground surrounding the colony is thoroughly moistened and the pesticide is applied under and around the hive in all directions extending out 90-180 cm from the colony. Permethrin is a synthetic pyrethroid insecticide, which structure is based on the natural substance *pyrethrum*, derived from dried chrysanthemum flowers (NPIC, 2009). Permethrin acts on the nervous system of insects, interfering with the sodium channels to disrupt the function of the neurons, causing muscles to spasm, resulting in paralysis and death (Tomlin, 2006). Permethrin is highly toxic to bees, as such the use of hand pump sprayers has been forbidden and if the treatment is administered when hives are present only a sprinkler can be used to avoid contact by spray or spray drift with bees or hive equipment. The drawback of using a ground drenches alone is that protection is offered to an individual hive but does very little to control the spread of *A. tumida* (Hood, 2004).

### 1.5.3 Mechanical control

Alternatives to chemical controls include the use of entrance reducers, which have been shown to significantly affect the average number of *A. tumida* invading the hive (Frake et al., 2009). Traps such as Beetle Blaster™, Beetle Eater™, West Trap™, Freeman™ and Hood Trap™ act as an adequate means to control *A. tumida* adults (Figure 1.6). The Beetle Blaster™ and Beetle Eater™ function in a similar manner, whereby they are placed in the frame top bar, filled with oil, vinegar or soapy water. The traps are filled to approx. 1/3$^{rd}$ of the trap height with the “drowning liquid” and upon entering the trap to hide from the worker bees, they drown in the solution. The West Trap™ and Freeman™ trap are positioned onto the bottom board and contain a shallow basin of oil, which is covered by a honey bee excluder. Similarly, the adult beetles will enter the traps to escape from worker bees and drown in the oil solution (Hood, 2011; Zawislak, 2014). The Hood Trap™ also serves as a mechanism to drown adults. It is attached to the side of a beehive frame and has three compartments that

are filled with apple cider, serving as an attractant, or oil. This trap tends not to be as efficient as the previously mentioned traps as due to is position on the frame it is visited less frequently and the space around the trap often gets filled with drone brood (Hood, 2011; Zawislak, 2014). Another method being employed in Australia is the use of disposable cleaning cloths know as Chux® Superwipes®. The cloths are folded and secured using a queen excluder to the top of the frames in the brood box, workers bees attack the cloth creating a fibrous material and when *A. tumida* adults are chased by the workers bees, they seek refuge in the folds of the cloth becoming imprisoned in the material (EFSA, 2015).

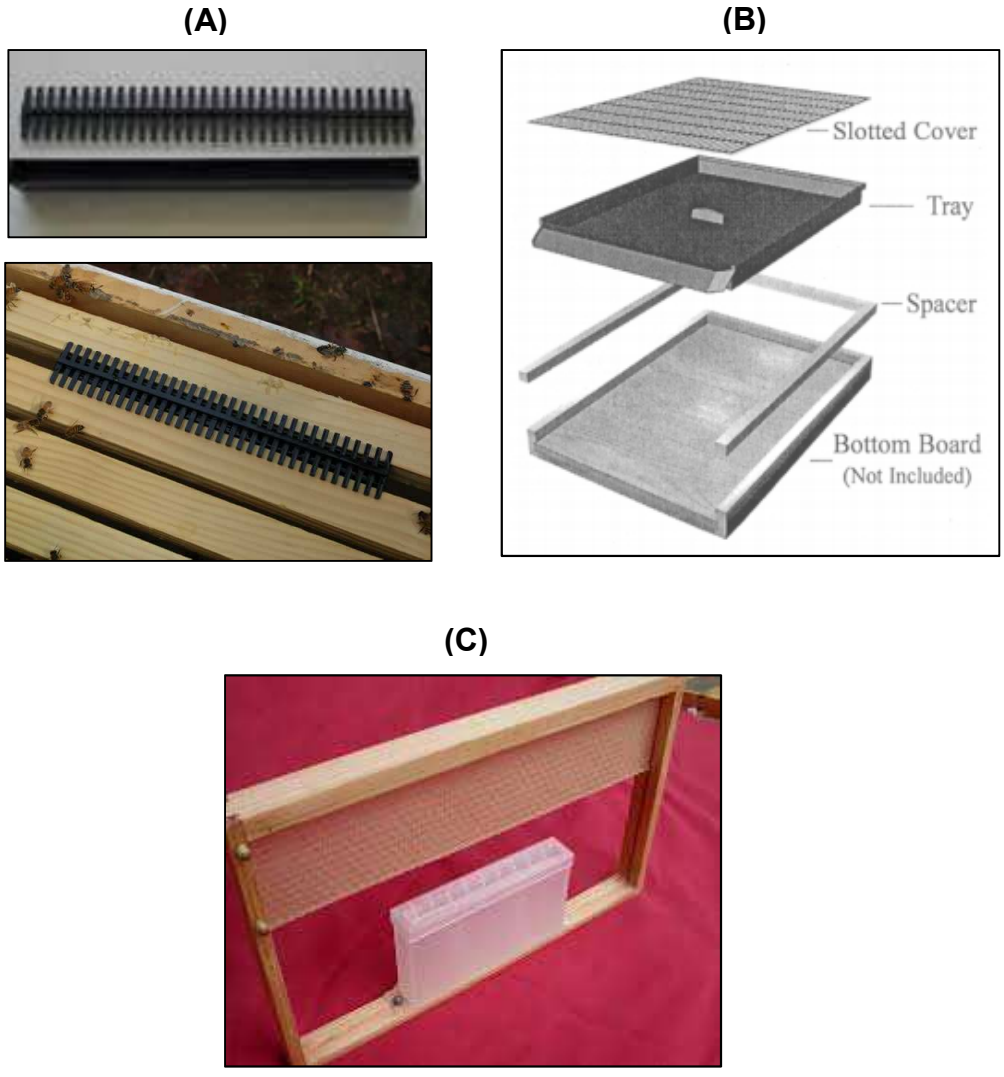



**Figure 1.6.** (A) Beetle Eater™, (B) Freeman™ trap and (C) Hood Trap™. (Source: Animal and Plant Health Agency, 2017).

### 1.5.4 Biological control

Lundie (1940) noticed during the laboratory rearing of *A. tumida* that a proportion of larvae that entered the soil to pupate did not emerge as adult beetles. Upon closer examination of the larvae an unknown pathogen was identified as the possible cause of the mortality. Ellis et al. (2004) identified five fungal species isolated from the pathogen-killed pupae: two soil dwelling fungal species (*Aspergillus niger* and *A. flavus*) and three saprotrophic fungi (*Clonostachys rosea, Glioclaadium catenulatum* and *Mucor plumbeus)*. Both *A. niger* and *A. flavus* are known to attack other soil dwelling insects and it was strongly speculated that either or both of the fungi were responsible for the mortality observed. Later Richards et al. (2005) inoculated *A. tumida* larvae with *A. niger* and *A. flavus* and found that the latter fungi caused significant levels of mortality in pupae. Muerrle et al. (2006) identified a naturally occurring fungal pathogen *Metarhizium anisopliae v. anisopliae* from South African adult *A. tumida*. The newly identified fungal pathogen together with three different entomopathogenic fungal isolates *(Beauveria bassiana*, *Metarhizium anisopliae* and *Hirsutella* illustris) were tested against adult *A. tumida*, with 74 % and 28 % mortality being recorded in *B. bassiana* and *M. anisopliae v. anisopliae* treated beetles, respectively. Leemon and McMahon (2009) screened a range of isolates of *B. bassiana* and *M. anisopliae* against both larval and adult *A. tumida*. The results indicated that *B. bassiana* isolates tend to be more effective against adults (up to 100 % mortality), whereas *M. anisopliae* are more effective against larvae, with over 90 % mortality being recorded. *Metarhizium anisopliae* spores were subsequently added to a colony and it was found that there were short-term negative effects towards adult honey bees and they tended to remove the spores from the hive (Leemon and McMahon, 2009). These results show great promise for the use of fungal pathogens as a benign control method. However further screening of entomopathogenic fungal is needed to develop an efficient mycoinsecticide for *A. tumida*.

The use of commercially available entomopathogenic nematodes *Steinernema carpocapsae*, *S. riobrave* and *Heterorhabditis megidis* against wandering larvae was investigated by Cabanillas and Elzen (2006). The authors reported the larvae to be susceptible to all three nematodes species and it was suggested that mortality may be enhanced by targeting the pupal stages of *A. tumida*. More recently Cuthbertson et al. (2012) demonstrated that application of *S. kraussei* and *S. carpocapsae,* resulted in

100 % mortality of wandering larvae directly exposed to the entomopathogenic nematodes. The authors also demonstrated that sequential applications of nematodes to the pupation substrate, following larvae entering the sand to pupate, significantly reduced adult emergence for up to 3 weeks. Entomopathogenic nematodes have enormous potential to form an integral component of Integrated Pest Management (IPM) strategies for *A. tumida*, but further improvements are required to make their application simple and cost effective (Bell, 2016).

## 1.6 Available chemical control methods in the UK

Effective pest control in the UK is coming under increased pressure due to the loss of effective chemical control options resulting from withdrawals arising from EU Directive 91/414, therefore limiting the range of products that could be employed in the event of *A. tumida* outbreak. The recent replacement of this directive with the Plant Protection Products Regulation (Regulation EC No 1107/2009), which introduced hazard-based cut-off criteria, is likely to further restrict the availability of chemical control products. All chemical compounds must be authorised under Regulation EU No 528/2012 prior to being made available on the market and must show efficacy towards the targeted pest without a risk to the environment, humans and animals (EFSA, 2015).

Currently, no veterinary medicines have been authorised for the control and/or eradication of *A. tumida* in Europe or the UK. Any product that is placed in a hive to control pests or pathogens are regarded as medicines and therefore must be registered under the Veterinary Medicines Regulations 2013 to ensure the products are safe to the user, colony and the environment. There are seven in-hive treatments that are approved exclusively for the control of *V. destructor* including, two synthetic pyrethroids, containing either tau-fluvalinate or flumethrin and the remaining five containing naturally occurring materials such as thymol, essential oils, oxalic acid and formic acid (APHA, 2017). Apistan™, contains tau-fluvalinate, is the only product that has shown to be toxic to feeding and wandering larvae, however no effects on adults *A. tumida* have been reported. However, Apistan™ is not licensed for use as a control method for *A. tumida* and would require a Special Treatment Authorisation, to be issued by the Veterinary Surgeon (EFSA, 2015). CheckMite +™, used for the control

of *A. tumida* in USA and Canada is authorised in six MS for the control of *V. destructor*, therefore it could be used for control in the UK, however it would also require a Special Treatment Authorisation prior to use (EFSA, 2015; FERA, 2014).

Of the 45 compounds approved for use in UK agriculture approx. 27 compounds (spinosyns, pyrethroids, carbamates, neonicotinoids and organophosphates) have traits that could be useful in the control of *A. tumida*. Several of these compounds show efficacy against some coleopteran pests, however these compounds are not registered for use as a soil drench against *A. tumida* and they are mostly not suitable for application to soils. (Bell, 2016). Synthetic pyrethroid permethrin ground drench is widely used for *A. tumida* control as they are highly effective against larvae, pupae and adult life stages and the mode of application minimises direct contact with honey bees. However permethrin is not approved for use within the EU (Delaplane, 1998; Hood and Miller, 2003). Currently there are 12 pyrethroids approved for use in UK agriculture, with only tefluthrin and lambda-cyhalothrin having the potential to be used as soil drenches for the control of *A. tumida*. Tefluthrin when used as a seed treatment is effective against the click beetle larvae (wireworms) and corn root worm (*Diabrotica spp.*), furthermore it has been suggested that this product has the potential to be applied to the soil for the control of these insect pests (Schwarz et al., 2002; van Herk et al., 2015). Similarly, lambda-cyhalothrin can be formulated for soil application and when used as a seed treatment is effective against click beetle larvae (Solomon and Emosairue, 1999; van Herk et al., 2015). In Italy, a 1 % solution containing two pyrethroids, cypermethrin and tetramethrin, has been used as a soil disinfectant (Mutinelli et al., 2014). Tetramethrin is not approved for use within the UK, whereas cypermethrin is suggesting that this insecticide could be used as a primary candidate in the absence of permethrin which is routinely used in USA and Australia. The major drawback of the above-mentioned pyrethroid is that it can be highly toxic to honey bees/non-target invertebrates and there is the potential for watercourse contamination if exploited as a ground drench (EFSA, 2015).

The use of pyrethroids as soil treatments is not considered within the framework of EU legislation on biocide products and safety of a given product in the soil environment would need to be considered. However, where a pyrethroid has been approved for insecticidal use, but not as a product to control *A. tumida* via soil treatment, special provision could be applied for under Article 55 or 56 of Regulation (EU) No 528/2012

(EFSA, 2015). As there are no immediate eradication options available in the UK, unless *A. tumida* was detected shortly after arrival, there is a high likelihood that eradication would be impossible due to the lack of chemical control products immediately available on the UK market.

## 1.7 Development of novel bio-pesticides: RNA interference

RNA interference (RNAi), is a post translational gene silencing phenomenon mediated by exogenous or endogenous double-stranded RNA (dsRNA). This mechanism is highly conserved in eukaryotes and has been widely used as a means to elucidate gene function (Hannon, 2002). RNAi silencing is a consequence of dsRNA molecules being taken up from the immediate environment by cells located for instance in the gut or haemocoel. If the signal then spreads from these cells to the neighbouring cells and tissues, then systemic RNAi is triggered (Winston et al., 2007; Gu and Knipple, 2013). Systemic RNAi was thought to only occur in plants and nematodes, however research over the past 20 years has revealed that systemic transfer of the silencing signal occurs in arthropods. Consequently, RNAi is increasingly being recognised as having potential application for the control of insect pests, where genes vital to survival or development are targeted for down-regulation can result in death of the pest organism. RNAi has been proposed as a novel insecticidal strategy, as the high sequence specificity of RNAi predicts that the effects on non-target organisms will be minimal, if any (Gu and Knipple, 2013; Scott et al., 2013; Bachman et al., 2013).

The efficacy of RNAi varies among insect Orders and the success of this technology as a control method for agricultural pests depends heavily on target specificity, gene-silencing efficiency and systemic spread of the silencing signal. Arthropods are a diverse group of organisms, many of which are economically significant agricultural pest, vectors of livestock disease and parasites of commercially managed honey bees. Therefore, control using RNAi has been evaluated for a range of arthropod species, including Coleoptera (Western corn rootworm*, Diabrotica virgifera virgifera*; Southern corn rootworm, *Diabrotica undecimpunctata howardii;* Colorado potato beetle, *Leptinotarsa decemlineata;* the red flour beetle, *Tribolium castaneum*), Diptera (fruit fly, *Drosophila* spp.; tsetse fly, *Glossina morsitans morsitans*; the housefly, *Musca*

*domestica*; cabbage root fly, *Delia radicum*), Hemiptera (pea aphid, *Acyrthosiphon pisum*; Triatomid bug, *Rhodnius prolixus*), Isoptera (eastern subterranean termite, *Reticulitermes flavipes*) and Lepidoptera (light brown apple moth, *Epiphyas postvittana*; cotton bollworm, *Helicoverpa armigera*; tobacco hornworm, *Manduca sexta*; diamondback moth, *Plutella xylostella*), with varying levels of success (Bischoff et al.,2006; Fujita et al.,2006; Hossaini et al., 2008; Zhou et al., 2008; Walshe et al., 2009; Aronstein et al., 2011; Powell et al., 2017). Honey bees are among the few recognised beneficial insects with large economic and ecological impacts and RNAi is increasingly being used as a commercial and research tool for fighting pests and pathogens in apiculture. For example, the administration of dsRNA has been successfully applied to lower infection levels of several pathogens, such as the intracellular parasite of honey bee adults, *Nosema ceranae*, *V. destructor,* responsible for vectoring DWV, Israeli Acute Paralysis Virus and Chinese Sacbrood Virus, however these are yet to be commercialised (Maori et al., 2009; Campbell et al., 2010; Paldi et al., 2010).

### 1.7.1 A brief history of the discovery of RNAi

The first report of an RNAi type phenomenon was by Napoli et al. in 1990, where they studied chalcone synthase (CHS), which is an enzyme involved in the biosynthesis pathway responsible for petunia colouration. In an attempt to produce purple petunia flowers, CHS was over-expressed and this unexpectedly generated white petunias. Transcript analysis revealed that the levels of endogenous and introduced CHS were 50-fold lower than wild-type petunias. It was hypothesised that this effect was a consequence of the introduced transgene suppressing the endogenous CHS gene and this phenomenon was termed “cosuppressing”. In 1992, Romano and Macino reported the same phenomenon in *Neurospora crassa* (ascomycete fungus), noting that introduction of portions of the *albino-3 (al-3)* and *albino-1 (al-1)* genes sequences caused a reduction in the expression levels of the endogenous gene. The term “quelling” was coined to describe this process in fungi (Romano and Macino 1992).

Several years after these unexplained gene silencing phenomena were described in plants and fungi, Guo and Kemphues (1995) observed the same phenomenon in

animals. It was observed that the introduction of sense or antisense RNA encoding for par-1 mRNA resulted in degradation of the par-1 message in the nematode *Caenorhabditis elegans.* At that time, the introduction of antisense RNA was favoured as it was thought to act by hybridising with endogenous mRNAs resulting in the formation of double-stranded RNA (dsRNA), which either inhibited translation or marked RNA for degradation. However, control experiments using only the sense par-1 RNA, which was thought not to hybridize with the endogenous par-1 transcript, surprisingly still targeted the par-1 transcript for degradation.

Fire et al*.* in 1998 first described RNAi and provided an explanation for the previously reported silencing of endogenous genes by “cosuppression”, “quelling” and sense mRNA. They hypothesised, using *C. elegans*, that the trigger for gene silencing was dsRNA and not single-stranded RNA (ssRNA). The findings of Guo and Kemphues (1995) were explained by reasoning that the introduction of the single-stranded sense and antisense RNA resulted in gene silencing as the preparations of ssRNA could have been contaminated with dsRNA, as the ssRNA samples were generally prepared using bacteriophage RNA polymerases. Fire and Mello (1998) tested this hypothesis by studying interference towards the *C. elegans unc-22* gene by using highly purified sense and antisense ssRNA preparations and comparing the effects to dsRNA. They showed that the introduction of purified ssRNAs (sense or antisense) was less effective than dsRNA or a sense-antisense RNA mixture targeting the *unc-22* mRNA, concluding that dsRNA and not ssRNA was the cause of this gene silencing.

The first RNAi success in insects was achieved in the fruit fly, *D. melanogaster*, and performed by *in vivo* injection of embryos (Kennerdell and Carthew 1998) and *in vitro* by soaking of Schneider Drosophila (S2) cells in dsRNA medium (Clemens et al., 2000). The majority of RNAi screens for this insect are performed with S2 cells, as RNAi is ineffective in most tissues due to an inability to take up dsRNA from the environment (Ulvila *et al.*, 2006). In 2000 the *D. melanogaster* genome was published by Adams et al. (2000) and RNAi became a popular research tool in functional genomics for this dipteran model insect. Several years after the *D. melanogaster* genome was sequenced, other insects such as *A. mellifera* (Honeybee Genome Sequencing consortium, 2006) and the red flour beetle, *T. castaneum* (Richards and Consortium, 2008), had their genomes sequenced. As such, these insects received

much attention with respect to RNAi, as a plethora of gene information became available with very little knowledge of their function. *Tribolium castaneum* soon become another model organism, as in contrast to *D. melanogaster*, they exhibit a robust systemic RNAi response that can be transmitted to their progeny (Bucher et al., 2002).

### 1.7.2 RNAi Mechanism

RNAi refers to the post-transcriptional silencing of gene expression by small non-coding RNA molecules, predominantly by the cleavage of a target mRNA in a sequence-specific manner (Fire et al.,1998). Three RNA silencing pathways are known to exist in insects which are mediated by siRNA, microRNAs (miRNAs) and piRNA (PIWI-associated RNA). The siRNA has evolved as a response to viral infection and plays a vital role in endogenous regulation of gene expression via translational suppression (Shabalina and Koonin, 2008; Ding, 2010; Gu and Knipple, 2013). The miRNA pathway uses endogenous gene products transcribed from the cell's genome and functions in the regulation of gene expression. The piRNA pathway has been identified as the main protection mechanism against the activity of transposable elements (TEs) in animal genomes and plays a role in fertility, evidenced by fertility defects in mutants lacking PIWI (Carmell et al., 2007).

The experimental use of RNAi exploits the siRNA pathway, as it makes use of the cell's natural machinery that allows for the degradation of mRNA with sequence identity to the administered dsRNA molecule. Functional RNAi machinery has two essential components. The first component is the core components within the cell, comprising of Dicer enzymes, RNA-binding factors and Argonaute protein, and secondly systemic components that are responsible for amplifying the initial dsRNA trigger, subsequently exporting it to other tissues in the organism (Price and Gatehouse 2008; Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Figure 1.7 shows a general overview of the siRNA pathway. Upon cell entry and recognition, the RNA pathway is initiated by the cleavage of long dsRNA into siRNA by dsRNA-specific RNase-III type ribonucleases called Dicers (Elbashir et al., 2001). In *D. melanogaster*, Dicer-1 is mostly used to produce miRNAs, while Dicer-2 functions is to cleave dsRNA into siRNAs (Lee et al., 2004). The siRNAs are approx. 21-23

nucleotide (nt) in length, with phosphate groups at their 5' ends and 2 nucleotide (nt) overhangs at their 3' ends (Hannon and Rossi, 2004; Meister and Tuschl, 2004). The siRNAs are incorporated into the RISC complex in conjunction with the argonaute multi-domain protein, containing an RNase H-like domain. RNase H-like domain is responsible for cleaving and removing the passenger (sense) strand from the siRNA duplex resulting in an active RISC. The remaining strand (antisense) of the siRNA duplex serves as the guide stand, directing the RISC to its targeted mRNA leading to its specific suppression and degradation (Shabalina and Koonin, 2008). RNA-dependant RNA polymerase (RdRP), interacts with RISC complex, allowing new dsRNA to be generated based on the partially degraded target template (Sijen et al., 2001). These RdRPs have been identified in gene products of eukaryotic microorganisms, fungi, plants, nematodes and the primitive vertebrate *Branchiostoma floridae*, but not in insects, molluscs or other vertebrates (Price and Gatehouse, 2008).

The RNAi effect is considered systemic if the RNAi signal spreads throughout the insect and this requires specific machinery to amplify the initial dsRNA trigger and to subsequently export it to other tissues in the organism (Price and Gatehouse 2008; Whangbo and Hunter, 2008; Huvenne and Smagghe, 2010). Amplification of the dsRNA relies on the presence of RdRP enzymes, which have been extensively researched in plants and *C. elegans*. RdRP can interact with RISC complex, allowing the production of secondary siRNAs acting as an amplification step, which greatly sustains the RNAi effect (Sijen et al., 2001; Price and Gatehouse 2008). In *C. elegans* amplification takes places in three steps. Firstly, cleavage of the long dsRNA (500 bp) by Dicer produces siRNA, resulting in a molar ratio from target to trigger increasing 20-fold. Secondary amplification of the initial signal occurs because of siRNAs being recycled after the degradation of the targeted mRNA. The third amplification step involves the antisense siRNAs with 3' hydoxy group annealing to the ssRNA (target) and the activities of RdRP enables elongation, resulting in longer stretches of dsRNA. The new dsRNA is cleaved by RNase III, or Dicer activity producing numerous siRNA allowing for the persistent and systemic RNAi effect (Sijen et al., 2001). In contrast to *C. elegans*, *T. castaneum* exhibits a very strong RNAi response, including systemic RNAi, however lacks any *C. elegans* like-RdRP, suggesting the presence of alternative mechanism(s) that elicit the amplification and spreading of the RNAi signal (Tomoyasu and Denell, 2004; Gu and Knipple, 2013).

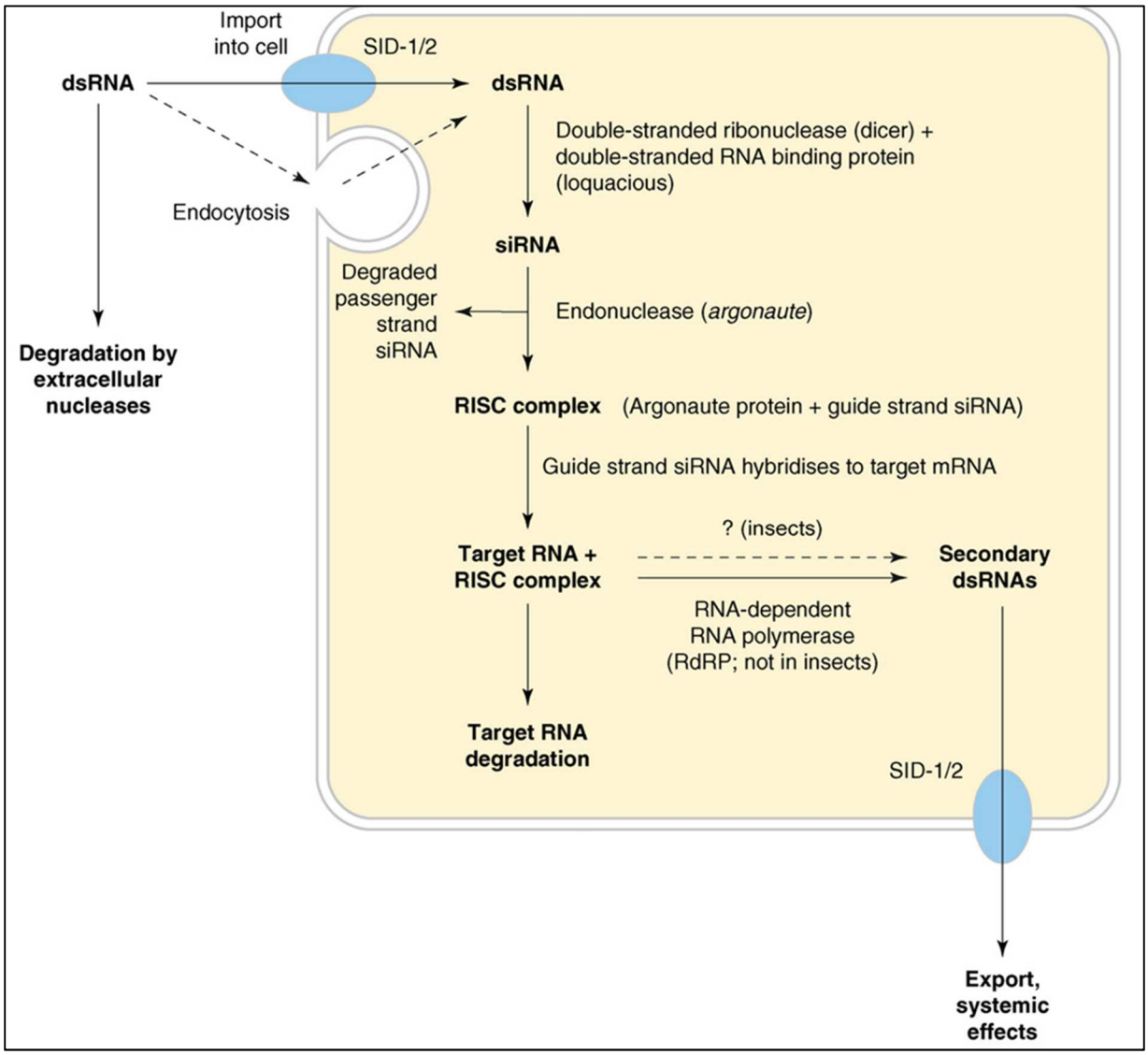



**Figure 1.7.** General overview of the siRNA pathway. Exogenous dsRNA is imported into cells, processed by dicer into small interfering RNA (siRNA; 21 bp + 2-base 30 extensions on each strand) and assembled with the argonaute protein into the RNA-induced silencing complex (RISC). The RISC complex targets and degrades specific mRNAs based on the siRNA sequence. Systemic RNAi effects are mediated through the production of new dsRNAs by RNA-dependent RNA polymerase (RdRP), which uses the target RNA as a template and is primed by siRNA strands. The secondary dsRNAs can be exported from the cell to spread the RNAi effect to other cells. Gene names in italics have been identified in *Drosophila melanogaster*. The transport proteins SID-1 and SID-2 have been identified in *Caenorhabditis elegans*, as has the RdRP enzyme. Transport mechanisms might differ between different organisms (Source: Price and Gatehouse, 2008).

### 1.7.3 RNAi Effects

The RNAi effect can occur through two different pathways, referred to as intracellular RNAi and extracellular RNAi. Intracellular RNAi involves the introduction of dsRNA into the cells using transfection or electroporation and also delivery by injection straight into a cell or a syncytial embryo (Yu et al., 2013). Whereas extracellular RNAi occurs via delivery of dsRNA via soaking, feeding or injection into the hemocoel and requires cellular uptake of the dsRNA (Yu et al., 2013). Whangbo and Hunter (2008) have further defined the RNAi mechanism into three types of RNAi response: cell autonomous, environmental and systemic, with the latter two being referred to as non-cell autonomous. Cell autonomous RNAi effects occur within the cell where dsRNA is constitutively expressed or exogenously introduced (Whangbo and Hunter, 2008). Non-cell autonomous RNAi was first documented by Fire et al. (1998) who injected dsRNA into the body cavity of *C. elegans* which spread from the site of application and resulted in the silencing of the targeted gene throughout the nematode body and its progeny. Non-cell autonomous RNAi has been further divided into environmental RNAi and systemic RNAi. Environmental RNAi silencing is a consequence of dsRNA molecules being taken up from the immediate environment by cells located for instance in the gut or haemocoel. If the signal then spreads from these cells to the neighbouring cells and tissues, then systemic RNAi is triggered (Winston et al., 2007; Gu and Knipple, 2013).

### 1.7.4 dsRNA uptake mechanism

Sensitivity to RNAi in insects remains variable between genes, organisms and life stages. This variability could be a consequence of the presence/absence of the core RNAi machinery (Miller et al., 2008), the cellular uptake of RNAi molecules and/or the spreading of the silencing effect (Xiao et al., 2012). Where systemic RNAi exists, the mechanisms responsible for the uptake of dsRNA and the systemic spreading of the RNAi effect remains undefined in different organisms (Guan et al., 2017).

Two pathways have been suggested as a means to explain systemic RNAi in insects; namely trans-membrane channel-mediated uptake and endocytosis-mediated uptake. Three trans-membrane proteins involved in the trans-membrane channel-mediated uptake mechanism have been identified in *C. elegans*, known as SID-1, SID-

2 and SID-5 (Feinberg and Hunter, 2003; Winston et al., 2002; Xu and Han, 2008). The *sid-1* gene product is responsible for mediating systemic RNAi effects. SID-1 is a multispan transmembrane protein that is expressed on the cell surface, and acts as a channel allowing passage of dsRNA between cells and thereby facilitates the uptake of dsRNA (Winston et al., 2002). SID-2 enables environmental RNAi, it is a gut-specific transmembrane protein with a single transmembrane region. It functions solely in the uptake of dsRNA from the environment via an endocytic pathway and is not involved in the subsequent spreading of the RNAi effect. The function of SID-2 was demonstrated in a related nematode (*C. briggae*), lacking the ability to take up dsRNA from the gut lumen, by transforming it with the *C. elegans* SID-2 gene, systemic RNAi phenotype was restored (Winston et al., 2007). SID-5 is an endosome-associated protein that mediates transport of both ingested and endogenous dsRNA between cells, however it is not involved in dsRNA uptake from the intestine. Thus, non-cell autonomous RNAi (environmental and systemic) is dependent on the involvement of SID-1, SID-2 and SID-5 (Hinas et al., 2012).

Homologues of the *C. elegans sid-1* gene have been identified in some insects including *T. castaneum*, *B. mori*, *A. mellifera* and more recently in aphids, but so far no *sid-2* or *sid-5* encoding genes have been found (Tomoyasu et al., 2008; Xu and Han, 2008; Zha et al., 2011). In contrast, dipterans such as *D. melanogaster* seem to lack *sid-1*-like genes altogether in their genome. Tomoyasu et al. (2008) carried out phylogenetic analysis revealing that *T. castaneum sid-1* like genes may not be orthologous to *sid-1* gene, but rather to the *C. elegans Tag-130* gene which is not associated with systemic RNAi in nematodes. This indicated that the *sid-1* gene is unlikely to play a major role in cellular uptake of dsRNA in insects. Where robust systemic RNAi responses are observed in insects such as *T. castaneum* additional or different genes with similar functions, or possibly even different mechanisms are responsible for the cellular uptake of dsRNA (Tomoyasu et al., 2008; Zhang et al., 2010).

The endocytosis-mediated uptake mechanism involves the insect cells absorbing the silencing signal from the environment via inward budding of the plasma membrane and spreading of the signal via vesicle-mediated intracellular trafficking (Tomoyasu et al., 2008; Ulvila et al., 2006; Saleh et al., 2006). This mechanism involves secretion of siRNA signal after it is produced in the form of a vesicle and the uptake of siRNAs for

the execution of siRNA silencing mechanism. This mechanism has been identified in *D. melanogaster* S2 cells, the desert locust (*Schistocera gregaria*) (Wynant et al., 2014), and the predatory mite (*Metaseiulus occidentalis*) (Wu and Hoy, 2014).

### 1.7.5 dsRNA delivery into insects

The RNAi effect can be triggered by several approaches: direct injection of dsRNA, soaking, oral delivery, topical administration and spraying of dsRNA (Aronstein et al., 2011). The preferred delivery method in the majority of insect studies relies on microinjection of dsRNA, synthesised *in vitro*, into the insect haemocoel. This method is preferred as most insects lack the enzyme RdRP which is required for the persistent and systemic RNAi effect. Microinjection allows for a known amount of dsRNA to be administered directly into the circulatory system allowing the selected cells to be targeted and short-term gene knockdown can be monitored (Price and Gatehouse, 2008). The disadvantages of this methodology are that injection into the insect hemocoel can result in cuticle damage, which in turn elicits a wounding response which can compromise gene expression investigations (Yu et al., 2013).

Soaking is not a widely used approach but has value when studying the RNAi efficacy in cell cultures. This technique was first reported in *C. elegans* and has since been used to assess RNAi efficiency in *D. melanogaster,* flatworms and nematodes (Tabara et al., 1998; Orii et al., 2003).

Oral delivery of dsRNA is convenient, causes less damage to the insect and is a natural and practical method of delivering dsRNA to an insect. Oral delivery of dsRNA incorporated into diet or transgenic plants expressing dsRNA has proved successful in achieving transcript suppression in Coleoptera (Baum et al., 2007), Lepidoptera (Baum et al., 2007; Kumar et al., 2012) and Hemiptera (Pitino et al., 2011; Zha et al., 2011). The use of RNAi has shown to significantly shorten the lifespans of a number of dipteran vector species, including the tsetse fly, *G. morsitans* (Walshe et al., 2009) sand fly, *Lutzomyia longipalpis* (Sant'Anna et al., 2008), and mosquitoes such as *Anopheles gambiae* (Magalhaes et al., 2008) However, oral delivery of dsRNA to some dipteran species has achieved less consistent results than microinjection and certain lepidopteran species require high doses of orally delivered dsRNA in order to trigger RNAi (Terenius et al., 2011). Such inconsistencies are likely to be attributed to

factors such as: stability during delivery, low or inconsistent dose taken up by the individual insect, the actual dose that reaches the midgut epithelium and inherent difference in the gut lumen of insect species potentially affecting the stability of dsRNA in this harsh environment (Scott et al., 2013).

An alternative to oral delivery has been demonstrated by Pridgeon et al. (2008) where dsRNA encoding for an inhibitor of apoptosis protein 1 gene in *A. aegypti* (AaeIAP1) was administered topically resulting in mortality of female mosquitos. More recently, Wang et al. (2011) has shown that topical application of dsRNA genes DS10 and DS28 (highly expressed during larval development) caused 40-50% mortality in the Asian corn borer, *Ostrinia furnalalis*, larvae. Furthermore, the author confirmed that dsRNA penetrated the body wall of *O. furnalalis* larvae and circulated in the body cavity by fluorescently labelling dsRNA.

Recently Miguel and Scott (2016) showed that spraying dsRNA onto the leaf surface afforded plant protection from the Colorado potato beetle (*L. decemlineata*) under green-house conditions. The observation that RNAi can be triggered by topical application, spraying or diet containing dsRNA has made this technology a potentially viable control strategy for the implementation of RNAi based pesticides (Gordon and Waterhouse, 2007).

### 1.7.6 RNAi against Coleoptera

RNAi efficacy varies from being generally high in coleopterans, to relatively low in lepidopterans. Systemic RNAi responses have been documented in the majority of coleopteran studies (Huvenne and Smagghe, 2010; Scott et al., 2013). Whereas in lepidopteran studies effects tend to vary, with a tendency towards a less efficient non-systemic RNAi responses. Where an RNAi response has been observed in lepidopterans a relatively large amount of dsRNA is required in comparison to coleopteran insects (Terenius et al., 2011). This insensitivity to RNAi is thought to be attributed to several factors: degradation dsRNA in the midgut and haemolymph, reduced uptake and transport of dsRNA, inability to process dsRNA into siRNA and the absence of core RNAi components (Arimatsu et al., 2007; Allen and Walker, 2012; Wynant et al., 2014; Garbutt et al., 2013; Kobayashi et al., 2013; Swevers et al., 2011).

As previously mentioned *T. castaneum* has a robust systemic RNAi response and the completion of its genome sequence has led to *T. castaneum* becoming an

accepted model for studying systemic RNAi in insects (Richards and Consortium, 2008). *Tribolium castaneum* sensitivity to RNAi has resulted in functional genomic screens to elucidate gene function and to identify potential pest control targets. This has allowed researchers to select candidate genes from other insects and identify orthologs via blast analysis of the *T. castaneum* genome database, BeetleBase (http:/beetlebase.org/). Biotargets identified for arthropod specific pest control include chitin/cuticle genes, chitin synthesis, molting, tanning of the epidermal cuticle and genes considered essential for survival such as *V-type ATPases* (Arakane et al., 2008; Hogenkamp et al., 2007; Zhu et al., 2008; Baum et al., 2007).

RNAi by feeding plant material expressing hairpin dsRNAs has proved to be highly effective against the Western corn rootworn, *D. v. virgifera* (Baum et al., 2007). Initially, larvae were fed on artificial diet supplemented with defined concentrations of dsRNA targeting 290 insect genes vital for survival. As a consequence of this high-throughput screening 14 genes were shown to have high insecticidal activity at low dsRNA concentrations, with gene suppression being confirmed for the corresponding genes via Northern blot analysis. For the gut-expressed gene *V-type ATPases subunit A*, mRNA suppression was complete one day after exposure to dsRNA. By contrast feeding α-*tubulin*, a housekeeping gene resulted in a slower rate of mRNA suppression and the slow onset of mortality strongly suggested the occurrence of systemic RNAi as export and amplification of the signal seemed to be taking place in this insect. Finally, a 246 nt region of the coding sequence of *V-type ATPases subunit A* was used to prepare a construct which was transformed into maize (Baum et al., 2007). In the same study two related coleopteran plant pests, Southern corn rootworm (*D. undecimpunctata howardi*) and *L. decemlineata* larvae were fed on three dsRNA targeting genes identified in *D. v. virgifera* (β-*tubulin*, *V-type ATPases subunit A* and *E*). Oral delivery of dsRNA caused mortality in *D. undecimpunctata howardi* and *L. decemlimeata* larvae, however the dsRNA concentration was approx. ten-fold higher than the dose administered to *D. virgifera*. However, when *L. decemlimeata* was fed on dsRNA based on their own sequences of *V-ATPase subunit A* and *E* the RNAi effect was enhanced when compared with the afore-mentioned feeding trial. The nucleotide sequence identities between *D. v. virgifera* and *L. decemlineata V-ATPase subunit A* and *E* were 83 % and 79 %, respectively. Non-target effects were attributed

to the identification of approx. 3 identical regions of 20-29 nucleotides in the published sequence alignment (Baum et al., 2007).

Other coleopteran pests showing sensitivity to RNAi include the striped flea beetle (*Phyllotreta striolata*), red palm weevil (*Rhynchophorus ferrugineus*), African Sweetpotato Weevil (*Cylas puncticollis*) and Asian Longhorned Beetle (*Anoplophora glabripennis*). Zhao et al. (2008) showed that feeding of dsRNA encoding for Arginine kinase, a phosphotransferase playing a critical role in cellular energy metabolism, resulted in significant impairment of *P. striolata* development. Zhao et al. (2011) went on to disrupt the host-preference of this beetle, mediated via a seven transmembrane-domain odorant receptor (Or) family. After injection of PsOr1 into *P. striolata* they were unable to sense attractant and odour stimulus, this was further evidenced by a host-preference test confirming that the suppression of the PsOr1 transcript impaired their ability to show preference towards cruciferous vegetables. In the study conducted by Laudani et al. (2017) RNAi was investigated through the suppression of α-amylase, V-ATPase and Ecdysone receptor in *R. ferrugineus* by inject and ingestion of target dsRNA. Injection and ingestion of α-amylase and Ecdysone receptor dsRNA resulted in varying levels of suppression of the targeted gene, interestingly this was not the case for V-ATPase. V-ATPase is a commonly targeted gene and RNAi silencing has been well documented in numerous insect studies. It has been suggested that the lack of an RNAi response was a consequence of targeting one of the numerous V-ATPase subunits, which was not sufficient to induce an RNAi effect, and/or it could reflect that different lineages of Coleoptera are not equally susceptible to introduced dsRNA. In *C. puncticollis* transcriptomic analysis determined the presence of core RNAi machinery. Sensitivity to RNAi was evidenced by the injection of dsRNA targeting *Laccase 2* into larvae, resulting in ineffective cuticle tanning in adults and persistent down regulation of the targeted gene was observed, indicating systemic RNAi (Prentice et al., 2015). Similarly, in *A. glabripennis* core RNAi genes were identified, and the injection of dsRNA targeting *iap* (inhibitor of apoptosis) into either larvae or adults caused gene suppression and mortality (Rodrigues et al., 2017).

In summary coleopterans tend to exhibit a robust systemic RNAi response and the sequence-specificity of this approach shows enormous potential for RNAi as a novel control method for other coleopteran pests such as *A. tumida*.

## 1.8. Development of novel bio-pesticides: Insecticidal Fusion proteins

### 1.8.1 Molecular targets for insect control

The major class of chemical insecticides act on one of six molecular targets in the insect nervous system, namely acetylcholinesterase, voltage-gated sodium channel ($Na_V$), γ-aminobutyric acid (GABA) receptor, the glutamate-gated chloride channel, the insect nicotinic acetylcholine receptors (nAChR), and ryanodine receptors (RyRs) (Fukuto, 1990; Raymond-Delpech et al., 2005). Consequently, the limited range of molecular targets for insecticides has lead to the development of resistance being reported in over 600 arthropod species (Bass and Field, 2011). Insecticidal resistance can develop in several ways via increases in metabolic detoxification resulting from an increase in esterase, glutathione S-transferase or monooxygenase levels; decreased target sensitivity and/or increase sequestration or lowered insecticidal bioavailability (Brogdon and McAllister, 1998; Feyereisen, 1995). The molecular mechanisms responsible for these increases in resistance are point mutation on the site of action in the ion channel of GABA receptor or $Na_V$ channel, mutation in acetylcholinesterase active site, amplification of the esterase gene and mutations causing the up-regulation of detoxifying enzymes (Brogdon and McAllister, 1998; Feyereisen, 1995; Hemingway and Ranson, 2000; Hemingway et al., 2004).

Insecticidal venom peptides derived from insect predators such as scorpions (Wugargiri et al., 2001), parasitic wasps (Gould and Jeanne, 1984), predatory mites (Tomalski et al., 1988) and spiders (Lipkin et al., 2002; Tedford et al., 2004) have received a great deal of interest as they provide an extensive source of highly insecticidal toxins. Many of these insecticidal toxins are active against neuronal voltage dependent sodium ($Na^+$), potassium ($K^+$), calcium ($Ca^{2+}$) or chloride ($Cl^-$ channels (Fajloun et al., 2000). $Na_V$ channels are the target of existing insecticides such as pyrethroids, dihydropyrazoles and oxadiazines and venom peptides targeting these channels could hold potential for the control of an insect population that has developed resistance to $Na_V$ channel insecticides (Raymond-Delpech et al., 2005; Smith et al., 2013). In contrast to $Na_V$ channel, voltage-gated calcium ($Ca_V$) channels re not highly conserved between insect orders and hold enormous potential for the development of biopesticides that target only the pest insect without harm to beneficial species (King et al., 2008; Smith et al., 2013).

### 1.8.2 Voltage-gated calcium channels as targets for insecticides

$Ca_V$ channels form membrane pores that open in response to membrane depolarization to allow the influx of extracellular calcium ions. $Ca_V$ channels are responsible for mediating a range of intracellular processes such as muscle contraction, hormone and neurotransmitter release, patterns of gene expression and regulation of a range of enzyme activities (Catterall, 2000). The biophysical and biochemical properties of $Ca_V$ channels are mainly defined by their $\alpha_1$ subunit and can be divided into two broad superfamilies referred to as low-voltage-activated (LVA) or high-voltage-activated (HVA) $Ca_V$ channels. LVA $Ca_V$ channels are activated by small membrane depolarization and show rapid voltage-dependant inactivation, in contrast HVA $Ca_V$ channels are only activated by large depolarizations and are inactivated at a slower rate. LVA $Ca_V$ channels are less complex than HVA $Ca_V$ channels and consists primarily of a pore-forming $\alpha_1$ subunit, whereas HVA $Ca_V$ channels typically comprising of 4-5 subunits. The pore-forming $\alpha_1$ subunit is made up of four repeat domains (I-IV) connected by an intercellular linker. Each domain consisting of six trans-membrane segments (S1-S6) and a re-entrant loop between S5-S6. S1-S4 region service as an independent voltage sensor and they are arranged in a circle, such that S5-S6 and the re-entrance loop from each domain forms the pore of the channel. HVA $Ca_V$ channels have in addition to the $\alpha_1$ subunit an extracellular $\alpha_2$ subunit; transmembrane δ subunit linked to $\alpha_2$ via a disulfide bond to form a $\alpha_2$-δ complex, an intracellular β subunit and in some cases a transmembrane γ subunit (Catterall, 2000; Kang and Campbell, 2003; Bourinet and Zamponi, 2005; Klint et al., 2012). The $\alpha_2$-δ complex, β and γ subunit are responsible for modulating activities such as the activation/inactivation kinetics and the voltage-dependence of activation of the $\alpha_1$ subunit (Doering and Zamponi, 2003). Additionally, β subunit assists in directing $\alpha_1$ subunit to the plasma membrane (Dolphin, 2003) (Figure 1.8). Both LAV and HAV $Ca_V$ channels can further be grouped according to one of three $\alpha_1$ subunit families $Ca_v1$, $Ca_v2$ and $Ca_v3$, with HVA currents being produced through $Ca_v1$, $Ca_v2$ while $Ca_v3$ channels are responsible for LVA currents (Catterall et al., 2005).

Mammalian genes encode for 10 $\alpha_1$ subunit, four β subunit, four $\alpha_2$δ complexes and seven γ subunit. In contrast insects have fewer genes coding for $\alpha_1$ subunit and in *D. melanogaster* genome only three $\alpha_1$ subunit, one β subunit, three $\alpha_2$ δ complexes and possibly one γ subunit have been identified (Littleton and Ganetzky, 2000; Rieckhof et

al., 2003; King, 2007). However, insects are able to expand the range of expressed functional $Ca_v$ channels through alternative slicing and editing (King, 2007).

In the *D. melanogaster* genome only three $\alpha_1$ subunits have been identified, Dmca1D, Dmca1A and Ca-$\alpha_{1T}$ and can be classified as $Ca_v1$, $Ca_v2$ and $Ca_v3$, respectively (King, 2007). Eberl et al. (1998) and Kawasaki et al. (2002) both demonstrated that a loss of function of Dmca1D and Dmca1A in mutant flies lead to embryonic lethality, highlighting that each of these channels have a distinct non-redundant physiological role that cannot be compensated for by another $Ca_v$ channel subunit (King, 2007). $Ca_v$ channels are less well conserved amongst insect Orders, compared to $Na_v$ channels, and comparison of *D. melanogaster* and *T. castaneum* $Ca_v1$ channel revealed that the level of amino acid identity was 74 %, which is 6 % more than the level of identity between *Drosophila* and human $Ca_v$ $\alpha_1$ subunit. The critical physiological role $Ca_v$ channels play in insects together with <68 % homology with vertebrates makes these channels highly suitable targets for the development of biopesticides (King, 2007). Additionally, the weak conservation of insect $Ca_v$ channels means that these targets can be used to selectively target specific insects, with having potentially minimal impact on beneficial insects (King et al., 2008).

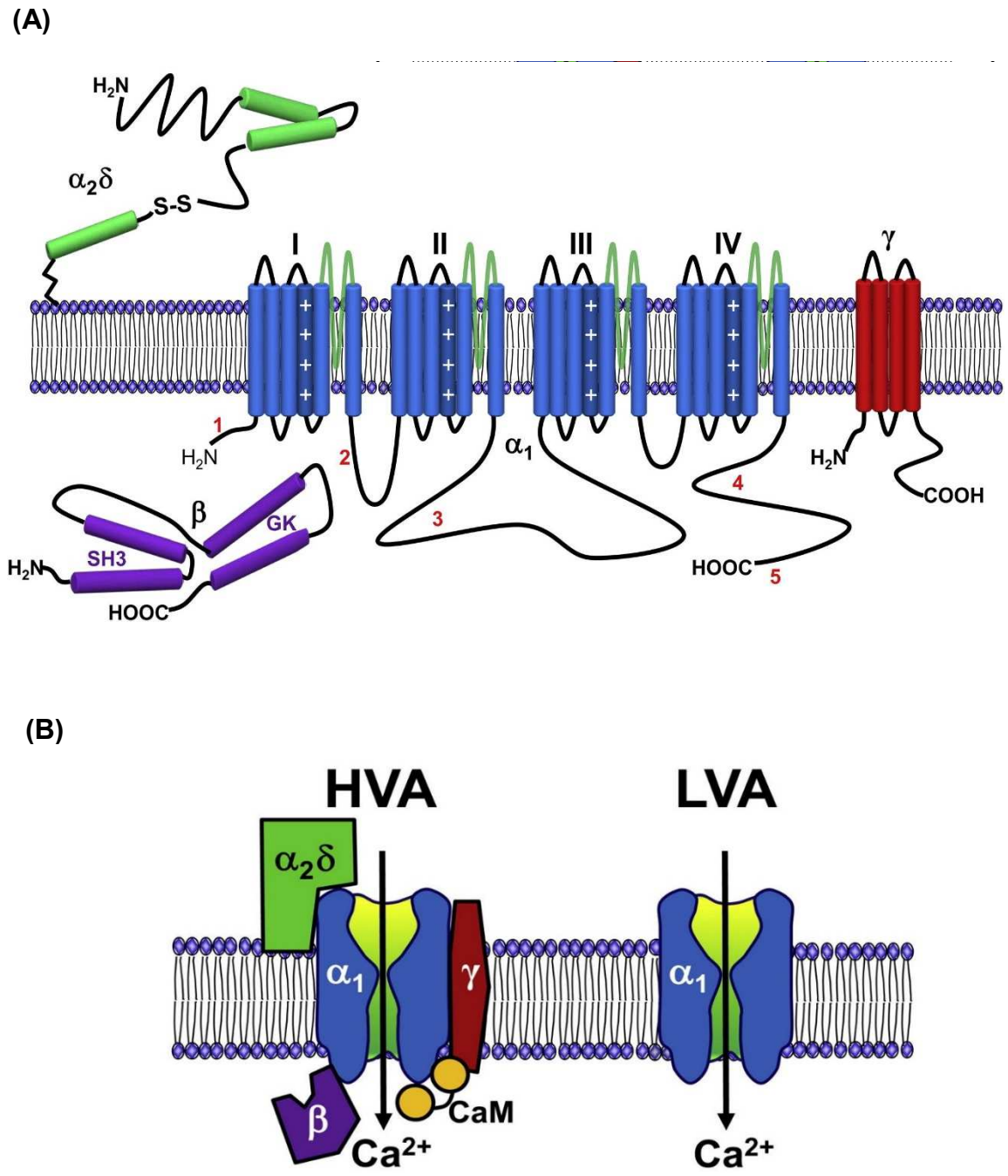



**Figure 1.8.** CaV channels structure indicating subunits. (A)Two-dimensional structure of HVA CaV channels, $\alpha_1$ subunit is made up of four repeat domains (I-IV) consisting of six trans-membrane segments (S1-S6); an extracellular $\alpha_2$ subunit; transmembrane δ subunit linked to $\alpha_2$ via a disulfide bond to form a $\alpha_2$-δ complex, an intracellular β subunit and a transmembrane γ subunit. (Source: Catterall and Few, 2008). (B) Three-dimensional structure of HVA and LVA CaV channels, indicating calmodulin (CaM) and the subunits mentioned above (Source: Simms and Zamponi, 2014).

### 1.8.3 CaV1 channel blockers – ω-Hexatoxin-Hv1a (Hv1a)

Spider venom is exceptionally diverse, and some spiders express as many as 600-1000 different peptides in a venom gland, providing a valuable source of insecticidal compounds. Spider venom is made up of a complex mixture of inorganic salts, low molecular organic molecules, disulfide-rich polypeptides and a high molecular mass protein including enzyme, which is responsible for incapacitating the central and peripheral nervous system of their prey, namely insects (Rash and Hodgson, 2002; Escoubas et al., 2006). Insect neurophysiology is partially controlled by $Ca_v$ channels and it is most likely that peptide toxins present in spider venoms interfere with the functioning of these channels (King, 2007). ω – Hexatoxin-1 (ω –HXTX-1; formerly ω-atracotoxin-1) peptides have been isolated from the Australian funnel-web spiders, *Atrax spp.* and *Hadronyche spp.* and belong to a family of peptides that consist of 36-37 amino acid residues (Fletcher et al., 1997). These peptides have been shown to be highly toxic to ticks and a range of insects, belonging to the Orders Lepidoptera, Coleoptera, Diptera and Dictyoptera, but have been shown to be non-toxic to newborn mice and have no effect on vertebrate nerve-muscle preparations (Fletcher et al., 1997; Tedford et al., 2004; Bloomquist, 2003; Mukherjee et al., 2006). Neurotoxic venoms tend to be highly toxic when injected, however they are largely ineffective when orally applied and this is due to only a fraction of the toxin being able to pass through the gut epithelium and target the site of action in the CNS (Fletcher et al., 1997).

One of the most studied spider venom toxin is ω-hexatoxin-Hv1a peptide (ω-HXTX-Hv1a formerly known as ω-atracotoxin-Hv1; referred to as Hv1a hereafter), which is a 37 amino acid residue toxin that has been isolated from *H. versuta*. Hv1a has been shown to be highly toxic to a range of arthropods, with no effect in vertebrates at doses some 10 000-fold higher than those shown to be effective in insects. Hv1a causes neuroexcitatory effects in affected insects, resulting in spastic paralysis, followed by flaccid paralysis and subsequent death. Hv1a acts on the CNS and the characteristic delay in paralysis after injection of the toxin is thought to be a consequence of the time require for the toxin to cross the nerve sheath and enter the CNS. This small peptide is able to bypass the nerve sheath, which is evident from the fact it targets the CNS and not the interganglionic or peripheral neuromuscular junction (Fletcher et al., 1997; Bloomquist, 2003). As shown in Figure 1.9 the structure of Hv1a consists of a

disordered N-terminal region (residue 1-3), a disulfide-rich globular core (residue 4-21) and a C-terminal region β-hairpin (residue 22-37) that protrudes from the disulfide-rich core (Fletcher et al., 1997). The three disulfide bonds form an inhibitory cystine knot (ICK) motif (King et al., 2002) in which the $Cys^{17}$ -$Cys^{36}$ disulfide bond is threaded through a closed loop formed by $Cys^{4}$ -$Cys^{21}$ and $Cys^{11}$ -$Cys^{22}$ disulfide bridges and the intervening section of the polypeptide backbone (Fletcher et al., 1997). The ICK motif is common in toxins isolated from venomous animals (Pallaghy et al., 1994) and provides these toxins with a high degree of chemical, thermal and biological stability affording resistance to proteolytic degradation (Saez et al., 2010; Herzig and King, 2015). Tedford et al. (2001) have shown that the β-hairpin region is vital for insecticidal action and the amino residues Pro10, Asn27 and Arg35 are responsible for the interaction of the toxin with insects $Ca_v$ channels (Tedford et al., 2004). Although the N-terminal residues are structurally disordered, two of the three residues are highly conserved across the ω-hexatoxins family. Wang et al. (1999) demonstrated that the first three amino acid residues are important for insecticidal potency, as deletions caused a reduction in insecticidal potency.

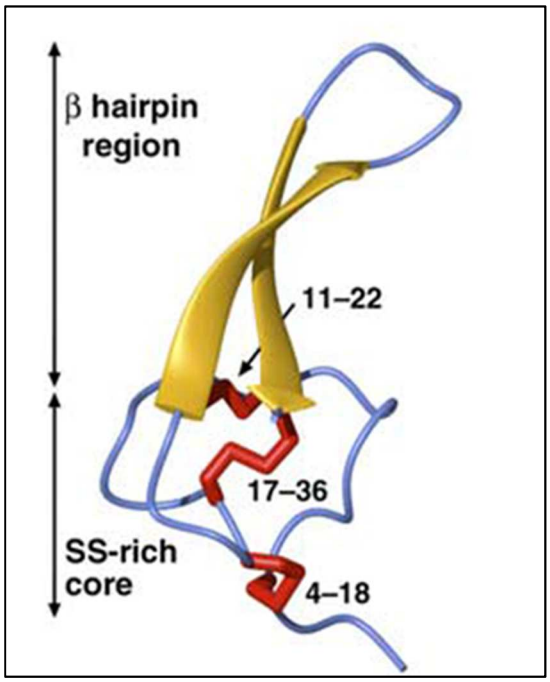



**Figure 1.9.** The structure of the ω-hexatoxin-Hv1a (ω-HXTX-Hv1a). The disulfide bonds between cysteine residues within the globular core are indicated in and the β-strands are indicated in yellow. The toxin structure can be divided into two regions: β-hairpin region and SS-rich core (Source: Tedford et al., 2004).

Tedford et al. (2007) confirmed that Hv1a targets $Ca_v1$, as *D. melanogaster* engineered to express an inducible Hv1a transgene often failed to successfully inflate and harden their wings after emerging from their puparia. In addition, this phenotype was similar to that observed in *D. melanogaster* carrying a hypomorphic Dmca1D allele ($Ca_v1$ gene) (Eberl et al., 1998). Hv1a binds with high affinity to $Ca_v1$ channels, which was evidenced by its ability to bind strongly to *P. americana* neuronal membranes and the elicitation of excitatory responses in *Drosophila* CNS preparations at subnanomolar concentrations (Bloomquist, 2003). In contrast Hv1a has been shown to have no effect at 10 µM concentration on cloned rat $Ca_v1.2$, $Ca_v2.1$ and $Ca_v2.2$ HVA channels and calcium currents in rat trigeminal neurons (Fletcher et al., 1998; Tedford et al., 2004).

As previously mentioned, Hv1a is mostly toxic to insects via injection although Mukherjee et al. (2006) reported oral toxicity against the tick *Amblyomma americanum*. In addition, Khan et al. (2006) reported that topical application of Hv1a was toxic to *H. armigera* and *Spodoptera littoralis* larvae; however, Hv1a was applied in a solution containing elevated levels of imidazole, a compound known to have contact insecticidal activity (Pence, 1965). Given their high specificity and potency towards a range of invertebrates, $Ca_v$ channel neurotoxins serve as ideal candidates for the development of novel bioinsecticides. However, potential application of Hv1a alone as a bioinsecticide is limited, without a suitable delivery system enabling transport of the toxin from the insect gut into the haemolymph.

### 1.8.4 *Galanthus nivalis* Agglutinin (GNA) as delivery agent for insecticidal peptides

Lectins are a large group of proteins, isolated from numerous organisms including bacteria, fungi, higher plants, vertebrates and invertebrate animals that bind to carbohydrates (Peumans and van Damme, 1995). Plant lectins are proteins that have at least one non-catalytic domain that bind reversibly and specifically to mono- or oligosaccharides (Peumans and van Damme, 1995). Plant lectins tend to be concentrated in seeds or vegetative storage tissues, serving as a source of amino acids for growth and development, and as a defence mechanism against biological stresses such as insect herbivory (Peumans and van Damme, 1995).

The snowdrop lectin, *Galanthus nivalis* agglutinin (GNA), is one of the most widely studied lectins and belongs to a group of mannose binding lectins isolated from the Amaryllidaceae plant family. GNA is a 50 kDa tetrameric protein composed of four identical 12.5 kDa subunits that shows specificity to α-D-mannose, particularly those containing α-1, 3 linked mannose oligosaccharides (Van Damme et al., 1987; Shibuya et al., 1988) (Figure 1.10). Although different isoforms have been isolated and cloned, typically each subunit consists of a 157 amino acid polypeptide, with a 23 residue N-terminal signal sequence and 29 residue C-terminal extension. The mature GNA lectin monomer consists of 105-residues and contains three carbohydrate binding sites (Van Damme et al., 1991).

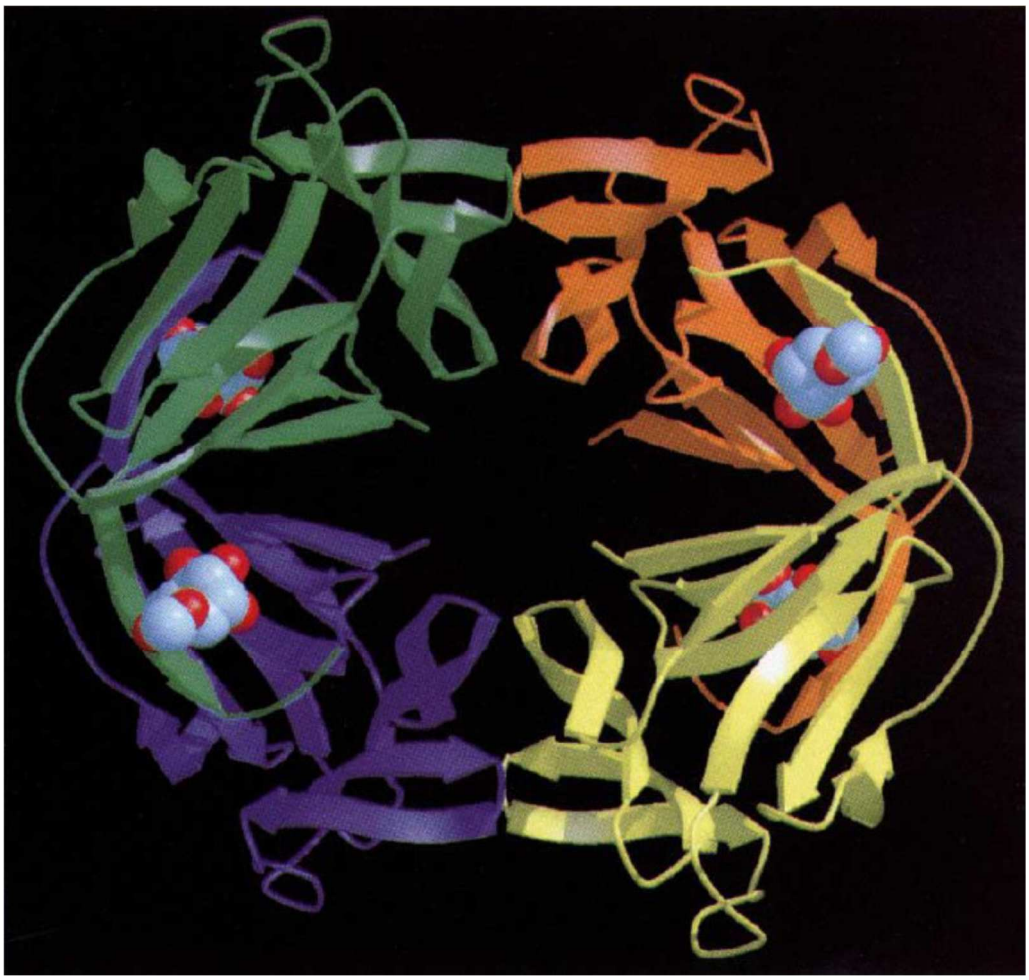


**Figure 1.10.** Structure of *Galanthus nivalis* agglutinin (GNA) tetramer; each subunit is represented by a different colour (green, orange, yellow and purple). The red and white circles represent mannose residues bound to the subunit binding site (Source: Hester et al., 1995).

*Bacillus thuringensis* (*Bt)* is a gram-positive soil dwelling bacterium that possesses entomopathogenic properties, and the insecticidal activity is due to crystal toxins being formed during sporulation in the membranes of gut epithelial cells (Angus, 1956; Morse et al., 2001). Cry and Cyt *δ*-endotoxin have shown to have high insecticidal activity against Lepidopterans and Coleopteran pests, however Hemipteran insects such as aphids, whiteflies, plant bugs and stink bugs, are not affected by these toxin (Sanahuja et al., 2011). In contrast, GNA has shown insecticidal effects against these sap-feeding insects as well as Lepidoptera, Coleoptera and Acari, having effects on fecundity, weight gain, pupation and in some cases survival (Gatehouse et al., 1996; Sauvion et al., 1996; Fitches et al., 1997; Stoger et al., 1998; Sétamou et al., 2003; McCafferty et al., 2008; Li et al., 2009). Consequently, GNA has received a great deal of attention particularly due to its toxicity towards Hemiptera and genes that encode GNA have been incorporated into potato, rice, maize, tobacco, wheat, tomato and sugarcane, affording partial protection against these pests (Li and Romeis, 2009). The toxicity of GNA, is thought to be a consequence of binding to many different glycoconjugate expressing recognised carbohydrate moieties, which causes morphological changes in the gut epithelium, resulting in the disruption of microvilli and brush border region, ultimately interfering with nutrient uptake and absorption (Powell et al.,1998; Du et al. 2000; Fitches et al., 2001; Sadeghi et al. 2008). Machuka et al. (1999) screened 16 lectins against the legume pod-borer, *Maruca virata*, with all of them effecting at larval survival, weight, feeding ability, pupation, adult emergence and/or fecundity, however GNA was deemed the most effective lectin affecting all the parameters previously mentioned. Powell et al. (1998) first confirmed that GNA binds to the gut of rice brown plant hopper, *Nilaparvata lugens* and can cross the gut barrier which was evidenced by the presence of GNA in the fat bodies, ovarioles and haemolymph. Thereafter Du et al. (2000) identified ferritin (involved in iron transport) as a receptor for GNA binding in the gut of *N*. *lugens*. Sadeghi et al. (2008) further confirmed the role of ferritin as a receptor of GNA in *S. littoralis*. Fitches et al. (2001) provided additional evidence for GNA ability to bind to the gut epithelium of the tomato moth *Lacanobia oleracea* and subsequent transport into the haemolymph, via receptor-mediated endocytosis, following oral delivery of this lectin. Additionally, Fitches et al. (2012) demonstrated that GNA delivered orally or via injection bound with high specificity to the nerve cord of the cabbage looper *Mamestra brassicae*.

Many lectins such as Black bean (*Phaseolus vulgaris*) phytohemagglutinin (PHA) and wheat germ N-acetylglucosamine-specific agglutinin (WGA) are known to be toxic towards mammals. The toxic effects can be attributed to resistance to proteolytic degradation and GNA like other lectins are not readily broken down enabling it to bind to the cells expressing recognised carbohydrate moieties (Pusztai et al.,1991). Pusztai et al. (1991) demonstrated this resistance to proteolytic degradation by feeding rats known amounts of three lectins, Concanavalin A (ConA), PHA and GNA, by subsequently recovering 90 % of the lectins from the rats faeces. In contrast to PHA and WGA, GNA is deemed to be safe to higher mammals having only a slight effect on the growth of rats at the dietary levels that would be encountered in the field (Pusztai et al.,1996). The negligible effects were thought to be attributed to the scarcity of the α-1, 3 linked mannose moieties in the brush border of the small intestine membrane of the rat, which is required for high specificity binding of GNA and subsequent interference with nutrient uptake and absorption. More recently, Poulsen et al. (2007) carried out a 90-day feeding study to assess the safety of transgenic rice expressing GNA on male and female Wistar rats. No toxic effects were recorded over the bioassay period, however several significant differences were observed between rats fed on diets containing genetically modified and parental rice, but none of the effects were considered to adversely affect clinical, biological, immunological, microbiological and pathological parameters.

### 1.8.5 Alternative carrier protein: Soybean Kunitz trypsin inhibitor-SKTI

Plant proteinase inhibitors (PIs) are small proteins, ranging from 4 to 25 kDa, that are rapidly synthesised in plant tissue as a defence molecule under stress-prone conditions such as insect attack and mechanical wounding. PIs can be divided into four main types; serine, cysteine, aspartic or metallo-proteases, based on their digestive protease activity. Lepidopteran larvae depend predominantly upon enzymes similar to the serine proteases (e.g., trypsin, chymotrypsin and elastase), whereas coleopterans typically rely upon enzymes similar to cysteine proteases for protein digestion (Schuler et al., 1998; Hilder and Boulter, 1999). PIs act by inhibiting the gut proteases of insects by irreversibly binding tightly to the active sites preventing utilisation of the ingested protein and consequently resulting in amino acid deficiency

which affects growth, development and survival (Ryan 1990; Richardson, 1991; Gatehouse et al., 1993; Solomon et al., 1999; Carlini and Grossi-de-Sá, 2002).

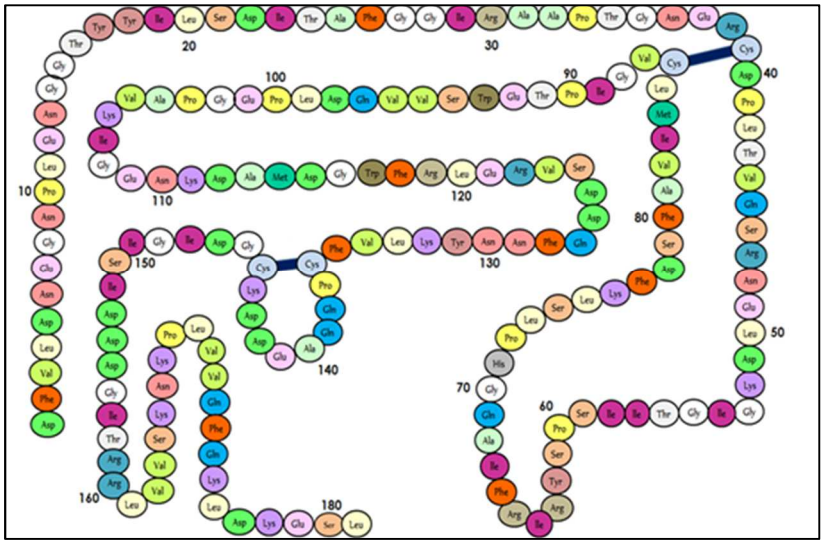
Interest in the effect of plant serine PIs (SPIs) was evoked in 1947 when Mickel and Standish observed that certain insect larvae did not develop normally when fed on soybean products (Mickel and Standish, 1947). Thereafter, Lipke et al. (1954) observed that the larvae of *Tribolium confusum* failed to develop on raw soybean inhibitors. Applebaum et al. (1964) went on to demonstrate that lima bean inhibitor, ovomucoid, soybean Kunitz trypsin inhibitor (SKTI) and soybean Bowman-Birk inhibitor (SBBI) inhibited mealworm, *Tenebrio molitar* midgut proteases known to contain both trypsin and chymotrypsin like enzymes. Additionally, Cowpea trypsin inhibitor (CpTI) has been shown to be insecticidal to a range of economically important pest species including members of the Orders Lepidoptera, Coleoptera and Orthoptera (Gatehouse et al., 1997). Direct evidence for the protective function of PIs against insects was demonstrated by Green and Ryan (1972), who showed that wounding caused by *T. castaneum* feeding on potato and tomato plants induced the synthesis and accumulation of PIs. Prior to 1987 the use of PIs was limited to the incorporation into artificial diet due to the lack of available PI sequence data. The first successful report of insect control making use of transgenic plants expressing PIs was documented by Hilder et al. (1987). Hilder et al. (1987) demonstrated that transgenic tobacco plants expressing CpTI caused 50 % mortality and stunted growth of tobacco budworm larvae *H. virescens*. Comparable effects of CpTI were subsequently observed against corn ear worm, *H. zea* (Hilder and Boulter, 1999; Schuler et al., 1998).

SKTI administered via artificial diet and transgenic plants, has shown to inhibit the development and growth of Coleoptera and Lepidoptera larvae (Gatehouse *et al.,* 1993; Johnson *et al.,* 1995). SKTI, first isolated from soybean seeds by Kunitz (1945) is a 21.5 kDa monomeric, nonglycosylated protein containing 181 amino acids. SKTI is a sphere of about 3-5 nm in diameter consisting of 12 criss-crossing antiparallel β-strands proteins that are highly resistant to thermal and chemical denaturation, with two disulfide bridges involving Cys $^{39\text{-}86}$ and Cys $^{138\text{-}145}$ critical for the inhibitory function (Steiner et al., 1965; Lehle et al., 1994; Tetenbaum and Miller, 2001) (Figure 1.11). Gatehouse et al. (1993) transformed tobacco plants with SKTI and demonstrated high growth inhibitory effects in *H. virescens* larvae. SKTI has also been transformed into

rice plants and resulted in 40-60 % mortality of brown plant-hopper *Nilaparvata lugens*, (Lee et al., 1999). Additionally, the SKTI gene was introduced into the white clover affording protection against attack by the black field cricket, *Teleogryllus commodus*, and grass grub, *Costelytra zealandica* (McManus et al., 2005). Transgenic tobacco plants expressing elevated levels of SKTI have shown greater resistance against the bollworm, *Helicoverpa armgera* than plants expressing CpTI, and this is thought to be attributed to SKTI being more effective in reducing proteolytic activity within the gut (Shukla et al., 2005).

Although PIs expressing transgenic plants can confer resistance to attack by crop pests, insects have been shown to be able to overcome toxic effects either by synthesising new proteases insensitive to the ingested PIs and/or by degradation of PIs (Brown et al., 1997; De Leo et al., 1998; Giri et al., 1998). For example, in *T. castaneum* that depends predominantly upon cysteine proteases upregulates the expression of serine proteases in response to ingestion of CPIs (Oppert et al., 2005). To overcome resistance to PIs synergistic effects of plant lectins and PIs such as CpTI are being evaluated and results have demonstrated higher mortality rates and lower larval weights against *H. virescens* in comparison to plants expressing only a lectin or a PI (Macedo et al., 2015). Zhu-Salzman et al. (2003) constructed a recombinant fusion protein encoding for soybean cysteine protease inhibitor N and *Griffonia simplicifolia* lectin II and after delivery to the cowpea bruchid, *Callosobruchus maculatus*, the fusion protein induced 100 % mortality, whilst proteins delivered separately led to on average 50 % mortality. Down et al. (1999) has shown evidence for the transport of SKTI, whereby SKTI was detected in the hemolymph of *L. oleracea*, after oral administration of the protein in artificial diet. These results suggest that the effects of SKTI could be enhanced by using it as an alternative carrier protein to GNA for the delivery of Hv1a to the circulatory system of *A. tumida*.

**(A)**



**(B)**

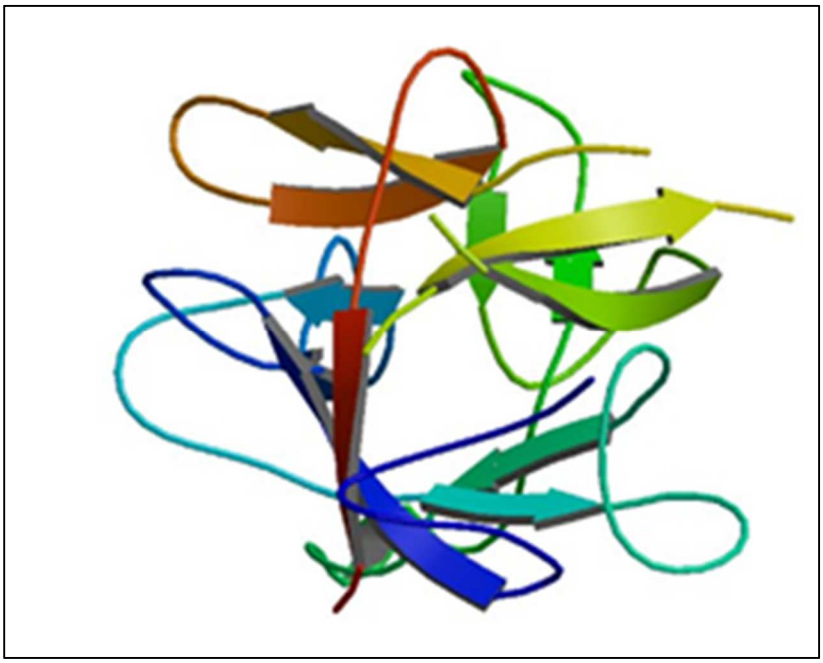


**Figure 1.11.** Structure of soybean Kunitz trypsin inhibitor (SKTI). (A) Primary structure, disulfide bonds are shown in black. (B) 3-dimentional structure, disulfide bonds are shown in blue. (Source: Onesti et al., 1991; De Meester et al., 1998).

### 1.8.6 Fusion of spider neurotoxin peptide to GNA to produce a recombinant fusion protein

Hv1a is one of the most toxic spider venom peptides towards to a range of insect pest belonging to the Orders Lepidoptera, Coleoptera, Dipteran and Dictyoptera (Tedford et al., 2004; Mukherjee et al., 2006; Khan et al., 2006). It's potency and specificity towards invertebrates, makes it an ideal candidate for the development of bioinsecticides (Bloomquist, 2003; Tedford et al., 2004; Mukherjee et al., 2006). Hv1a is toxic by injection, but typically lacks insecticidal effects after oral delivery and this has been ascribed to poor absorption by the insect and degradation of the peptide in the gut, preventing the delivery of the toxin to the action site in the CNS (Atkinson et al., 1998; Bloomquist, 2003; Tedford et al., 2004). GNA is the ideal carrier protein for Hv1a as it has shown to be highly resistant to proteolytic degradation and is able to cross the gut epithelium, (Fitches et al., 2001).

The potential of GNA as a carrier protein for the transport of insecticidal peptides into insect haemolymph, was first demonstrated by Raemaekers (2000). A recombinant fusion protein consisting of GNA fused to the green fluorescent protein (GFP), was fed to *Lacanobia oleracea* larvae and following ingestion the fusion protein was detected in the haemolymph. Fitches et al. (2002) subsequently produced an insecticidal fusion protein by fusing tobacco hornworm *Manduca sexta* allatostatin (Manse-AS; a neuropeptide hormone) to GNA. Oral delivery of the fusion protein significantly reduced growth and feeding in fifth stadium *L. oleracea* larvae. and delivery of Manse-AS was evidenced by the detection of intact protein in the haemolymph of fusion-fed insects. The use of GNA as a carrier protein to deliver fused peptides has further been demonstrated for the spider toxin SF11 isolated from *Segestria florentia* and ButaIT a toxin isolated from the red scorpion, *Mesobuthus tamulus*. Oral delivery of both fusion proteins caused decreased survival and growth in *L. oleracea*, *T. castaneum* and *M. domestica*, larvae fed on diets containing the respective fusion proteins (Fitches et al., 2004; Fitches et al., 2010). Fitches et al. (2012) showed that an Hv1a/GNA fusion protein had significant oral activity when fed to lepidopteran *M. brassicae* larvae. Furthermore, the authors provided evidence for GNA binding to the nerve cord of *M. brassicae* following injection and feeding of fluorescently labelled fusion protein, suggesting GNA can act as an anchor binding to the nerve cord, increasing the local concentration of the Hv1a peptide dramatically

enhancing the activity of the peptide (Fitches et al., 2012). Yang et al. (2014) further demonstrated the carrier and binding capabilities of GNA by fusing it to another spider venom peptide δ-amaurobitoxin-PI1a (PI1a). It was demonstrated that injection of PI1a/GNA fusion was approx. six times more toxic to *M. brassicae*, than injection of PI1a alone. Furthermore, oral delivery of the PI1a/GNA fusion protein resulted in 100 % mortality of *M. brassicae* larvae after 6 days, whereas PI1a alone showed no toxicity towards larvae after ingestion.

## 1.9. Expression of fusion proteins: The yeast expression system

The methylotrophic yeast *Pichia pastoris*, is a single-celled eukaryotic microorganism that is a capable of the post-translational modifications performed by higher eukaryotic cells such as proteolytic processing, folding, disulfide bond formation, and glycosylation. Hence *P. pastoris* has become a widely used expression system for the large-scale production of eukaryote recombinant proteins that typically end up as insoluble inclusion bodies in *Escherichia coli* (prokaryotic) expression systems (Cregg et al., 2000). Thus, *P. pastoris* is an ideal host for producing small proteins with a high content of disulfide bridges that require complex post-translational processing dependant on the presence of endoplasmic reticulum (ER) compartment, enabling correct protein folding allowing for maintenance of biological activity (Daly and Hearn, 2005). Additionally, this system is considered easy to manipulate, grows rapidly on inexpensive media achieving high cell densities expression levels and subsequently high protein yields (Cregg et al., 2000). Lectins and fusion proteins have successfully been produced in *P. pastoris* as fully active folded proteins allowing for direct recovery of soluble protein from culture media overcoming issues associated with recovery and refolding of protein from insoluble inclusion bodies from expression in *E. coli*. (Raemaekers et al., 1999; Fitches et al., 2004; 2012; Trung et al., 2006).

The expression of a foreign gene in *P*. *pastoris* consists of three steps: (a) insertion of the target gene into an expression vector; (b) introduction of the expression vector into the P. *pastoris* genome and (c) screening of clones for the expression of the foreign gene (Patrick et al., 2005). The expression of the incorporated coding sequence in *P*. *pastoris*, is driven by the glyceraldehyde-3-phosphate dehydrogenase gene (*GAPH*) promoter. The *GAPH* promotor makes use of a continuous glycerol feed

as a source of carbon and is favoured over the inducible promoter derived from the alcohol oxidase I gene (*AOXI*) which utilises methanol for induction and requires a shift from one carbon source to another to induce expression of a foreign gene (Cregg et al., 2000). The most commonly used secretory signal in commercially available *P. pastoris* vectors (pGAPZα) is the *Saccharomyces cerevisiae* α-factor prepropeptide. During post-translational processing, the signal peptide is most often cleaved from the expressed protein prior to leaving the yeast cell (Brakes, 1990). The α-factor prepropeptide used in *P. pastoris* enables proteins to be secreted into the culture supernatant and as few endogenous proteins are secreted into the media, this makes purification and down-stream processing straight forward for large-scale production (Cereghino and Gregg, 2000; Goodrick et al., 2001).

The wild-type X33 *P. pastoris* strain produces extracellular proteases during fermentation which can lead to the degradation of recombinant proteins. As such the *P. pastoris* strain SMD1168H is sometimes used to produce recombinant proteins as it is deficient in the extracellular vacuole peptidase A (pep4) responsible for protein cleavage (Gleeson et al., 1998). This was illustrated by Fitches et al. (2004) whereby expression of SFI1/GNA fusion protein in the wild type X33 strain resulted in 1:1 ratio of intact protein to cleaved GNA. Expression of the same fusion protein in SMD1168H significantly reduced but did not completely prevent proteolytic cleavage (thought to occur at or near the linker region between SFI1 and GNA) during fermentation. Similarly, Hv1a/GNA when expressed using SMD1168H resulted in the production of 75-100 % intact fusion protein, as compared to 50-60 % intact protein was recovered from the wild type strain X33. Hv1a/GNA cleavage occurs between the C-terminus of the toxin and the N-terminus of GNA (Fitches et al., 2012). The C-terminus of Hv1a, residue 33-36, has an amino acid sequence VRKC which is similar to the EKRE signal sequence present in the α-factor prepropeptide which is cleaved between the R and E by the KEX2 gene product (Fitches et al., 2012). Pyati et al. (2014) altered the 34$^{th}$ amino acid of Hv1a by point mutation from lysine to glutamine residue (K34 to Q34) and showed that the modified version of Hv1a/GNA enhanced the expression of intact fusion protein by almost 10-fold, without effecting the biological activity. Additionally, a C-terminal (His)6 tag was incorporated at the C-terminus of GNA, allowing for rapid purification by nickel affinity chromatography (Fitches et al., 2010; Pyati et al., 2014). Fitches et al. (2010) also showed that linkage of the ButaIT toxin to the C-terminus (as

opposed to the N-terminus) of GNA (GNA/ButaIT) and the incorporation of a tri-alanine linker region appeared to improve stability during expression and purification, increasing yield of intact fusion protein. Furthermore, it was demonstrated biological activity towards *S. littoralis*, *M. domestica* and *T. castaneum* was not significantly reducing, as compared to ButaIT/GNA.

## 1.10 Aims and objectives

The aims of the project were to explore potential for the development of next generation biopesticides, including RNAi and fusion protein technology, as an alternative control method for *A. tumida*. Assessment of non-target effects upon honey bee adults and larvae was also a key component of this research.

The objectives were:

1. Identification of suitable target genes and subsequent evaluation of RNAi effects in *A. tumida.* Assessment of non-targets effects against honey bees.
2. To produce and analyse the biological activity of recombinant GNA, Hv1a and the fusion proteins Hv1a/GNA, GNA/Hv1a, Hv1a(K>Q)/GNA and GNA/Hv1a(K>Q) towards *A. tumida*. Assessment of non-targets effects against honey bees.
3. Identification and characterisation of the digestive proteases of *A. tumida* feeding larvae.
4. Evaluation of SKTI as an alternative carrier to GNA to transport Hv1a into the circulatory system of *A. tumida*. To clone, express and analyse biological activity of recombinant SKTI and SKTI/Hv1a fusion proteins towards *A. tumida*.

# CHAPTER 2 | MATERIALS AND METHODS

## 2.1 General molecular biology methods

### 2.1.1 Chemicals and reagents

All the chemicals and reagents used in these experiments were supplied by VWR or Sigma unless otherwise stated. Restriction enzymes and other molecular biology reagents were supplied by Fermentas, ThermoFisher Scientific, Bioline Reagents Ltd, Promega or Qiagen, unless otherwise stated.

### 2.1.2 Oligonucleotides

Oligonucleotides used to synthesise dsRNA or expression constructs were obtained from Eurofins MWG (Ebersberg, Germany). Primers were re-suspended in molecular grade water to a concentration of 100 pmol/µl. Melting temperature (*Tm*) for the primers was determined using Eurofins MWG (Ebersberg, Germany) property check calculator. Primers were used at *Tm* 50°C for Taq polymerase and at 60°C for Phusion HF polymerase.

### 2.1.3 RNA extraction and DNase treatment

Total RNA was DNase treated and isolated from *A. tumida* using SV Total RNA Isolation System. *Aethina tumida* larvae or pupae were placed into a 2 ml tube containing 0.5 ml of 0.5 mm zirconia/silica beads (Thistle Scientific) and the amount RNA Lysis Buffer (RLA) containing 2 % (v/v) 2-mercaptoethanol added to each sample was based on a recommended ratio of 1 ml of RLA to 171 mg of tissue. Samples were homogenised using a Precellys 24 tissue homogenizer (Bertin Technologies) for 2 min at 6800 rpm. After homogenisation the RNA extraction and DNase treatment continued according to manufacturer's instructions. Prior to cDNA synthesis RNA integrity was verified by agarose gel electrophoresis and quantified using a NanoDrop spectrometer (see section 2.1.9).

### 2.1.4 cDNA synthesis

cDNA was synthesised using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). For first strand synthesis 1 µg of total RNA was added to 500 ng of oligo(dT)18 primer (TTTTTTTTTTTTTTTTTT) (Life Technologies), 1 µl 10 mM dNTP mix (10 mM dATP, dCTP, dGTP and dTTP) and sterile water to a volume of 13

µl. Thereafter the mixture was heated to 65°C for 5 min and placed directly on ice for at least one min. 4 µl 5x First Strand Buffer, 1 µl 0.1 DTT, and 1 µl of Superscript III RT (200 units/ µl) was added and incubated at 50°C for 50 min. The enzyme was subsequently inactivated by incubating for 15 min at 70°C. First-strand cDNA was quantified using a NanoDrop spectrometer (see section 2.1.9) and stored at -80°C until use.

### 2.1.5 Taq Polymerase Chain Reaction (PCR)

*Taq* DNA Polymerase was used for standard PCR reactions. PCR reactions were set up in 0.2 ml thin-walled PCR tubes in a total volume of 50 µl. A typical reaction contained 25 µl of 2x PCR buffer (10x taq buffer; 3 mM $MgCl_2$, 0.4 mM dNTP's (dATP dCTP, dGTP, dTTP ), 0.2 µM of each of the forward and reverse primer, 0.32 µl (1.25 units) *Taq* DNA Polymerase, template cDNA and molecular grade water (MGW) to a volume of 50 µl. PCR conditions were: 95°C for 3 minutes (1 cycle), 95°C for 30 seconds, 50°C for 30 seconds, 72°C for 40 seconds (35 cycles) and 72°C for 10 minutes (1 cycle). *Taq* polymerase was used for PCR other than when sequences for RNAi constructs were being amplified, in this instance Phusion Polymerase was utilised.

### 2.1.6 High Fidelity PCR

Phusion® High-Fidelity DNA Polymerase was used for the amplification of sequences for RNAi constructs. Proof reading enzyme ensures that the sequence amplified is correct as the error rate is 50-fold lower than that of *Taq* DNA Polymerase. PCR reactions were set up 0.2 ml thin-walled PCR tubes in a total volume of 50 µl. A typical reaction contained 10 µl 5x Phusion buffer, 1 µl 10 mM dNTP mix, 0.5 µM of each of the forward and reverse primer, 0.5 µl (1 unit) Phusion® DNA Polymerase, plasmid DNA and MGW to a volume of 50 µl. PCR conditions were: 95°C for 3 minutes (1 cycle), 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds (20 cycles) and 72°C for 7 minutes (1 cycle).

### 2.1.7 Agarose gel electrophoresis

To check the integrity of RNA extractions or size of PCR products agarose gel electrophoresis was used. Gels were made up with 1.2 % (w/v) Agarose Molecular

Grade in 250 ml conical flask with 1xTBE (1.1 % (w/v) Tris, 0.55 % (w/v) Boric acid, 50 mM $Na_2EDTA$ (pH 8.0) prepared in distilled water) and ethidium bromide (final concentration 0.5 µg/ml). Samples were mixed with loading dye (40 mM EDTA pH 8.0, 15 % (w/v) Ficol 400, 0.25 % (w/v) Orange G, prepared in sterile distilled water) and loaded into the gel submerged in 1xTBE buffer. A MassRuler DNA Ladder Mix (0.8-10.0 kb) was run alongside samples at room temperature, at 120 V for 40-50 min. DNA fragments were visualised under a UV light source.

### 2.1.8 Purification of DNA from agarose gel

The bands visualised on the trans-illuminator were excised from the gel using a single edged razor blade. The DNA was extracted from the gel using a QIAquick gel extraction kit according to the manufacturer's instructions. All DNA was eluted in 30 µl of the suppled buffer and stored at -20°C until use.

### 2.1.9 Quantification of isolated RNA and DNA

RNA, cDNA, plasmid DNA, ssRNA and dsRNA was quantified using Thermo Scientific NanoDrop 1000 Spectrophotometer under highly accurate UV/V is and 1-2 µl samples were analysed. MGW or Ringers solution (125 mM NaCl, 1.5 mM $CaCl_2$, 5 mM KCl pH 7.31), depending on the sample type, was used as a blank measurement.

### 2.1.10 Restriction endonuclease digestion

Restriction enzyme digestions were carried out using commercially available enzymes and were carried out using buffers and temperatures recommended by the manufacturers. The digestions reactions were typically carried out in 20-100 µl reaction, using the recommended buffer supplied and 5-10 units of enzyme per 1 µg DNA. Where double digestions were conducted optimal buffers were selected via manufacturer's guidelines. Digestion reactions were incubated at 37°C overnight.

### 2.1.11 Ligation of DNA

Ligations were carried out using 1 unit of T4 ligase and 1 µl 10x ligase buffer in a 10 µl reaction. The ratio of plasmid DNA to insert was 1:3 based on concentration. Ligation reactions were incubated at 4°C overnight.

### 2.1.12 *E. coli* transformation

One Shot® TOP10 Chemically Competent *E. coli* were used as they provide a transformation efficiency of 1 x $10^9$ cfu/µg plasmid DNA and are ideal for high-efficiency cloning. Plasmids were transformed by adding 5 µl of ligation mixture to 50 µl of competent cells and gently mixed. Cells were chilled on ice for 30 min, thereafter heat shocked for 30 seconds at 42°C and then placed on ice for 2 min. 250 µl of pre-warmed Super Optimal Broth (2 % tryptone w/v, 0.5 % yeast extract w/v, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, and 20 mM glucose) was then added and the resulting mixture was incubated at 37°C for 1 hr with shaking at 225 rpm. For each transformation 20 µl and 200 µl of culture was spread onto Luria Bertani (LB) (1 % (w/v) NaCl, 1 % (w/v) peptone, 0.5 % (w/v) yeast extract, 1.5 % Bacto agar, prepared in distilled water) plates containing carbenicillin (100 µg/ml) antibiotic and incubated overnight at 37°C. Low salt Luria Bertani (LSLB) (0.5 % (w/v) NaCl) was used in place of LB when zeocin antibiotic was used.

### 2.1.13 Bacterial cultures

To grow bacterial cultures after transformation a single colony was picked from a LB or LSLB plate or 5-10 µl of glycerol stock and grown in 5-10 ml of autoclaved sterilized LB or LSLB liquid culture containing carbenicillin (100 µg/ml) or zeocin (25 µg/ml) antibiotic. Cultures were grown overnight at 37°C with shaking at 225 rpm. Carbenicillin anti-biotic was added to sterilized agar once cooled to 40-50°C to avoid antibiotic degradation. Sterile microbiology technique was used in all cases.

### 2.1.14 Glycerol stocks of *E. coli* clones

*E. coli* clones were grown as previously stated in section 2.1.13. 750 µl of overnight culture was added to 250 µl sterile 50 % (w/v) glycerol in a 1.5 ml micro centrifuge tube, and stored at -80°C until use.

### 2.1.15 Colony PCR

Colony PCR was carried out to verify if the selected DNA insert was cloned into the vector of choice. The volumes were reduced to 20 µl, with the volumes scaled down from those previously stated in section 2.1.5. MassRuler DNA Ladder Mix was used to verify the predicted molecular weight of the cloned products. For each

transformation seven bacterial colonies and a negative control were screened for the presence of the desired insert. Colonies were picked from LB plate with a sterile pipette tip, streaked onto a second plate (incubated overnight at 37°C), thereafter the individual pipette tips were mixed in an aliquot of PCR mastermix. The bacterial cells from the colonies were used as a template for PCR, as the cells are lysed in the first cycling step, and if the correct priming sites are present it allows for the clones to be assayed for the presence of an insert. PCR conditions were: 95°C for 3 minutes (1 cycle), 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 1 min (35 cycles). PCR products were visualised by agarose gel electrophoresis and positive clones were verified via DNA sequencing.

### 2.1.16 Plasmid purification from transformed *E.coli*

Isolation of plasmid DNA from small-scale *E. coli* cultures (5-10 ml) and large-scale cultures (50 ml) was carried out using QIAprep Spin Miniprep or Midiprep Kit according to the protocol supplied by Qiagen. The DNA from small-scale cultures was eluted in 50 µl, whereas DNA from large -scale cultures was eluted in 1 ml. DNA plasmid were stored at -20°C until use.

### 2.1.17 DNA sequencing and analysis

All purified plasmids were sequenced by Eurofins MWG (Ebersberg, Germany) using appropriate vector primers. Sequences were analysed using a combination of Applied Biosystems Sequencer Scanner v 1.0, MEGA 5 software package (Tamura et al., 2011) and CLUSTALW.

### 2.1.18 Linearization of plasmid DNA

In preparation for dsRNA synthesis, plasmid DNA was linearized with restriction enzyme *XhoI* and *XbaI* to generate two ssRNA. Typically, 20 µg of plasmid DNA was linearized for a single *in vitro* transcription reaction. Each linearization reaction contained 10x enzyme activity buffer, 20 µl *XhoI* or *XbaI* and water to make up the appropriate volume. Reaction were incubated at 37°C overnight.

In preparation for yeast transformation, plasmid DNA was linearized with restriction enzyme *Bln*I. Typically, reactions were 200-300 µl in volume consisted of 6 µg plasmid

DNA, 10x enzyme activity buffer, 6 µl of *Bln*I and water to make up the appropriate volume. Reaction were incubated at 37°C overnight.

### 2.1.19 Ethanol precipitation

Nucleic acid solutions were precipitated to isolated DNA from samples by adding 10 % v/v of 3M Sodium acetate pH 5.2 and 250 % absolute ethanol. Samples were mixed thoroughly by vortexing and incubated at -20°C overnight. Thereafter samples were centrifuged at 14, 000 g for 15 min at 4°C. The supernatant was carefully removed, and the resultant pellet was washed with 200/300 µl 70 % ethanol and centrifuge at the previously described conditions. The nucleic acid pellet was placed in a SpeedVac Plus (Savant) to dry and re-suspended in an appropriate amount of Ringers solution or MGW.

## 2.2 Cloning of *Laccase 2*, *V-ATPase subunit* A and *GAPDH* for synthesis of dsRNA

### 2.2.1 Design of degenerate primers

A PCR primer sequence is considered degenerate if some of its positions have several possible bases and degeneracy represents the number of possible combination of base pairs. To identify conserved regions, selected amino acid and mRNA sequences were aligned using CLUSTALW. Degeneracy levels were kept to a minimum increasing specificity of the PCR amplification and base pair C or G was incorporated at the 3' end to allow for efficient primer extension.

Degenerate primers were designed to amplify PCR products of 629 base pair (bp), 618 bp and 547 bp for *Laccase 2*, *V-ATPase subunit A* and *GAPDH*, respectively (Table 2.1). *Laccase 2* degenerate primers were designed based on conserved regions in *T. castaneum* (GenBank accession no. AY884061.2), *Monochamus alternatus* (accession no. EU093075.1) and *Bombyx mori* BmLac2 (accession AB379590.1). *V-ATPase subunit A* degenerate primers were designed based on conserved regions in *T. castaneum*, (accession no. XM 971095.2), *M. domestica* (accession no. XM 005179917.1) and *Ceratitis capitata* (accession no. XM 004533325.1). PCR reactions were performed using *Taq* DNA Polymerase under standard conditions. Amplified products were cloned into pJET1.2 (CloneJET PCR Cloning kit) as described in the manufacturer's protocol. Purified plasmids were

sequenced by Eurofins MWG (Ebersberg, Germany). Genbank accession numbers for cloned partial *Laccase 2*, *V-ATPase subunit A* and *GAPDH* sequences are KU696310, KU696311 and KU696309, respectively.

**Table 2.1.** Sequence of degenerate forward (F)/reverse (R) primers used for to isolate *Laccase* 2, *V-ATPase subunit A* and *GAPDH*.

| Primer | Sequence 5'-3' |
|---|---|
| *Lac* F1 | GACGTVGAGAACCAYATSGAAGG |
| *Lac* R2 | CGTATCKTTCMCCWGARAACG |
| *VTE* F2 | GKGARATYATYCGTYTGGARGGYGAHATG |
| *VTE* R1 | GMYTGYGAGATKACRGTYTTRCCRCA |

### 2.2.2 Isolation of *Laccase 2*, *V-ATPase subunit A* and *GAPDH* fragments

To isolate *Laccase 2*, *V-ATPase subunit A* and *GAPDH* total RNA was isolated, as previously described, from 3rd week pupae and "wandering" larvae (i.e. in search of a suitable site for pupation), respectively. First-strand cDNA was prepared and the primers shown in table 1 were used to carry out PCR using *Taq* DNA Polymerase as previously described. PCR products were separated on 1.2 % agarose and visualised bands were excised from the gel using a single edged razor blade. The DNA was extracted from the gel using a QIAquick gel extraction kit according to the manufacturer's instructions. CloneJET PCR Cloning Kit sticky-end cloning protocol was followed to ligate purified PCR products into pJET1.2/blunt Cloning Vector. The resulting ligation was transformed using One Shot® TOP10 Chemically Competent *E. coli*. Colony PCR was used to verify correct insert of DNA sequence, thereafter plasmid DNA was isolated from small-scale *E. coli* cultures (5 ml) using QIAprep Spin Miniprep Kit. Purified plasmids were verified via DNA sequencing.

### 2.2.3 Cloning of template for dsRNA synthesis

*Laccase 2* (301 bp) and *V-ATPase subunit A* (306 bp) templates for *in vitro* transcription were generated using gene specific primers including *XhoI* and *XbaI* restriction enzyme sites (Table 2). PCR was carried out using Phusion® High-Fidelity DNA Polymerase under the following conditions: 95°C for 3 minutes (1 cycle), 95°C

for 30 seconds, 60°C for 30 seconds, 72°C for 40 seconds (20 cycles) and 72°C for 7 minutes (1 cycle). The PCR products were excised and purified using QIAquick Gel Extraction kit. Purified PCR products and plasmid Litmus28i (New England BioLabs) were restricted with *XhoI* and *XbaI* and purified by agarose gel electrophoresis. PCR products and plasmid Litmus28i were ligated using T4 ligase and incubated overnight at 4°C. The ligation mixture was transformed into One Shot® TOPO10 Competent Cells, and selected clones were purified, and plasmids were verified by DNA sequencing.

**Table 2.2.** Sequence of gene specific forward (F)/reverse (R) primers used for the synthesis of *Laccase* 2 and *V-ATPase subunit A* dsRNA. Region in primer sequence underlined indicates restriction enzyme recognition site.

| Primer | | | Sequence 5'-3' |
|---|---|---|---|
| *Lac* | (RS) | F | TATCTCGACGTGGAACCCAATATTACGA |
| *Lac* | (RS) | R | ATATCTAGAGACCGGTGTTTACAGCCAAT |
| *VTE* | (RS) | F | TATCTCGAGGGTGTAACAGTTGGTGATC |
| *VTE* | (RS) | R | ATATCTAGACCCTTGGCTTTAGGTGGCA |

### 2.2.4 *In vitro* production of double stranded RNA (dsRNA)

*Laccase 2* and *V-ATPase subunit A* dsRNAs were prepared using Megascript T7 transcription kit (Ambion), according to the manufacturer's instructions. For control treatments dsRNA was prepared corresponding to a region of a bacterial nptII resistance gene (*nptII*). T7-RNA polymerase was used in transcription reactions, with target template linearized with *XhoI* and *XbaI* to generate ssRNA. Each ssRNA was precipitated by adding equal amounts of lithium chloride and nuclease-free water and re-suspended in Ringers solution. Finally, equal amounts of ssRNA were added together and annealed by heating the reaction to 80°C and allowing it to cool to room temperature overnight. The resultant dsRNA was quantified using a NanoDrop spectrometer and stored at -80°C until use.

### 2.2.5 Analysis of gene expression by quantitative PCR

Quantitative PCR (qPCR) was performed on *A. tumida* cDNA and relative expression of *Laccase 2* and *V-ATPase subunit A* was determined using ViiA™ 7 Real-Time PCR System (Life Technologies) with ΔΔCT methodology. In all cases, except for endogenous gene expression experiments, 3 biological replicates containing 5 pooled insects for each target gene and time point were analysed. qPCR primers were designed using Primer express software for real-time PCR v 2 (Applied Biosystems) (Table 2.3). Reaction mixtures (20 µl) contained 1x SYBR® Green JumpStart™ *Taq* ReadyMix™, ROX as a reference dye, 10 µM qPCR primers and 200 ng of cDNA or water as a negative control. Reactions were run in triplicate. Analysis of amplification profiles was performed using ViiA™M 7 software (Life Technologies), according to the manufacturer's guidelines. qPCR experiments were performed according to the MIQE guidelines outlined by Bustin et al. (2013). Expression of *A. tumida Laccase 2* and *V-ATPase subunit A* was normalized to *GAPDH*, whereas *A. mellifera* expression was normalized to *Elongation factor-1* (*EF-1*) (Martin et al., 2013).

### 2.2.6 Expression of *Laccase 2* and *V-ATPase subunit A* during the life-cycle of *Aethina tumida*

Total RNA was isolated at different developmental stages (eggs, larvae, wandering larvae, prepupae, pupae, non-emerged adult and emerged adult) and first-strand cDNA was synthesised as previously stated. In this case a single biological replicate containing 5 pooled insects or 50 mg wet weight of eggs were analysed in triplicate. Relative expression of targeted genes during the life-cycle of *A. tumida* was analysed using qPCR.

**Table 2.3.** Sequence of forward (F)/reverse (R) qPCR primers used to monitor expression of *A. tumida Laccase* 2, *V-ATPase subunit A* and *GAPDH.*

| Primer | Sequence 5'-3' |
|---|---|
| *A. tumida Lac* F | CCCATTGGAAGTGTTCACCAT |
| *A. tumida Lac* R | GAAGCGAAGGAGTTGATGATACG |
| *A. tumida VTE* F | TGTGGCCTGTACGTCAACCA |
| *A. tumida VTE* R | TCCGGTGAGAAGAGGATGATTC |
| *A. tumida GAPDH* F | TTCGAGATCGTGGAAGGTTTG |
| *A. tumida GAPDH* R | CAGAGGGACCGTCGACAGTT |

## 2.3 Standard biochemical techniques

### 2.3.1 Estimation of protein concentration: Bradford Assay

Protein concentration was estimated using the Coomassie Plus (Bradford) Assay Kit according to manufacturer's instructions. Bovine serum albumin (BSA) was used as a standard (0-7 µg/ml). In a microtitre plate 10 µl of sample, standard or water serving as a negative control was added to a well (in triplicate) and subsequently mixed with 290 µl of Coomassie Plus Protein Assay Reagent. Absorbance was measured at 595 nm and protein concentration was determined by plotting the standard curve versus the sample concentration in µg/ml.

### 2.3.2 Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) was used to analyse protein samples, which consisted of 17.5 % or 15 % resolving gel (17.5% or 15 % (v/v) acrylamide) (National Diagnostics) 0.375 M Tris/HCI (pH 8.8), 0.1 % (w/v) SDS, 0.2 % (w/v) ammonium persulphate, 0.05 % TEMED (N,N,N',N'-tetramethylethane) and 5 % and stacking gel (5 % (v/v) acrylamide, 0.125 M Tris/HCI (pH 6.8), 0.1 % (w/v) SDS, 0.25 % (w/v) ammonium persulphate, 0.075 % TEMED were cast in 1mm depth gel plate kits. All protein samples were prepared to the desired concentration by adding 20 mM sodium phosphate buffer (SP) (pH 7.4) and 5 X SDS sample buffer (0.2 M Tris-Base (pH 6.8); 5% (w/v) SDS; 20% (v/v) glycerol; 0.03% (w/v) Bromophenol blue; 25% (v/v) β-mercaptoethanol). Prior to being loaded into the wells of the gel, samples were boiled for 10 min in a heating block. A PageRuler™

Unstained Low Range Protein Ladder was used to enable protein Mw estimation (3.4 kDa, 5 kDa, 10 kDa, 15 kDa, 20, kDa 25 kDa, 30 kDa, 100 kDa). Samples were fractionated at 90 V through the stacking gel and 150 V through the resolving gel, in 1 x reservoir buffer (10 x reservoir buffer: 0.25 M Tris-HCI, 1.92 M Glycine, 1 % (w/v) SDS) using an ATTO AE-6500 gel tank apparatus.

### 2.3.3 SDS-PAGE staining with Coomassie Brilliant Blue and de-staining

After gel electrophoresis, proteins were visualised by the addition of Coomassie Brilliant Blue (CBB) (0.01 % (w/v) Coomassie Brilliant Blue G250, 12 % (v/v) glacial acetic acid, 20 % (v/v) methanol, prepared in distilled water), with agitation overnight at room temperature. Following staining, the gel was de-stained for 3-4 hr with 10 % (v/v) glacial acetic acid, 20 % (v/v) methanol, prepared in distilled water at the same conditions as stated above.

### 2.3.4 Western Blotting

Wet western transfer used in combination with chemiluminescent detection was performed following sample fractionation by SDS-PAGE (section 2.3.2). Nitrocellulose membrane (Protran BA85 nitrocellulose membrane, Whatman Ltd.) and 3MM blotting papers were cut to a similar size as the resolving gel and equilibrated in transfer buffer (25 mM Tris-Base; 192 mM glycine; 20% (v/v) methanol) together with foam pads for approximately and 1 hr prior to blotting. The aforementioned were prepared in a mini gel holder cassette for blotting in Mini Trans-Blot® Cell (Bio-Rad) in the following order: foam pad, three sheets of 3MM paper, the polyacrylamide gel, nitrocellulose membrane, 3 additional sheets of 3MM paper and finally the foam pad. The holder cassette was then secured and subsequently placed in the Mini Trans-Blot Central Core and electro-blotting was carried out at 100 V for 1 hr. Transfer of proteins and visualisation of the protein maker to the nitrocellulose membrane was confirmed by incubating the membrane in Ponceau S stain (Sigma) for 5 min and de-stained in blocking solution (1 x PBS, 0.05 % (v/v) Tween-20, 5 % (w/v) Milk powder). The nitrocellulose membrane was blocked in blocking solution for 1 hr at room temperature in 50 ml of blocking buffer with shaking. The proteins on the nitrocellulose membrane were probed 10 ml of blocking buffer containing GNA (1:2500) or SKTI (1:5500) primary antibodies and were incubated overnight at 4 °C. The primary antibody was

removed by 3 x 15 min washes in blocking buffer at room temperature with shaking. The secondary antibody was used in 10 ml blocking buffer at 1:5000 dilution for 2 hr. Membranes were then washed a further 3 times for 10 min in wash buffer (1 X PBS; 0.05% (v/v) Tween 20) and finally thoroughly rinsed in distilled water. Under dark room conditions, membranes were incubated in 5 ml chemiluminescent detection solution A (1 M Tris-HCI pH, 0.2 mM Coumaric acid, 1.25 mM Luminol) and 15 µl of solution B (100 µl / ml 30% (w/v) sterile water) for 1 min. The membrane was then transferred to an X-ray cassette and proteins were visualised by exposure to X-ray film (Fujifilm). The film was developed by incubation for approx. 3 min in Developer solution (Kodak) and 3 min in Fixer solution (Kodak).

## 2.4 Production of recombinant insecticidal proteins in *Pichia pastoris*

### 2.4.1 Synthesis of fusion protein constructs for expression in *Pichia pastoris*

2.4.1 (i) Recombinant GNA and SKTI protein

An expression construct containing the mature GNA nucleotide sequence (105 amino acids) was produced at Durham University and Fera Science Ltd essentially according to Raemakers *et. al.,* 1999. An expression construct containing the mature SKTI nucleotide sequence (189 amino acids) in pGAPZαB was designed at Durham University and produced at Fera Science Ltd.

2.4.1 (ii) Recombinant pro-Hv1a protein

An expression construct containing the mature pro-Hv1a (native pro-region) nucleotide sequence (59 amino acids) in pGAPZαB was designed and produced at Durham University.

2.4.1 (iii) GNA/Hv1a, Hv1a/GNA construct and K>Q variants

Four constructs containing either the Hv1a or Hv1a (K>Q) toxin linked to the N- or C- terminal of mature GNA nucleotide sequence in pGAPZαB were designed, at Durham University. The generation of a construct encoding for the mature Hv1a linked to the N-terminus of GNA has been previously reported (Fitches *et al.,* 2012). The C-terminus of the Hv1a peptide (residues 33–36) includes the sequence -VKRC-, which is similar to the sequence -EKRE- that is present in the α-factor signal sequence of the pGAP expression vector. -EKRE- is cleaved between R and E by the *P. pastoris*

KEX2 gene product. The inclusion of the 34th amino acid sequence K>Q modification in the Hv1a sequence, was altered by point mutation from a lysine to glutamine residual to remove a Kex2 cleavage site. The addition of this modification has shown to reduce cleavage resulting in more intact protein with no effect on toxicity after injection into *M. brassicae* (Pyati et al., 2014). Hv1a or Hv1a (K>Q) peptide was N- or C- terminal joined to the mature GNA nucleotide sequence by a 3-alanine linker region (*Not I* restriction site). All constructs had a (His)6 tag incorporated at the C-or N-terminal enabling a single step purification of the fusion protein from fermenter supernatants. Fermentation and purification took place at Durham University and Fera Science Ltd.

2.4.1 (iv) SKTI/Hv1a and SKTI/Hv1a with GGGGS extended linker (EL)

Two existing constructs one containing the mature SKTI coding sequence and the other the mature C-terminal Hv1a toxin coding sequence were used for the creation of construct encoding for SKTI/Hv1a and SKTI/Hv1a with an extended linker region including GGGGS. For both constructs the SKTI sequence was amplified by PCR using forward primer to include 5' *Pst I* restriction sites and (His)6 tag (5' TATCTGCAGCACATCATCATCATCATCATGATTTCGTGCTCG). For SKTI/Hv1a the reverse primer included only a 3' *Not I* restriction sites (5' ATATGCGGCCGC AGAAAGGCCATGATTTTTC), whereas SKTI(EL)/Hv1a reverse primer had the addition of 3' GGGGS linker followed by a *Not I* restriction sites (5' ATATGCGGCCGCGCTACCACCACCACCAGAAAGGCCATG). Following amplification by PCR, the purified PCR products were transferred to the yeast expression vector pGAPZαB containing a C-terminal Hv1a toxin coding sequence by digestion with *Pst I and Not I* restriction enzymes. Ligation and transformation was carried out as previously stated. Positive clones were verified by DNA sequencing.

2.4.1 (v) SKTI/Hv1a with X2 proline rich domain (PRD)

Two proline rich domains (PRD), each followed by the linker GAAG nucleotide sequence (Bonning et al., 2014) with codon usage optimised for yeast, was inserted in the pUC57 vector incorporating *Not I* and *Sal I* restriction sites, was synthesised and supplied by ShineGene Molecular Biotech, Inc. (Shanghai 201109 China) (TATGCGGCCGCAGGTGATGATGCTCCACCATCTCCAGGTCCAGATCCAGGTC

CACAACCACCACCACCACCACCACCATCTCCAACTCCAGTTGGTGGTGCTGCT
GGTGGTGATGATGCTCCACCATCTCCAGGTCCAGATCCAGGTCCACAACCACC
ACCACCACCACCACCATCTCCAACTCCAGTTGGTGGTGCTGCTGGTTCTCCAAC
TTGTATTCCATCTGGTCAACCATGTCCATATAATGAAAATTGTTGTTCTCAATCTT
GTACTTTTAAAGAAAATGAAAATGGTAATACTGTTAAAAGATGTGATTGAGTCGA
CTAT). Lyophilised plasmid DNA (4 µg) was re-suspended in sterile water. Plasmid DNA and expression vector pGAPZαB containing the mature SKTI coding sequence was digested with *Not I* and *Sal I* restriction enzymes. Ligation and transformation was carried out as previously stated. Positive clones were verified by DNA sequencing.

### 2.4.2 Yeast competent cell preparation: SMD1168H of the genotype *pep4* strains of *Pichia pastoris* (Invitrogen)

A 10 ml starter culture of YPG (2 % (w/v) trypticase peptone, 1 % (w/v) yeast extract, 4 % (v/v) glycerol) was inoculated with 2ul from glycerol stock and incubated overnight at 30ºC in a shaking incubator (220 rpm). An OD reading at 600 nm of the overnight culture was taken (1.1 to 1.3). Subsequently, 5 ml of the overnight culture was diluted to an OD600 of 0.1 to 0.3 in 50 ml of YPG. Thereafter the culture was incubated as above for approx. 4 to 6 hr until the optical density (OD) reached 0.6 to 1.0. The cells were pelleted by centrifugation at 500 g for 5 min at room temperature. The resultant supernatant was discarded, and the remaining cell pellet was re-suspended in 5 ml of Solution I. This suspension was further centrifuged as stated above, with the supernatant being discarded and the pelleted cells being re-suspended in 500 µl of Solution I. The competent cells were aliquoted into 50 µl aliquots in 1.5 ml tubes. The competent cells were frozen slowly by being stored at -20°C overnight. The following morning the competent cells were transferred and stored at -80°C until use.

### 2.4.3 Transformation of fusion protein constructs in *Pichia pastoris*

Sequence verified clones were cultured overnight in 50 mL LSLB and zeocin antibiotic, and plasmid DNA isolated using a QIAprep Spin Midiprep kit (section 2.1.16). A total of 6 µg of DNA was linearized overnight at 37°C using the *Bln1* restriction enzyme according to manufacturer's instruction. Linearized DNA was ethanol precipitated by adding 1/10 volume of 3 M sodium acetate and double the reaction volume of absolute ethanol and precipitated overnight at -20°C. The

precipitated product was centrifuged at 14 000g for 15 min at 4°C. The supernatant was discarded, and the pellet was washed with 300 µl of 70% ethanol. The precipitated produce was centrifuged as stated above, with the supernatant being discarded and the resulting pellet was dried using a SpeedVac® Plus SC110A (Thermo Savant). The pellet was re-suspended in 11 µl of sterile distilled water and 1 µl of DNA was fractionated by agarose gel electrophoresis to confirm the presence of DNA. The remaining linearised DNA plasmid was added to 50 µl competent SMD cells, thereafter 1 ml of Solution II was added. The cell mixture was incubated at 30°C for 1 hr with intermittent vortexing every 15 min. The cells were then heat shocked in a water bath at 42°C for 10 min. The cells were then transferred to a 50 ml falcon tube and 2 ml of YPG was added. The cell mixture was incubated at 30°C for 2 hr to allow for the expression of the zeocin resistance. The cell mixture was pelleted by centrifugation at 3000 g for 5 min at room temperature. The supernatant was discarded, and the pellet was washed with 1 ml of Solution III. The cells were re-pelleted by centrifugation as stated above and the pellet was re-suspended in 125 µl of Solution III. The transformation was plated onto YPG agar (containing zeocin antibody) and incubated at 30 °C for 3-4 days. Colonies were used to inoculate 10 ml YPG media, the cultures were grown in an incubator for 3 days (225 rpm; 30 °C). Cultures were centrifuged (10 000 g; 5 min; 4 °C) and the supernatants analysed for recombinant protein expression by western blotting (section 2.3.4).

### 2.4.4 Expression screening of *Pichia pastoris* colonies using western blot analysis

YPG cultures (10 ml) containing 100 mg/ml zeocin were inoculated with a single transformed colony. Cultures were grown for 3 days in a shaking incubator (220 rpm) at 30 °C. The cells were pelleted by centrifugation at 8 000 *g* for 10 min at room temperature. Each 20 µl sample of supernatant was fractioned by SDS-PAGE and proteins were visualised using western blot analysis.

### 2.4.5 Large-scale expression of fusion proteins via fermentation

Selected *P. pastoris* clones containing integrated expression cassettes were used to inoculate three 100 ml starter cultures of YPG, which were grown at 30 °C for 3 days on an orbital shaker at 220 rpm, in the absence of selective antibiotics.

Subsequently 250 ml of subculture was used to inoculate 2.5 l of sterile minimal media (Higgins & Creggs, 1998) supplemented with PTM1 salts (4.35 mL/ 1L minimal media; Cino, 1999) in a BioFlo/ CelliGen 115 Benchtop fermentor (New Brunswick Scientific). Cultivation was carried out at 30°C, 30% dissolved oxygen; pH 4.5-5.0 with continuous agitation and a ramped glycerol feed (5–10 ml/h) over 4 days (Fitches *et al.,* 2004).

### 2.4.6 Purification of fusion proteins using nickel affinity chromatography

Secreted proteins were isolated from fermented cultures by centrifugation (4 °C; 9500 g; 20 min). Yeast culture supernatant was clarified by filtration through 2.7uM and 0.7uM glass fibre filters (GFD and GFF; Whatmann) aided by a water vacuum system. The filtered supernatant was adjusted to 0.02 M sodium phosphate buffer, 0.4 M sodium chloride, pH 7.4 by adding 4x binding buffer (BB). Recombinant proteins were purified by nickel affinity chromatography on 5 ml HisTrap crude nickel columns (GE Healthcare Life Sciences) equilibrated in 1x BB. Culture supernatants were loaded with a flow rate of 4 ml/min during the day and 2 ml/min overnight. After loading columns were then washed with 1 x BB, followed by 1 x BB containing 0.01M imidazole and finally bound recombinant proteins were eluted with 1x BB containing 0.2M imidazole. Peak fractions and collective eluted proteins were analysed by SDS-PAGE gel electrophoresis.

### 2.4.7 Dialysis and freeze drying of fusion proteins

Dialysis was carried out using dialysis tubing (25.4mm Inflated; Scientific laboratory supplies) with a molecular weight cut off from 12-14 kDa. Dialysis tubing was prepared by boiling for 15 min in distilled water containing 2 % (w/v) sodium bicarbonate and 1 mM EDTA. Purified protein was diluted 1:1 with distilled water and 150 ml of diluted elution was dialysed against 8 L of $ddH_2O$ (4 °C; stirring) containing trace of ammonia bicarbonate. Pooled protein fractions were dialysed overnight, with the $ddH_2O$ being changed in total 3-4 times over a 24 hr period. Dialysed samples were snap frozen in liquid nitrogen and lyophilised using a One VirTris Benchtop BTP 8ZLFreeze dryer (Biopharma process systems Ltd). Lyophilised proteins samples were stored at 4 °C. Protein concentration was estimated using known amounts of recombinant GNA or SKTI fractioned via SDS-PAGE.

## 2.5 *Aethina tumida* insect culture

*Aethina tumida* cultures were maintained in the darkness at 20°C, with 65% relative humidity (RH), in the Quarantine Entomology Unit (Fera Science Ltd.). The culture was originally established from wandering larvae imported under three levels of containment supplied by the Plant Protection Research Institute, South Africa. Cultures were maintained.

As shown in Figure 2.1 adult beetles are allowed to emerge inside sealed containers of sand, in which they pupate. Each container has been converted to allow for an inverted plastic cylindrical tube to be attached, which act as a collection chamber. The plastic tube is baited with honey to lure the adults into the collection chamber, when a number of adults have congregated in the tube it was unscrewed, sealed and the adults were transferred into rearing boxes. Each rearing box contained suitable diet, consisting of honey and pollen and an egg slide to provide a surface for eggs to be deposited. After several days, the egg slides are removed and placed into a damp container and when the larvae begin to emerge honey bee brood was added as a food source. As the larvae enter the final stages of development they clear their guts and become morphologically distinct from actively feeding larvae, additionally their behaviour changes and they begin to wander *en masse*, this larval stage is referred to as the wandering stage. This distinct behavioural and morphological change signifies the need to find suitable substrate to allow for pupation. At this point they were transferred into a container filled with approximately 15 cm of damp soil, the wandering larvae burrow into the sand where they pupate and approx. 30 days later emerge as adults.

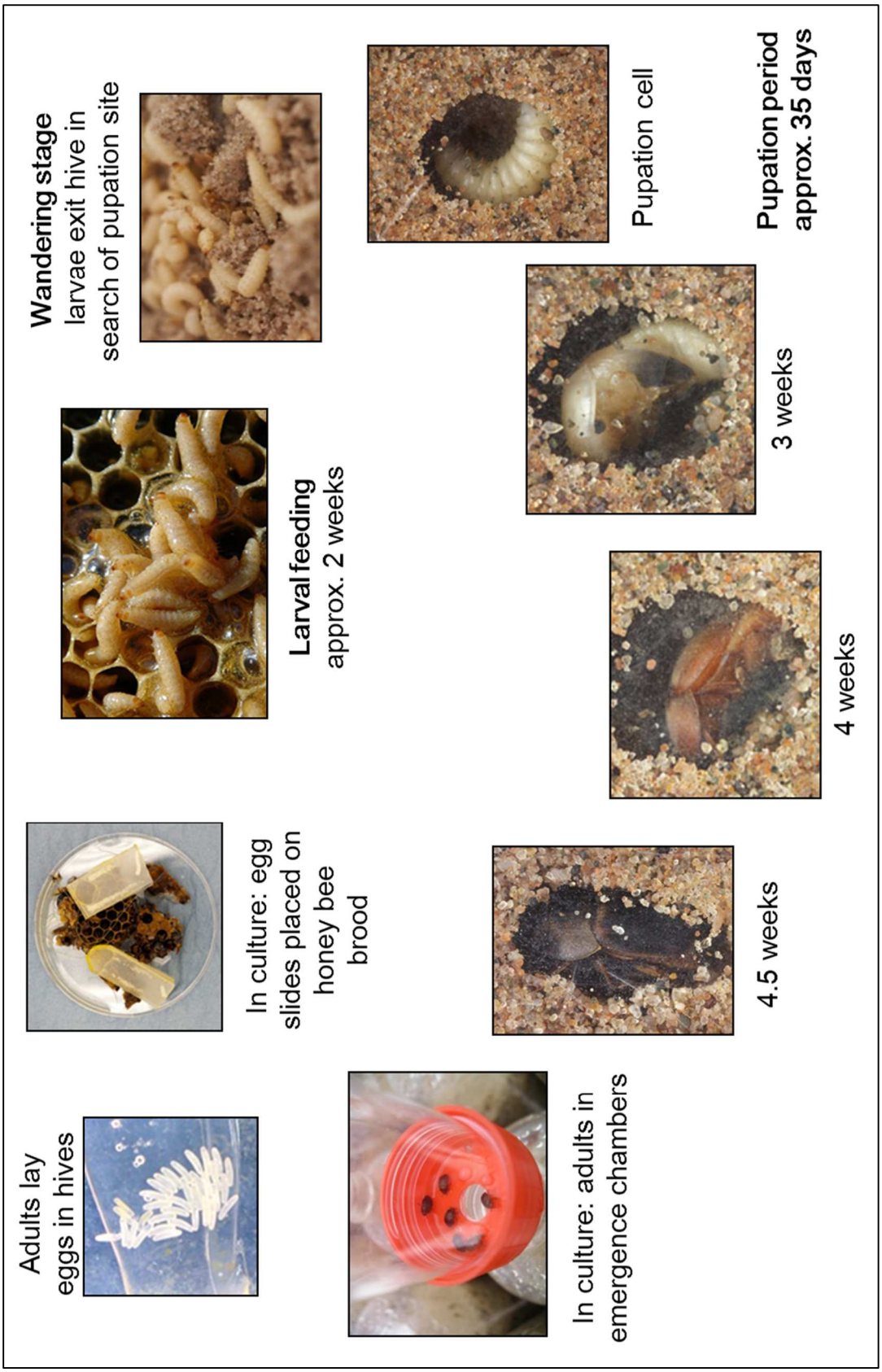



**Figure 2.1.** Development of *Aethina tumida* from egg to fully developed adult under culturing conditions (Cuthbertson et.al., 2013).

## 2.6 RNAi experiments

### *2.6.1 Aethina tumida* injection bioassays

Wandering or 7 day old *A. tumida* larvae were injected using a Hamilton micro-syringe fitted with a 26 gauge needle (Essex Scientific Laboratory Supplies Ltd) with doses ranging from 2-500 ng of *Laccase 2* or *V-ATPase subunit A* dsRNAs; *nptII* dsRNA or Ringers solution served as negative controls. *A. tumida* larvae were anesthetised using $CO_2$ and injected with 1 µl (wandering larvae) or 0.5 µl (7 day old larvae) of dsRNAs or Ringers solution. Larvae were injected in the 3$^{rd}$ dorsal segment and needles were left in the larvae for 30 s prior to withdrawal, to reduce the expulsion of fluid from the wound. Larvae were placed in a petri dish after injection to allow the wound to seal. Thereafter, wandering larvae were placed in tubs of sand and monitored for phenotype and/or emergence over a period of 35 days (n=10 per treatment) or removed after 48 hr, 1 week (*V-ATPase subunit A*) or 3 weeks (*Laccase 2*) (n=15 per treatment) for qPCR analysis. Seven day old larvae were treated in the same manner, although after injection they were returned to sandwich boxes containing brood food and left to feed until they entered the wandering stage. Time points for qPCR analysis were selected based on preliminary assays which indicated that insects injected with dsRNAs were still alive at the time of sampling.

### 2.6.2 *Aethina tumida* feeding bioassays

2.6.2 (i) Oral delivery in an artificial diet

Egg slides from *A. tumida* were placed onto artificial diet containing 50 % (v/w) aqueous honey solution (1 ml) and 2.5 g of crushed bee pollen (Figure 2.2 A). Larvae were allowed to feed for 7 days before being transferred to artificial diets containing *Laccase 2, V-ATPase subunit A,* control *nptII* dsRNA or Ringers solution. Thirty larvae per treatment were placed onto 900 mg of diet containing 30 µg of dsRNA (33 ng/mg), and fresh diet was provided after three days. After 6 days of feeding the wandering larvae were placed in tubs of sand and monitored for phenotype and/or emergence over a period of 35 days. For qPCR analysis 15 larvae (per treatment and time point) were treated as stated above. Samples were taken 48 hr after feeding on dsRNA or removed after 1 week (*V-ATPase subunit A*) or 3 weeks (*Laccase 2*) after the wandering stage had commenced.

2.6.2 (ii) Oral delivery via a drinking bioassay

Larvae were reared as previously described. Seven day old larvae (n=20) were transferred into sterile 5 ml tubes containing 300 µl of 50 % (w/v) sterile sucrose solution (prepared with Ringers solution) containing 20 µg of each dsRNA or sucrose solution serving as a negative control (Figure 2.2 B). After 24 hr the larvae were transferred to artificial diet to feed until wandering, thereafter the larvae were placed in tubs of sand and monitored for phenotype and/or emergence. qPCR analysis was conducted only for *V-ATPase subunit A* dsRNA treated larvae (n=15 per treatment) with samples being removed after 1 week.

2.6.2 (iii) Oral delivery via a sterile agar diet

In a third feeding bioassay 5 day old larvae (n=20) were transferred sterile agar diet – 1 % (w/v) agar (Oxoid Agar Technical *Agar technical* [*Agar No. 3*]) containing 40 % (w/v) sucrose (Figure 2.2 C). The sterile agar was melted and 350 µl of agar solution was added to a 2 ml microcentrifuge, this was subsequently placed in a heat block preheated to 37 ºC and allowed to cool to the aforementioned temperature. Thereafter 100 µl of dsRNA (200 ng/µl) was added to the agar solution and subsequently vortexed to ensure the dsRNA was evenly distributed, thereafter the agar solution was left to solidify at room temperature, with a final weight of approx. 450 mg (57 ng/mg). The diet was replenished daily and after 3 days of feeding the larvae were transferred onto artificial diet until the wandering stage. Subsequently, larvae were placed in tubs of sand and monitored for phenotype and/or emergence. qPCR analysis was conducted for *V-ATPase subunit A* dsRNA treated larvae (n=15 per treatment) with samples being removed after 1 week.

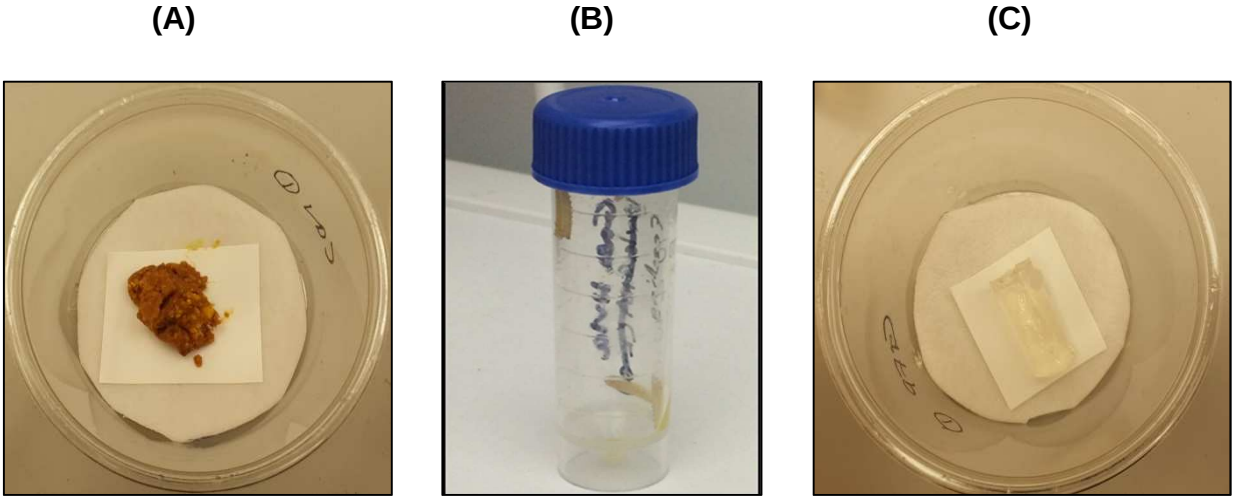



**Figure 2.2.** *Aethina tumida* larvae were placed onto 3 diets containing 1 µg/insect of *Laccase 2 or V-ATPase subunit A* dsRNAs, control *nptII* dsRNA, or Ringers solution serving as a negative control: (A) Diet 1: artificial diet comprising of pollen and 40 % (w/v) sterile sucrose solution; (B) Diet 2: 300 µl of 50 % (w/v) sterile sucrose solution (prepared with Ringers solution) containing 20 µg of each dsRNA or Ringers solution.; and (C) Diet 3: sterile agar diet containing 1 % (w/v) agar made up with 40 % (w/v) sucrose.

### 2.6.3 dsRNA stability assays

2.6.3 (i) Persistence of dsRNA in sucrose solutions containing *Aethina tumida* larvae

The stability of dsRNA in sucrose solution was evaluated by incubating 1 µg of *V-ATPase subunit A* dsRNA in 10 µl of 50 % (w/v) sucrose solution at 20°C for 22 hr. Following confirmation that dsRNA was stable under these conditions two 7 day old larvae were incubated in 100 µl of 50 % sucrose solution containing 10 µg of *V-ATPase subunit A* dsRNA, with 10 µl aliquots taken at the following time points: 0, 1, 2, 4, 6, 8, 18 and 22 hr. The integrity of the dsRNA was analysed by separation on 1.2 % (w/v) agarose gels and bands were visualised by ethidium bromide staining under UV.

To determine whether *A. tumida* larvae produced extracellular ribonucleases, 7 day old (i.e. feeding stage) larvae were incubated for 12 hr in sucrose solution as described above. Thereafter the larvae were removed, 10 µg of *V-ATPase subunit A* dsRNA was added and incubated for 8 hr at 20°C. A second assay was carried out to identify the possible source of ribonuclease activity. Wandering larvae (i.e. non-feeding, cleared guts) and frass were separately incubated for 8 hr, as described previously, in the

presence of 10 µg of *V-ATPase subunit A* dsRNA. The integrity of the dsRNA was analysed by agarose gel electrophoresis.

2.6.3 (ii) Persistence of dsRNA in agar diet fed on by *Aethina tumida* larvae

The stability of dsRNA in sterile agar diet was evaluated by incubating 10 µg of *V-ATPase subunit A* dsRNA in 450 mg of agar at 20°C for 22 hr. Five larvae were allowed to feed on the agar diet for the following time points: 0, 1, 2, 4, 6, 8, 18 and 22 hr. The integrity of the dsRNA was analysed by separation on 1.2 % (w/v) agarose gels and bands were visualised by ethidium bromide staining under UV.

2.6.3 (iii) *In vitro* stability of dsRNA in larval gut extracts

Gut samples dissected from 10 feeding stage larvae were re-suspended in 100 µl Ringers solution and homogenised using a sterile pestle. Protein content was estimated using Coomassie Plus (Bradford) Assay Kit using Bovine serum albumin as standards. The samples were centrifuged for 5 min at 14 000 g and the resulting supernatant was used in the assay. Gut extract samples (10 µg total protein in 20 µl; equivalent to approx. 1/10 of a larval gut) were incubated with 500 ng of *V-ATPase subunit A* dsRNA at room temperature for 5, 15, 30 and 60 min. The integrity of the dsRNA was analysed by agarose gel electrophoresis.

### 2.6.4 *Apis mellifera* injections bioassays

Newly emerged *A. mellifera* workers were anesthetized by cooling on ice and subsequently injected under the 5th abdominal segment with 2 µl containing 50 ng of *A. tumida Laccase 2*, *V-ATPase subunit A,* control *nptII* dsRNAs or Ringers solution. Injections were conducted using a Hamilton micro-syringe fitted with a 33 gauge custom fine needle (Essex Scientific Laboratory Supplies Ltd). Following injection, worker bees were grouped in cohorts of 10 or 15 individuals, supplied with 50 % (w/v) sucrose solution and placed in an environmental chamber (night cycle, 34°C, with 60 % R.H). Thereafter, worker bees were monitored for phenotype (n=20 per treatment) for 10 days or removed after 48 hr and 1 week (n=15 per treatment) post-injection for qPCR analysis. Additionally, qPCR analysis was carried out on 2 day old pharate adults (part of the pupal stage) injected with *Laccase 2* dsRNA as, according to Elias-Neto *et al*. (2013) this gene is significantly up-regulated at this stage in the life-cycle.

Pharate adults were injected as previously described and after injection individuals (n=15 per treatment) were carefully positioned in a well of a microtitre plate and removed from the environmental chamber after 48 hr for qPCR analysis. All samples for qPCR analysis were snap frozen in liquid nitrogen and stored at -80°C until use.

### 2.6.5 CLUSTALW analysis of dsRNA sequences

*Aethina tumida* dsRNA sequences were compared with *A. mellifera Laccase 2* (Genbank: FJ470292) and *V-ATPase subunit A* (GenBank: XM 006567414) using CLUSTALW.

## *2.7* Fusion proteins experiments

### 2.7.1 *Aethina tumida* wandering larvae injection bioassays

Purified recombinant Pro-Hv1a, GNA, SKTI and fusion proteins were tested for biological activity by injecting 1 µl of protein resuspended in SP solution, into wandering *A. tumida* larvae (average weight 17.64 mg), as previously described in section 2.6.1. For each concentration tested, 10 larvae were injected, with SP solution serving as a negative control. After injection larvae were placed in a petri dish line with moist filter paper and mortality was monitored over 7 days.

### 2.7.2 *Aethina tumida* larvae feeding bioassays

2.7.2 (i) Oral delivery in an artificial diet

Seven day old larvae (n=20), reared on artificial diet, were placed onto 900 mg of artificial containing recombinant GNA and GNA/Hv1a at a concentration of 5000 ppm. Negative control larvae were fed on artificial diet containing SP buffer. Larvae were allowed to feed for approx. 6 days, with fresh diet being provided after 3 days, and survival was monitored daily.

2.7.2 (ii) Development of a drinking bioassay

Five day old larvae (n= 10), reared on artificial diet, were transferred into plastic pot containing moist filter paper and two 1.5 ml microcentrifuge lids or larvae were maintained on artificial diet as a control. Each lid contained 75 µl of SP/sucrose solution (13 % v/w) with 2 µl of red food dye, with the solution being changed every 24 hr for 72 hr. Thereafter larvae were supplied with artificial until they entered the

wandering stage (i.e. approx. 4 days post liquid feed). Mortality was recorded daily, and weight was recorded on day 7.

2.7.2 (iii) Oral delivery via a drinking bioassay

Larvae were reared and assayed as described above. Larvae (n=20) were exposed to recombinant GNA(5mg/ml), Pro-Hv1a (1.25 mg/ml GNA/Hv1a, Hv1a/GNA or K>Q variants at a final concentration of 0.132-5 mg/ml. Recombinant SKTI fusion proteins were delivered at 10 mg/ml concentration. Negative control larvae were fed on SP/sucrose solution without recombinant proteins. The diet was changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial until they entered the wandering stage (i.e. approx. 4 days post liquid feed).

2.7.2 (iv) Treatment of bee brood or egg and bee brood with GNA/Hv1a, to assess oral toxicity

The oral toxicity of GNA/Hv1a was assessed by carrying out two separate assays, whereby bee brood (two replicates; SP control n=370 and 290 eggs; GNA/Hv1a n=350[+/- 10]), or an egg slide and bee brood was treated were treated with GNA/Hv1a at 5 mg/ml or SP solution serving as a negative control (one replicate; SP control n=210 eggs; GNA/Hv1a n=360 eggs). For the bee brood assay, egg slides (composed of two Perspex sheets glued together and adults lay the eggs between the slides) from *A. tumida* adults were placed onto approx. 4 cm by 3 cm piece of honeybee brood (Figure 2.3). The following day the slide was carefully removed, and the bee brood was sprayed with 1 ml of GNA/Hv1a at a final concentration of 5 mg/ml. Negative control consisted of brood sprayed with a SP solution without recombinant protein. The brood was sprayed every 24 hr for 72 hr and thereafter larvae were supplied with brood ad lib until they entered the wandering stage and survival was assessed. The egg and bee brood assay were conducted as stated above, except egg slides were split open to expose the eggs and subsequently sprayed with 250 µl of GNA/Hv1a at a final concentration of 5 mg/ml or SP solution as a negative control.

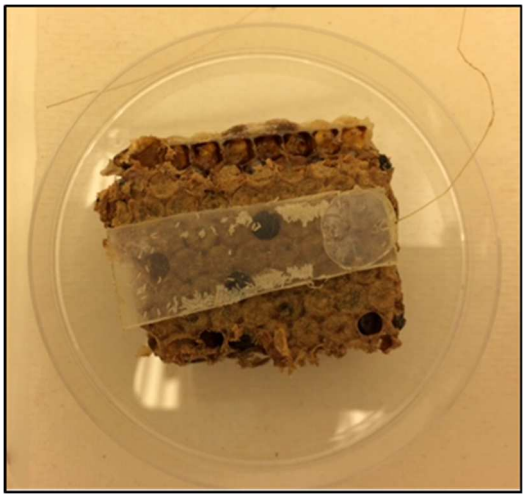


**Figure 2.3.** Eggs slides from *Aethina tumida* adults were placed onto approx. 4 cm by 3 cm piece of bee brood, which was subsequently sprayed with GNA/Hv1a at 5 mg/ml concentration.

### 2.7.3 Adult *Aethina tumida* feeding bioassays

Adults (approx. 1 week old) were transferred into a 50 ml collection chamber (Figure 2.1) containing two 1.5 ml microcentrifuge. Each lid contained 75 µl of SP/sucrose solution (13 % v/w) containing recombinant GNA and Pro-Hv1a at 5 and 1.25 mg/ml and GNA/Hv1a, Hv1a/GNA or K>Q variants at a final concentration of 0.625-5 mg/ml. Negative control adults were fed on SP/sucrose solution without recombinant proteins. The diet was changed every 48 hr for 6 days and thereafter adults were supplied 50 % honey solution until day 14.

### 2.7.4 Preparation of *Aethina tumida* samples for Western analysis

To assess internalization and transport of recombinant proteins, haemolymph and gut samples were collected from *A. tumida* larvae, whereas only gut samples were collected from adults 24 h post feeding on recombinant proteins as previously described.

#### 2.7.4 (i) Haemolymph Extraction

Haemolymph samples were extracted from chilled *A. tumida* by piercing the dorsal region with a fine needle. Haemolymph samples were re-suspended in 6 M urea and

boiled for 10 min at 90°C, to prevent coagulation. Samples were stored at -20°C until use.

2.7.4 (ii) Gut Extraction

Larval gut samples (n=10) were dissected out over ice from either feeding or wandering stage larvae or adults. Adults were flash frozen in liquid nitrogen and gut samples (n=10) were dissected under a microscope, with the gut samples being maintained on ice. Larvae and adult guts were re-suspended in 100 or 40 µl SP solution, respectively and homogenised using a sterile pestle. Protein content was estimated using Coomassie Plus (Bradford) Assay Kit using Bovine serum albumin as standards. The samples were boiled for 10 min at 90°C, then centrifuged for 5 min at 14 000 g and the resulting supernatant was stored at -20°C until use.

### 2.7.5 Stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants

2.7.5 (i) *In vitro* stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants in sucrose solutions containing *Aethina tumida* larvae

The stability of fusion proteins in sucrose solution was evaluated by incubating 5 day old larvae (n=10) in 100 µl of SP/sucrose solution (13 % [v/w]) containing 2.5 mg/ml of GNA/Hv1a, Hv1a/GNA or K>Q variants, with 5 µl aliquots taken at the following time points: 0,1, 2, 4, 6, 8 and 24 hr. Positive control included the relevant fusion protein incubated for 24 hr in the absence of larvae. The samples were boiled for 10 min at 90°, then centrifuged for 5 min at 14 000 g and the resulting supernatant was stored at -80°C until use.

2.7.5 (ii) *In vitro* stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants in larval gut extracts

Gut samples dissected from 10 feeding stage larvae were re-suspended in 200 µl SP solution and homogenised using a sterile pestle. Protein content was estimated using Coomassie Plus (Bradford) Assay Kit using Bovine serum albumin as standards. The samples were centrifuged for 5 min at 14 000 g and the resulting supernatant was used in the assay. The equivalent of two larval guts (40 µl) were incubated with 75 µg of GNA/Hv1a, Hv1a/GNA or K>Q variants at room temperature with 5 µl aliquots taken at 0,1, 2, 4, 6, 8 and 24 hr. Controls included fusion protein incubated for 24 hr without

guts and in the presence of denatured (boiled) guts. The samples were boiled for 10 min at 90 °C, and the resulting supernatant was stored at -80°C until use.

### 2.7.6 Electrophoretic zymogram

To analyse the digestive proteases within the gut of feeding *A. tumida* larvae three protease inhibitors were use: soybean Kunitz trypsin inhibitor (SKTI), soybean Bowman-Birk inhibitor (SBBI) and Epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64) to inhibit trypsin, chymotrypsin and trypsin and cysteine proteases, respectively. Non-reducing SDS-PAGE was performed using resolving and stacking polyacrylamide gels of 12.5% (v/v) and 5% (v/v), respectively. A total of 15 µg of gut extract, in a volume of 10 µl, was mixed with 10 µl of SKTI, SBBI at a final concentration of 5 µM, 12.5 µM or 25 µM or E-64 a final concentration of 0.5 µM, 1.25 µM or 2.5 µM. Control samples included gut preps of feeding larvae and wandering larvae without proteases inhibitor. After incubation at room temperature for 20 min, 8 µl of sample buffer without β-mercaptoethanol was added to each sample. Non-reducing SDS-PAGE was carried out at 4°C at a constant voltage of 100 V. Thereafter, the gel was placed in phosphate buffer pH 8 containing 2.5% Triton X-100 for 20 minutes, with shaking. After this step, the gel was immersed in 1% casein and shaken for 3 hours. The gel was washed in distilled water and stained with 0.1% Coomassie Brilliant Blue R-250 in methanol-acetic acid-water (50:10:40). After 2 h, the gel was washed in water and destained in methanol-acetic acid-water (50:10:40) for 3 h until clear bands could be visualised against the dark blue background (Chitgar et al., 2013).

### 2.7.7 Native Polyacrylamide gel electrophoresis (Native-PAGE)

Native Polyacrylamide gel electrophoresis (Native-PAGE) used to analyse *A. tumida* gut samples, as previously stated in section 2.3.2. With the exception that 12.5 % resolving gel were used and SDS was removed gels and reservoir buffer. Samples were prepared in 5x sample buffer, without the addition of SDS or β-mercaptoethanol.

### 2.7.8 *In vitro* stability of GNA/Hv1a and Hv1a/GNA in *Aethina tumida* larval gut extracts with the addition of SKTI

The stability of GNA/Hv1a and Hv1a/GNA was assessed *in vitro* by incubating the equivalent of two larval guts (40 µl) with 75 µg of GNA/Hv1a or Hv1a/GNA. Total

protein content was estimated using Coomassie Plus (Bradford) Assay Kit using Bovine serum albumin as standards. For every 15 µg of gut extract protein present 500 µM SKTI was added. Samples were incubated at room temperature and samples were taken at 0 and 24 hr. Controls included fusion protein incubated for 24 hr and fusion protein incubated with denatured (boiled) guts and SKTI for 24 hr. The samples were boiled for 10 min at 90°C, and the resulting supernatant was stored at -80°C until use. Samples were analysed for the presence of fusion proteins by western blotting, using anti-GNA antibodies.

### 2.7.9 Adult *Apis mellifera* injections bioassays

Acute toxicity was assessed by injecting newly emerged adult honey bees, as previously stated in section 2.6.4, with 10 µg Pro-Hv1a, 40 µg of recombinant GNA, and GNA/Hv1a, with SP injected serving as a negative control. Adult honey bees (n=20 per treatment) were supplied with 50 % (w/v) sucrose solution and placed in an environmental chamber (night cycle, 34°C, and with 60 % R.H), survival was monitored daily for 7 days.

### 2.7.10 *Apis mellifera* larval feeding bioassay

Standard operating procedures established for the *in vitro* testing of pesticides were used to test for acute toxicity of recombinant GNA and GNA/Hv1a towards honeybee larvae (Aupinel et al., 2007). A single oral dose of 100 µg/larva of recombinant GNA or GNA/Hv1a 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. Thirty larvae were treated with recombinant GNA, GNA/Hv1a or SP serving as a control treatment. Five larvae from each treatment was sacrificed at 24 and 92 hr to obtain haemolymph, whole larval and diet samples for western blot analysis. Haemolymph was obtained by piercing pre-chilled larvae with a fine needle and collecting haemolymph directly into a tube containing N-phenylthiourea (anti-oxidising agent) to prevent melanisation. The survival of the remaining 20 larvae was monitored for four days subsequent to the single acute dose of recombinant GNA or GNA/Hv1a.

## 2.11 Statistical analysis

The qPCR results are presented as the mean ±SD of three independent biological replicates and the relative levels of mRNA expression was analysed by One-way ANOVA followed by Tukey test for significant differences between mean values. All survival data was analysed using Kaplan-Meier survival analysis and $LD_{50}$ or $LC_{50}$ were calculated. Wandering larvae survival after brood assay experiments was analysed by Chi-square test for significant differences between single values GraphPad Prism version 7.00 for windows was used for all the analysis. $P < 0.05$ was taken as the level of statistical significance.

# CHAPTER 3 | RNAI-MEDIATED CONTROL OF *AETHINA TUMIDA*

## 3.1 Introduction

Evidence for systemic RNAi in *T. castaneum* and successful RNAi studies in other coleopteran insect pests (Nui et al., 2008, Baum et al., 2007) formed the rationale for our investigations into the potential use of RNAi as a target specific control strategy for *A. tumida*. The phenoloxidase gene *Laccase 2* and vacuolar-ATPase *V-type (V-ATPase) subunit A* were selected as target genes on the basis of previous RNAi studies (Arakane et al., 2005; Baum et al., 2007; Dittmer et al., 2004; Forgac, 2007; Nui et al., 2008).

Inhibition of exoskeleton formation has been previously identified as a target for pesticide action, and a range of genes involved in chitin biosynthesis and the formation of the cuticle have been functionally characterized, revealing a range of potential arthropod-specific targets for control. Arakane et al. (2005) proved that *Laccase 2*, a phenoloxidase gene, is required for adult *T. castaneum* cuticle tanning and reported that injection of prepupae with *Laccase* 2 dsRNA exhibited a dose and time-dependant phenotype. Delivery of 200 ng of dsRNA per prepupa resulted in inhibition of tanning in adults, severe developmental abnormalities and mortality on day 2 or 3. When the dose was reduced by 100-fold to 2 ng per prepupa, this resulted in more normal looking adults, but there was still a degree of malformation and the tanning process was slowed taking several additional days to complete. *V-ATPase* is required to pump protons across intracellular and plasma membranes in the insect gut and other tissues; this ATP-powered pump is vital for survival as the proton gradient created is used to drive a range of transport processes (Forgac, 2007). RNAi targeting the genes encoding *V-ATPase subunits A* and *E* has proved to be effective against several coleopteran species. In *D. v. virgifera* oral delivery of 5.2 ng dsRNA /cm² of *V-ATPase subunit A* dsRNA demonstrated rapid knockdown of endogenous mRNA within 24 h of ingestion and resulted in significant larval mortality and/or stunting (Baum et al., 2007). To our knowledge this is the first study to provide evidence for target specific systemic RNAi in *A. tumida*

## 3.2 Results

### 3.2.1 Cloning of *Laccase 2*, *V-ATPase subunit A* and *GAPDH*

Partial sequences obtained from degenerate PCR for *Laccase 2* (621 bp) and *V-ATPase subunit A* (618 bp) were used as a template to design gene specific primers to amplify a 301 and 306 bp region for *in vitro* transcription of dsRNA. Additionally, the 3' end of the sequence, including *GAPDH* (547 bp), was used to design qPCR primers to monitor gene expression (Figure 3.1, 3.2 & 3.3). Genbank accession numbers for cloned partial *Laccase 2*, *V-ATPase subunit A* and *GAPDH* sequences are KU696310, KU696311 and KU696309, respectively.

```
DNA: GGTCGCTTCAAGGGCAAAGTCACCACCGACGGTACCAGCCTCATCGTCAACGGCAAGGCT
+1FR:·G··R··F··K··G··K··V··T··T··D··G··T··S··L··I··V··N··G··K··A·
DNA: ATCCAGGTCTTCCAAGAGAGAGACCCAGCCAACATTCCATGGGGCAAAGCCGGTGCCGAA
+1FR:·I··Q··V··F··Q··E··R··D··P··A··N··I··P··W··G··K··A··G··A··E·
DNA: TACATCGTTGAATCCACCGGTGTGTTCACCACCATCGAGAAGGCCTCCGCCCACTTGAAG
+1FR:·Y··I··V··E··S··T··G··V··F··T··T··I··E··K··A··S··A··H··L··K·
DNA: GGTGGTGCTAAGAAAGTCATCATCTCTGCACCATCTGCCGATGCCCCAATGTACGTCTGC
+1FR:·G··G··A··K··K··V··I··I··S··A··P··S··A··D··A··P··M··Y··V··C·
DNA: GGTGTCAACTTGGACAAATACAACCCATCTGACAAGGTAATCTCCAACGCCTCCTGCACC
+1FR:·G··V··N··L··D··K··Y··N··P··S··D··K··V··I··S··N··A··S··C··T·
DNA: ACCAACTGCTTGGCCCCATTGGCCAAAGTAATCCACGACAACTTCGAGATCGTGGAAGGT
+1FR:·T··N··C··L··A··P··L··A··K··V··I··H··D··N··F··E··I··V··E··G·
DNA: TTGATGACCACCGTACACGCTACCACCGCCACCCAGAAAACTGTCGACGGTCCCTCTGGC
+1FR:·L··M··T··T··V··H··A··T··T··A··T··Q··K··T··V··D··G··P··S··G·
DNA: AAGTTATGGCGTGATGGCCGTGGCGCCGCCCAGAACATCATTCCCGCTTCCACCGGAGCT
+1FR:·K··L··W··R··D··G··R··G··A··A··Q··N··I··I··P··A··S··T··G··A·
DNA: GCCAAAGCCGTCGGCAAGGTTATCCCAGCCTTGAACGGCAAACTTACCGGTATGGCATTC
+1FR:·A··K··A··V··G··K··V··I··P··A··L··N··G··K··L··T··G··M··A··F·
DNA: CGTGTTC
+1FR:·R··V·
```

**Figure 3.1.** Cloned partial DNA (547 bp) and amino acid (180 codons) sequences of *Aethina tumida GAPDH* (GenBank: KU696309). Highlighted in pink is the position of the forward/reverse qPCR primers generating an amplicon of 76 bp.

```
DNA:   GACGTAGAGAACCATATGGAAGGCATGGAGGTGACCATCCATTGGCACGGAATCTGGCAA
+1FR:  ·D··V··E··N··H··M··E··G··M··E··V··T··I··H··W··H··G··I··W··Q·
DNA:   CGTGGAACCCAATATTACGATGGTGTGCCATTCGTTACACAATGTCCCATCCAACAAGGA
+1FR:  ·R··G··T··Q··Y··Y··D··G··V··P··F··V··T··Q··C··P··I··Q··Q··G·
DNA:   AACACCTTCAGGTACCAGTGGGTAGCCGGTAACGCCGGAACGCACTTTTGGCATGCCCAC
+1FR:  ·N··T··F··R··Y··Q··W··V··A··G··N··A··G··T··H··F··W··H··A··H·
DNA:   ACCGGTCTGCAAAAGATGGACGGTTTGTATGGCAGCATCGTCATCCGTCAACCACCTTCC
+1FR:  ·T··G··L··Q··K··M··D··G··L··Y··G··S··I··V··I··R··Q··P··P··S·
DNA:   AAAGACCCCAACAGCAACTTGTACGACTACGATCTGACAACACACGTTATGCTGCTTTCC
+1FR:  ·K··D··P··N··S··N··L··Y··D··Y··D··L··T··T··H··V··M··L··L··S·
DNA:   GATTGGATGCACGAAGATGCTGCCGAAAGATTCCCAGGAAGATTGGCTGTAAACACCGGT
+1FR:  ·D··W··M··H··E··D··A··A··E··R··F··P··G··R··L··A··V··N··T··G·
DNA:   CAGGATCCCGAGAGCTTGCTGATCAACGGCAAAGGCCAGTTCAGAGACCCCAACACCGGT
+1FR:  ·Q··D··P··E··S··L··L··I··N··G··K··G··Q··F··R··D··P··N··T··G·
DNA:   TTCATGACCAACACCCCATTGGAAGTGTTCACCATGACCCCAGGCAACCGCTACCGATTC
+1FR:  ·F··M··T··N··T··P··L··E··V··F··T··M··T··P··G··N··R··Y··R··F·
DNA:   CGTATCATCAACTCCTTCGCTTCTGTGTGTCCGGCTCAGCTGACCATACAGGGACACGAC
+1FR:  ·R··I··I··N··S··F··A··S··V··C··P··A··Q··L··T··I··Q··G··H··D·
DNA:   CTAACTTTGATCGCCACCGACGGAGAGCCCGTGCATCCTGTAAAAGTCAACACCATAATT
+1FR:  ·L··T··L··I··A··T··D··G··E··P··V··H··P··V··K··V··N··T··I··I·
DNA:   TCGTTCTCAGGTGAAAGATACGATCTTCT
+1FR:  ·S··F··S··G··E··R··Y··D··L·
```

**Figure 3.2.** Cloned partial DNA (621 bp) and amino acid (209 codons) sequences of *Aethina tumida Laccase 2* (GenBank: KU696310). Highlighted in blue is the position of the gene specific forward/reverse primers used to amplify a 301 bp product for *in vitro* transcription of dsRNA, which is depicted in orange. In pink is the position of the forward/reverse qPCR primers generating an amplicon of 69 bp.

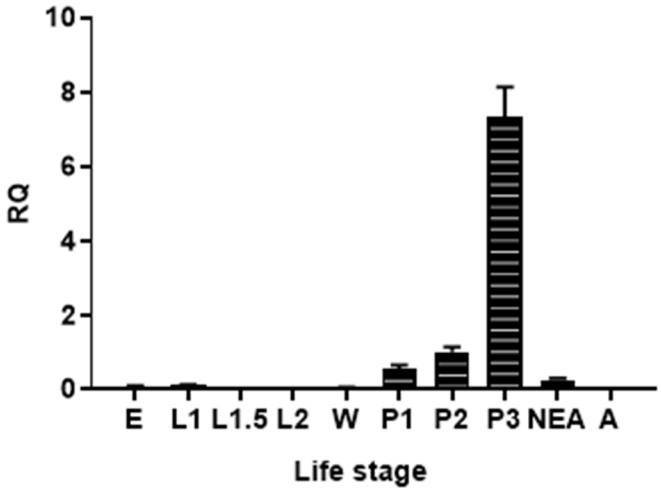
```
DNA:   GAGATCATCCGTTTGGAGGGCGAAATGGCCACTATCCAAGTATACGAAGAAACATCCGGT
+1FR: ·E··I··I··R··L··E··G··E··M··A··T··I··Q··V··Y··E··E··T··S··G·
DNA:   GTAACAGTTGGTGATCCGGTGTTGCGTACCGGTAAACCCTTGTCCGTCGAATTGGGACCT
+1FR: ·V··T··V··G··D··P··V··L··R··T··G··K··P··L··S··V··E··L··G··P·
DNA:   GGTATTATGGGTTCAATTTTCGACGGTATCCAACGTCCGTTGAAAGACATCAACGATTTG
+1FR: ·G··I··M··G··S··I··F··D··G··I··Q··R··P··L··K··D··I··N··D··L·
DNA:   ACCCAGAGCATTTACATTCCCAAGGGTGTGAACGTGCCCGCCCTTTCGAGGACGGCCAAA
+1FR: ·T··Q··S··I··Y··I··P··K··G··V··N··V··P··A··L··S··R··T··A··K·
DNA:   TGGGAATTCAATCCGTGGAACATCAAATTGGGAGCTCACTTAACGGGAGGTGACATCTAC
+1FR: ·W··E··F··N··P··W··N··I··K··L··G··A··H··L··T··G··G··D··I··Y·
DNA:   GGTATCGTCCACGAAAACACCCTGGTGAAACACAAAATCGTCCTGCCACCTAAAGCCAAG
+1FR: ·G··I··V··H··E··N··T··L··V··K··H··K··I··V··L··P··P··K··A··K·
DNA:   GGTACCGTTACATACGTAGCTGAGCCGGGTAATTACACAGTCGATGAAGTTGTATTGGAA
+1FR: ·G··T··V··T··Y··V··A··E··P··G··N··Y··T··V··D··E··V··V··L··E·
DNA:   ACGGAATTCGATGGCGAACGCACCAAATATTCTATGTTGCAAGTGTGGCCTGTACGTCAA
+1FR: ·T··E··F··D··G··E··R··T··K··Y··S··M··L··Q··V··W··P··V··R··Q·
DNA:   CCACGTCCGGTCAGCGAAAAGTTGCCAGCGAATCATCCTCTTCTCACCGGACAACGTGTC
+1FR: ·P··R··P··V··S··E··K··L··P··A··N··H··P··L··L··T··G··Q··R··V·
DNA:   CTGGACTCTTTGTTCCCTTGTGTACAGGGTGGTACCACCGCCATTCCCGGAGCTTTCGGT
+1FR: ·L··D··S··L··F··P··C··V··Q··G··G··T··T··A··I··P··G··A··F··G·
DNA:   TGCGGCAAAACCGTAA
+1FR: ·C··G··K··T··V·
```

**Figure 3.3.** Cloned partial DNA (618 bp) and amino acid (205 codons) sequences of *Aethina tumida V-ATPase subunit A* (GenBank: KU696311). Highlighted in blue is the position of the gene specific forward/reverse primers used to amplify a 306 bp product for *in vitro* transcription of dsRNA, which is depicted in orange. In pink is the position of the forward/reverse qPCR primers generating an amplicon of 69 bp.

### 3.2.2 Expression of *Laccase 2* and *V-ATPase subunit A* during development of *Aethina tumida*

As shown in Figure 3.4 A *Laccase 2* transcripts were detected at significantly higher levels during the three week pupal phase as compared to levels at all other developmental stages, although the mRNA was detectable throughout the insect life-cycle. The peak in *Laccase 2* mRNA levels during the third week of the pupal stage coincides with the onset of cuticle tanning, and subsequently declines to a level close to the detection limit in the emerged adult. By contrast, *V-ATPase subunit A* transcripts were readily detectable during all developmental stages (Figure 3.4 B). *V-ATPase subunit A* transcript levels were generally higher in the later stages of development (i.e. late larval through pupal stage to adult) with the highest levels detected in wandering larvae and 3 week old pupae.

**(A)**




**(B)**

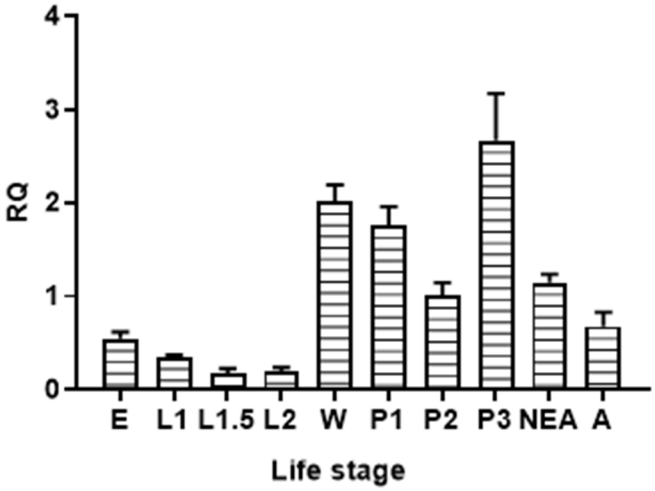


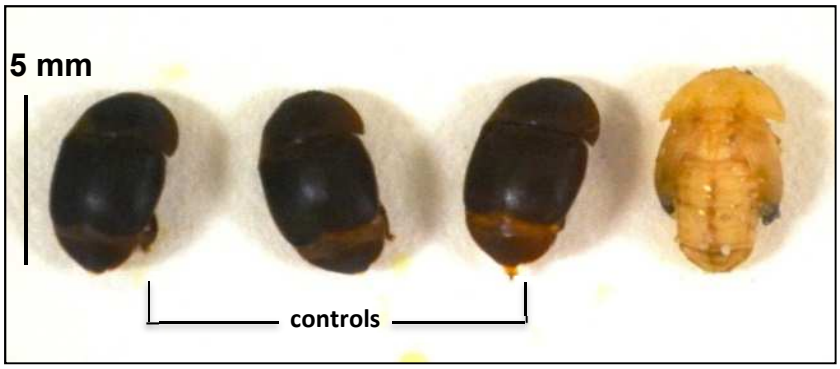

**Figure 3.4.** Expression of: (A) *Laccase 2* and (B) *V-ATPase subunit A* genes throughout the life-cycle of *Aethina tumida*, assayed by quantitative PCR. Developmental stages as follows: E denotes eggs; L1-W, different stages of larval growth (1, 1.5 & 2 week old larvae; W=wandering stage); P1-P3 are samples taken at 1, 2, and 3 week intervals during the pupal stage; NEA are non-emerged adults and A are emerged adults. Expression levels are normalised to *GAPDH* mRNA; RQ was set to 1 for P2 stage samples. All error bars represent the ±SD of the mean, as determined from one replicate (n=5 per insect replicate or 50 mg wet weight of eggs), each with three technical replicates.

### 3.2.3 Injection of dsRNA to assess phenotype in wandering *Aethina tumida* larvae

The phenotypes observed in wandering *A. tumida* following injections with 500 ng target dsRNAs are shown in Figure 3.5 A & 3.5 B. All insects injected with *Laccase 2* dsRNA died and were albino-like in appearance, exhibiting a distinct lack of melanisation in comparison to control treatments where tanning was evident 3 weeks after injection (Figure 3.5 A). Whilst a phenotype (i.e. lack of melanisation) was evident 3 weeks after injection of *Laccase 2* dsRNA, mortality did not occur until approx. 4 weeks post-injection and was recorded as failure to emerge as adults from sand at approx. 35 days post-injection. The injection of *V-ATPase subunit A* dsRNA also resulted in a lethal phenotype with treated larvae failing to develop into normal pupae (Figure 3.5 B). A failure to develop from the wandering to pupal stage was observed approx. 2 weeks after injection of *V-ATPase subunit A* at which point mortality was not always evident; as for *Laccase 2* treated insects mortality was recorded when the controls emerged as adults.

Reducing the dose of *Laccase 2* dsRNA from 500 ng to 12.5 ng did not reduce lethality in wandering stage larvae with 100 % of the adults failing to emerge. A further reduction in injection doses to 10 ng, 5 ng and 2 ng *Laccase 2* dsRNA did provide a dose response, with a respective 90 %, 20 % and 10 % of the adults failing to emerge. *Aethina tumida* injected with *V-ATPase subunit A* dsRNA at a dose of 12.5 ng, 10 ng and 5 ng resulted in 100 % mortality, with 90 % mortality observed in the 2 ng treatment, assessed as a failure to emerge as adults. Control mortality was 10 % in either Ringers solution or *nptII* dsRNA treatments and 100 % survival was recorded in the non-injected control group (Table. 3.1). $LD_{50}$ of 7.49 ng (95 % C.I 2.35-9.35 ng) could only be determined for *Laccase 2* due to the high level of mortality recorded in the *V-ATPase subunit A* treatment.

**(A)**




**(B)**


**Figure 3.5.** *Aethina tumida* phenotype after injection with dsRNAs: (A) & (B) Wandering stage larvae injected with 500 ng *Laccase 2* (35 days post-injection) and *V-ATPase subunit A* (approx. 2 weeks post-injection.) dsRNA, respectively. Controls from left to right are not injected; injected with Ringers solution and *nptII* dsRNA. Scale bars are indicated for larvae, pupae and adults.

**Table 3.1.** Survival of *Aethina tumida* wandering larvae (n=10 per treatment) injected with different doses of *Laccase 2 or V-ATPase subunit A* dsRNAs, controls were non-injected; injected with *nptII* dsRNA or Ringers solution.

| Treatment | Dose (ng) | Survival (%) | 95 % C.I (ng) |
|---|---|---|---|
| Non-injected | - | 100 | - |
| Ringers Control | - | 90 | - |
| *nptII* | 12.5 | 90 | - |
| | | | |
| *Laccase 2* | 12.5 | 0 | 2.35-9.35 |
| | 10.0 | 10 | - |
| | 5.0 | 80 | - |
| | 2.0 | 90 | - |
| | | | |
| *V-ATPase subunit* | 12.5 | 0 | - |
| | 10.0 | 0 | - |
| | 5.0 | 0 | - |
| | 2.0 | 10 | - |

### 3.2.4 Effect of injected dsRNA on gene expression in wandering *Aethina tumida* larvae

To confirm that lethality was a result of a reduction in mRNA levels the expression of target genes in injected insects was assessed by qPCR. For *Laccase 2*, expression levels were analysed for wandering larvae 48 hr and 3 weeks after the injection of 10 ng dsRNA (Figure 3.6 A). Larvae injected with *Laccase 2* dsRNA exhibited a significant 25-45 % decrease in *Laccase 2* mRNA levels 48 hr post-injection relative to the control groups ($P < 0.01$; One-way ANOVA followed by Tukey test). Analysis of larvae 3 weeks after injection also showed a significant reduction in *Laccase 2* transcript levels (by approx. 70 to 87 %) as compared to the control treatments ($P < 0.0001$; One-way ANOVA followed by Tukey test). Furthermore, mean mRNA levels in *Laccase 2* treated insects were significantly lower 3 weeks post-injection as compared to 48 hr post-injection ($P < 0.01$; One-way ANOVA followed by Tukey test). Transcript levels in wandering larvae injected with 2 ng of *V-ATPase subunit* dsRNA were analysed in samples extracted 48 hr and 1 week post-injection (Figure 3.6 B). A significant 31-54 % decrease in relative levels of *V-ATPase subunit A* mRNA was observed 48 hr post-injection relative to the control groups ($P < 0.001$; One-way ANOVA followed by Tukey test) increasing to 67-85 % in samples taken 1 week after injection ($P < 0.0001$; One-way ANOVA followed by Tukey test). Additionally, mean mRNA levels in *V-ATPase subunit A* injected insects were significantly lower 1 week post-injection as compared to 48 hr post-injection ($P < 0.01$; One-way ANOVA followed by Tukey test).

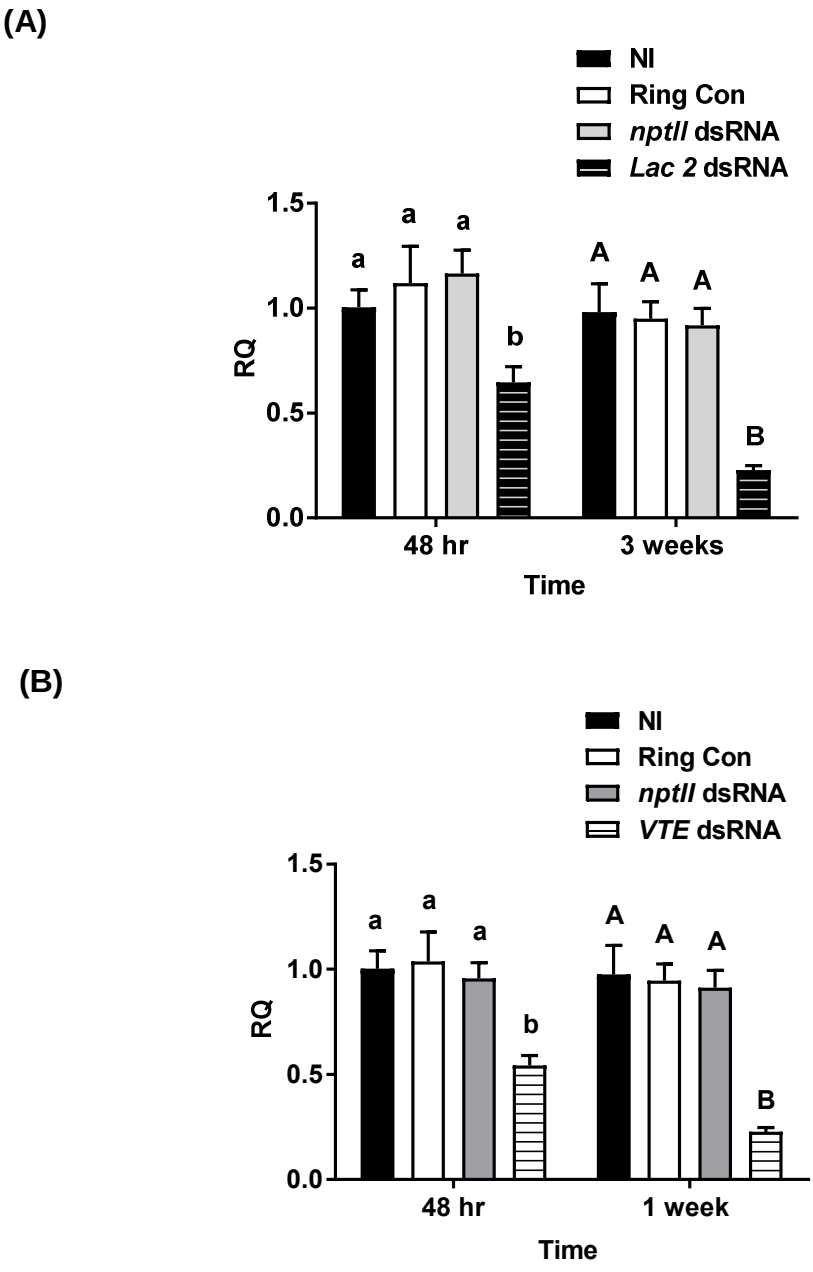



**Figure 3.6.** Relative expression of: (A) *Laccase 2* mRNA in *Aethina tumida* wandering larvae 48 hr and 3 weeks after injection of 10 ng *Laccase* 2 dsRNA (*Lac 2*); (B) *V-ATPase subunit A* mRNA in wandering larvae 48 hr and 1 week after injection of 2 ng *V-ATPase subunit A* dsRNA (*VTE*). Controls are non-injected (NI); Ringers solution (Ring Con) and *nptII* dsRNA injected. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for NI. All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insects per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

### 3.2.5 Injection of dsRNA to assess phenotype and effect on gene expression in 7 day old *Aethina tumida* larvae

Larvae were initially injected in the wandering non-feeding phase as this was an appropriate stage for administering dsRNAs, given endogenous expression of the target genes, and it was also a convenient developmental stage for injection. It has previously been reported that RNAi efficiency can be affected by the developmental stage of an insect (Huvenne and Smagghe, 2010).

To verify persistent and systemic RNAi (prior to oral delivery bioassays) within actively feeding insects, 7 day old larvae were injected with 50 ng of *Laccase 2* and *V-ATPase subunit A* dsRNA. As for wandering larvae, mortality was not evident for *Laccase 2* treated insects until approx. 4 weeks post-injection and 2 weeks after injection of *V-ATPase subunit A* dsRNA; and in both cases was recorded as a failure to emerge as adults 35 days post-injection. Control survival ranged from 90-100 % whereas 80 % and 100 % mortality was recorded for *Laccase 2* and *V-ATPase subunit A* treated insects, respectively (Table 3.2). For insects injected with *Laccase 2* dsRNA tanning was delayed and adults that emerged exhibited developmental abnormalities (Figure 3.7 A). The transcript levels of *Laccase 2* (assessed 3 weeks after injection of 7 day old larvae with 50 ng dsRNA) were a significant 68-78 % lower than controls (Figure 3.8 A; $P < 0.0001$; One-way ANOVA followed by Tukey test). For 7 day old larvae injected with 50 ng of *V-ATPase subunit A* dsRNA mRNA levels were significantly reduced (by 72-92 % and 55-90 %, respectively) in samples taken 48 h and 1 week post-injection (Figure 3.8 B; $P < 0.0001$ and 0.01; One-way ANOVA followed by Tukey test). As observed in insects injected in the wandering phase, development was arrested at the larval stage (Figure 3.7 B). It is clear from these data that the RNAi effect was persistent and systemic regardless of life stage.

**Table 3.2.** Survival of *Aethina tumida* feeding stage larvae (n=10 per treatment) injected with different doses of *Laccase 2 or V-ATPase subunit A* dsRNAs, controls were injected with *nptII* dsRNA or Ringers solution.

| Treatment | Dose (ng) | Survival (%) | 95 % C.I (ng) |
|---|---|---|---|
| Non-injected | - | 100 | 74 – 100 |
| Ringers Control | - | 100 | 74 – 100 |
| *nptII* | 50 | 90 | 62 - 99 |
| *Laccase 2** | 50 | 20 | 4 - 50 |
| *V-ATPase subunit* | 50 | 0 | 0 - 26 |

*Both of the emerged adults had deformities, but were included in the survival %

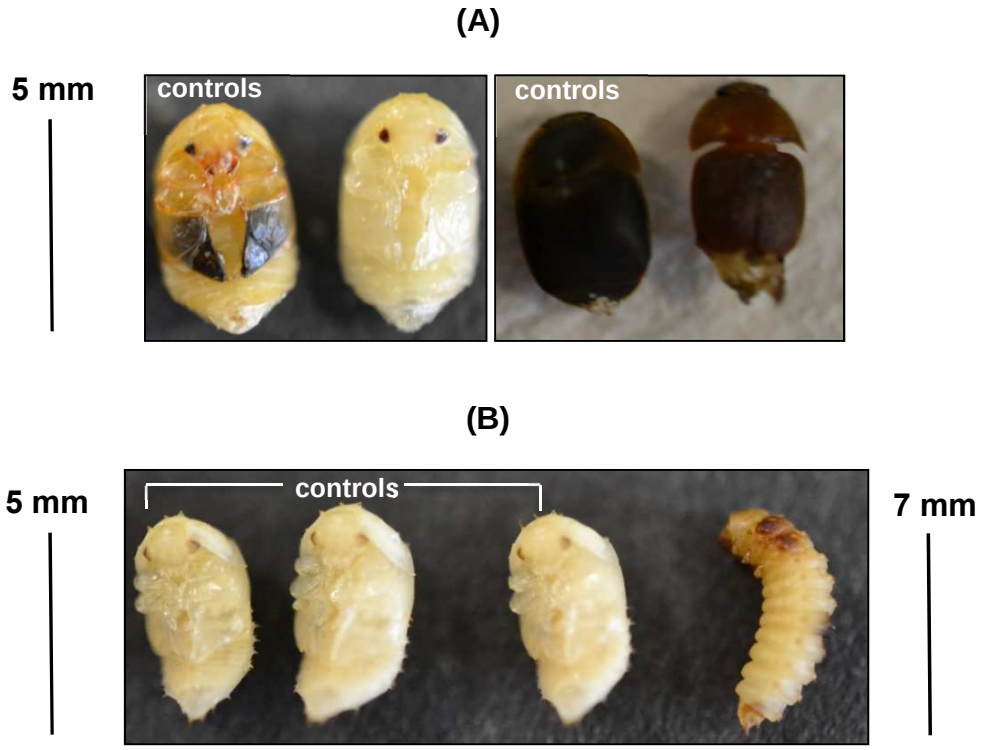
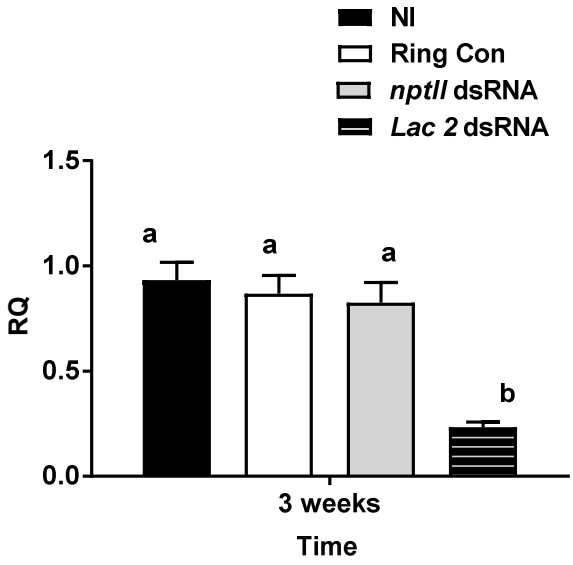



**Figure 3.7.** Phenotype of feeding *Aethina tumida* larvae after injection with dsRNAs: (A) Feeding stage larvae injected with 50 ng *Laccase 2* dsRNA (left 17 days and right 35 days post-injection); (B) Feeding stage larvae injected with 50 ng *V-ATPase subunit A* dsRNA, controls from left to right are not injected; injected with Ringers solution and *nptII* dsRNA (14 days post-injection). Scale bars are indicated for larvae, pupae and adults.

**(A)**




**(B)**

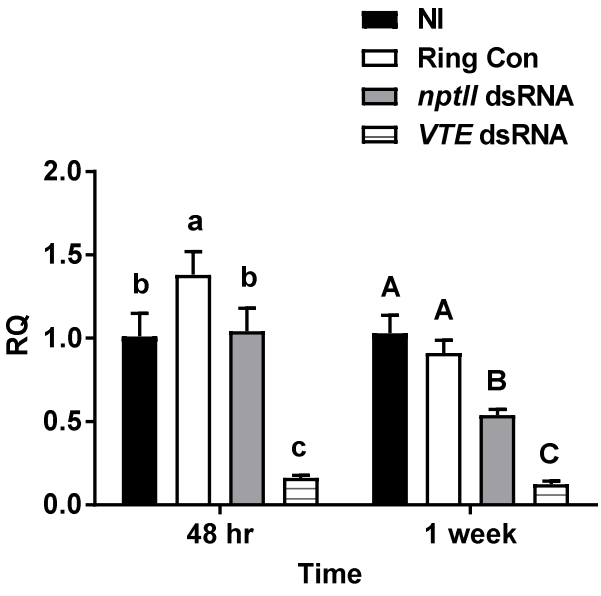



**Figure 3.8.** Relative expression of: (A) *Laccase 2* mRNA in feeding stage (7 day old) larvae 3 weeks after injection of 50 ng *Laccase* 2 dsRNA; (B) *V-ATPase subunit A* mRNA in 7 day old larvae 48 hr and 1 week after injection of 50 ng *V-ATPase subunit A* dsRNA (*VTE*). Controls are non-injected (NI); Ringers (Ring Con) and *nptII* dsRNA injected. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for NI. All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insects per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

### 3.2.6 Oral delivery of dsRNA in artificial diet

To determine if mRNA levels could be down-regulated via oral delivery of dsRNA, 7 day old *A. tumida* larvae (n=30) were fed on artificial diet containing target or control dsRNAs (or Ringers solution as a negative control) for 6 days. Adult emergence was monitored after approx. 35 days; Ringers control emergence was 100 %, whereas 93 % emergence was observed in both *nptII* and *Laccase 2* dsRNA treatments and 73 % emergence was recorded in the *V-ATPase subunit A* dsRNA treatment. The expression of *V-ATPase subunit A* mRNA in treated insects was assessed by qPCR analysis of larvae collected 48 hr and 1 week after feeding on artificial diets containing dsRNA. Larvae fed on *V-ATPase subunit A* dsRNA showed no significant decrease in transcript levels 48 hr or 1 week after feeding on dsRNA (Figure 3.9 $P > 0.05$; One-way ANOVA followed by Tukey test).

### 3.2.7 Stability and oral delivery of dsRNA in sucrose solution

Feeding dsRNA in artificial diets did not trigger RNAi effects and analysis of the stability of dsRNA in the diet proved problematic as separation of the pollen and honey from dsRNA by centrifugation was incomplete and hence it was unclear if the dsRNA remained intact or was degraded over time (results not shown). Prior to conducting soaking bioassays the stability of dsRNA in the presence of feeding *A. tumida* larvae was assessed by taking samples over a period of 0 to 22 hr. As shown in Figure 3.10 the dsRNA remained mostly intact for a period of 1 hr, showing a reduction in size indicative of exonuclease activity. After 2 hr there is approximately half the amount of dsRNA, as compared to time 0, present in the sucrose solution and after 8 hr the dsRNA is completely degraded.

Subsequently a second feeding assay whereby 7 day old larvae (n=20) were soaked for 24 hr (with the solution being renewed at 8 hr intervals) in sucrose solutions containing 100 ng/µl of target or control dsRNA, was conducted. Adult emergence was monitored after approx. 35 days; sucrose and *nptII* dsRNA control emergence was 80 % (n=20) and 82 % (n=17) respectively, whereas 100 % (n=16) emergence was observed in *Laccase 2* dsRNA treatments (Table 3.3). For *V-ATPase subunit A* dsRNA treatment 50 % (n=18) emergence was recorded, with 17 % of the emerged adults exhibiting morphological deformities (Figure 3.11 A). However, when this experiment was repeated, qPCR analysis of samples extracted 48 hr and 1 week after feeding on

*V-ATPase subunit A* dsRNA showed that transcript levels were not significantly decreased in comparison to the control groups (Figure 3.11 B; $P < 0.05$; One-way ANOVA followed by Tukey test).

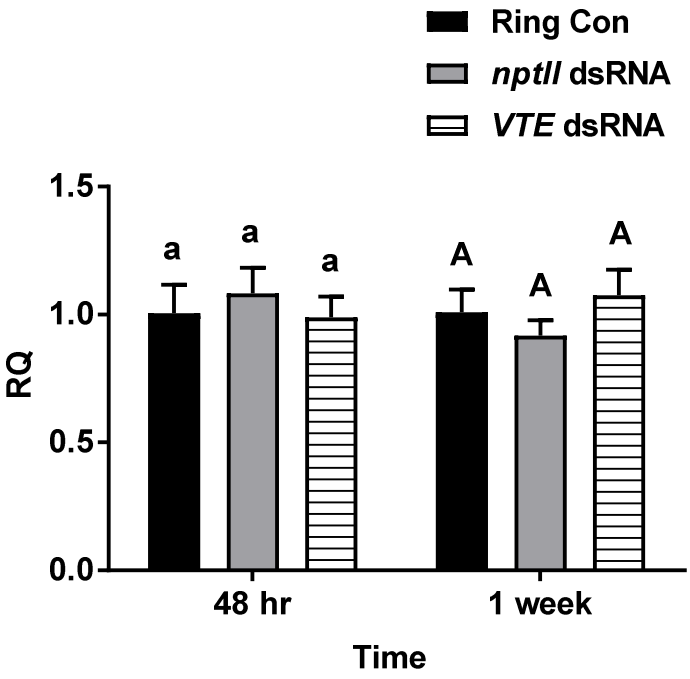



**Figure 3.9.** Expression of *V-ATPase subunit A* mRNA in *Aethina tumida* after feeding for 6 days on artificial diet containing 33 ng/mg of *V-ATPase subunit A* dsRNA (*VTE*), samples were removed 48 hr and 1 week after feeding. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for Ringers solution control sample (Ring Con). All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

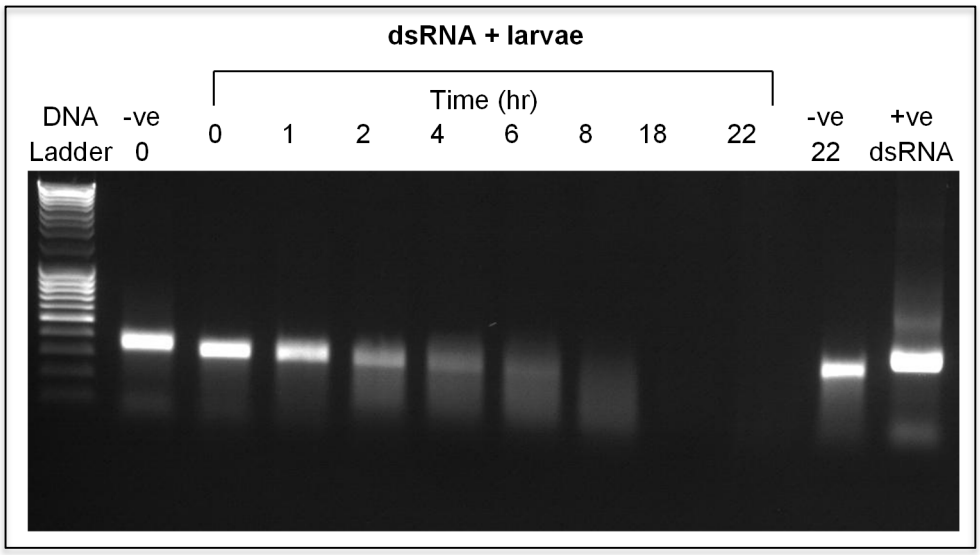



**Figure 3.10.** Stability of dsRNA in the presence of feeding stage *Aethina tumida* larvae. Larvae were immersed in sterile sucrose solution containing dsRNA and samples taken at specified time points were analysed for integrity by agarose gel electrophoresis. Negative controls are –ve 0 and –ve 22 which show dsRNA with no larvae present at time 0 and after 22 hr; +ve dsRNA denotes dsRNA re-suspended in Ringers solution.

**Table 3.3.** Survival of *Aethina tumida* soaked in 50 % sucrose solutions containing 10 µg of *Laccase 2, V-ATPase subunit A* or *nptII* dsRNA and sucrose solution served as an additional control.

| Treatment | Survival | Sample No. | 95 % C.I survival (%) |
|---|---|---|---|
| Sucrose Control | 80 | 20 | 59 – 92 |
| *nptII* | 82 | 17 | 60 - 95 |
| *Laccase 2* | 100 | 16 | 83- 100 |
| *V-ATPase subunit** | 50 | 18 | 28- 72 |

* 3 of the emerged adults had deformities, but were included in the survival

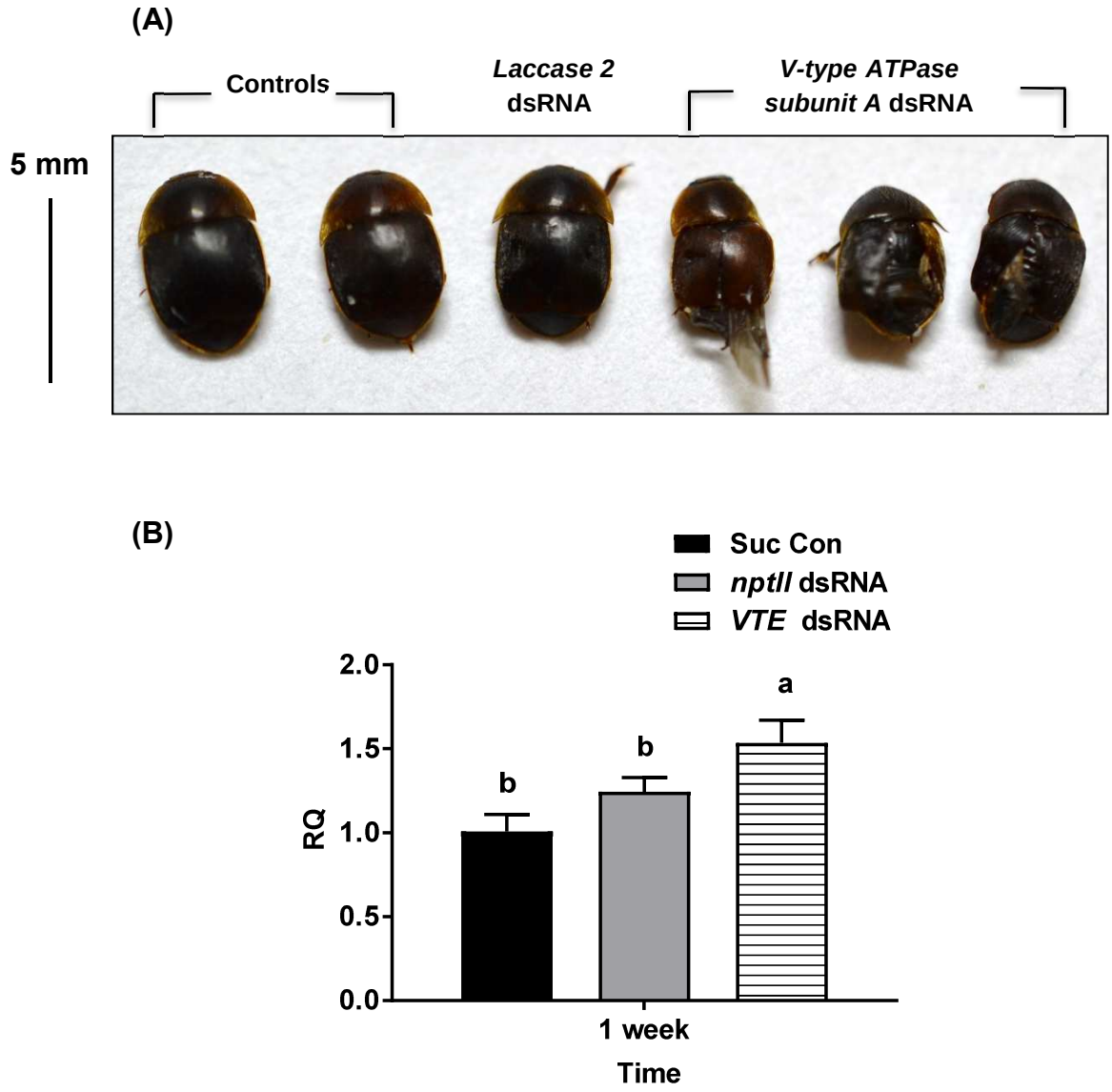



**Figure 3.11.** (A) Adult *Aethina tumida* 35 days after soaking for 24 hr in sucrose solutions containing 0.1 µg/µl of *Laccase 2* dsRNA or *V-ATPase subunit A* dsRNA; controls left and right are sucrose only treated and *nptII* dsRNA treated; Scale bars are indicated for adults. (B) Expression of *V-ATPase subunit A* mRNA in *A. tumida* after soaking for 24 hr in sucrose solutions containing 0.1 µg/µl of *V-ATPase subunit A* dsRNA (*VTE*), samples were removed 1 week after feeding. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for sucrose soaked control sample (Suc Con). All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

### 3.2.8 Stability and oral delivery of dsRNA in agar diet

The results of the soaking bioassay suggested that an RNAi effect had been triggered as 50 % mortality was recorded and 17 % of the emerged adults exhibiting morphological deformities. However, when the experiment was repeated, qPCR analysis failed to confirm a decrease in *V-ATPase subunit A* transcript levels. To enhance the stability of the dsRNA it was incorporated into a sterile agar diet with the aim of providing great protection from exonuclease activity.

Prior to conducting agar feeding bioassays the stability of dsRNA in the agar diet was assessed in the presence of *A. tumida* larvae by taking samples over a period of 0 to 22 hr. As shown in Figure 3.12 the dsRNA remained mostly intact for a period of 8 hr and after 18 and 22 hr, respectively, there was approximately one seventh of the amount of dsRNA, as compared to time 0. The results suggest that the incorporation of dsRNA into an agar diet significantly improved dsRNA stability as intact dsRNA was evident after 8 hr, whereas dsRNA remained intact for 1 hr during the soaking assay.

A second feeding assay whereby 5 day old larvae (n=20) were fed for 72 hr on agar diet (with the diet being renewed at 24 and 48 hr intervals) containing 20 µg of *V-ATPase subunit A* or *nptII* dsRNA, with Ringers solution serving as an additional control. Adult emergence was monitored after approx. 35 days; Ringers control and *nptII* dsRNA control emergence was 95 % (n=20) and 90 % (n=20) respectively, For *V-ATPase subunit A* dsRNA treatment there was not a significant decrease in survival, with 85 % (n=20) emergence being recorded. The expression of *V-ATPase subunit A* mRNA in treated insects was assessed by qPCR analysis of larvae collected 1 week after feeding on agar diet containing dsRNA. Larvae fed on *V-ATPase subunit A* dsRNA showed no significant decrease in transcript levels 1 week after feeding on dsRNA (Figure 3.13 $P > 0.05$; One-way ANOVA followed by Tukey test).

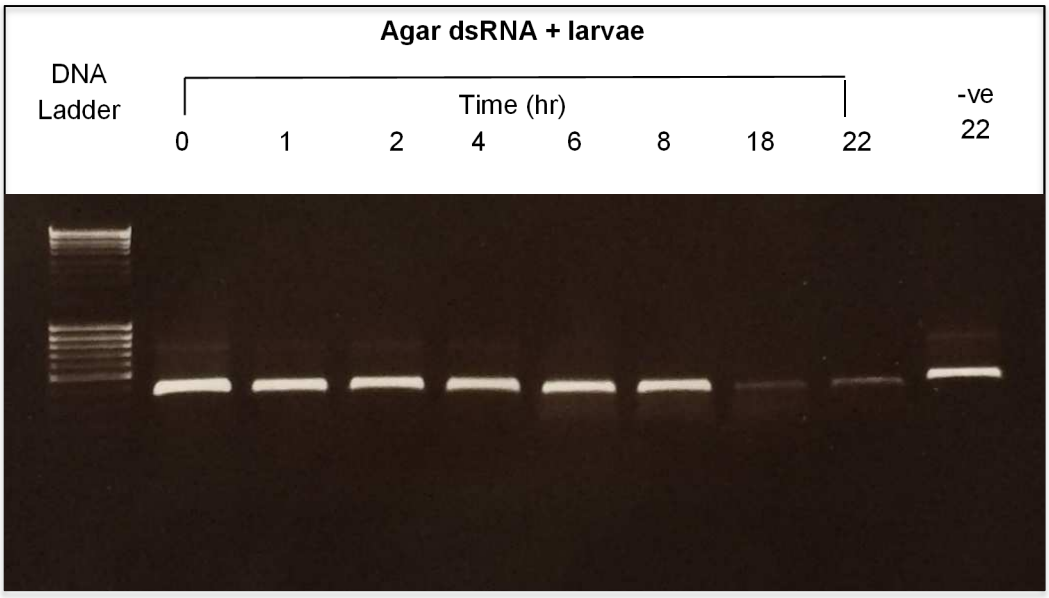



**Figure 3.12.** Stability of dsRNA, incorporated into agar diet, in the presence of feeding stage *Aethina tumida* larvae. Larvae were allowed to feed on the agar diet containing dsRNA and samples taken at specified time points were analysed for integrity by agarose gel electrophoresis. Negative control is –ve 22 which shows dsRNA with no larvae present after 22 hr.

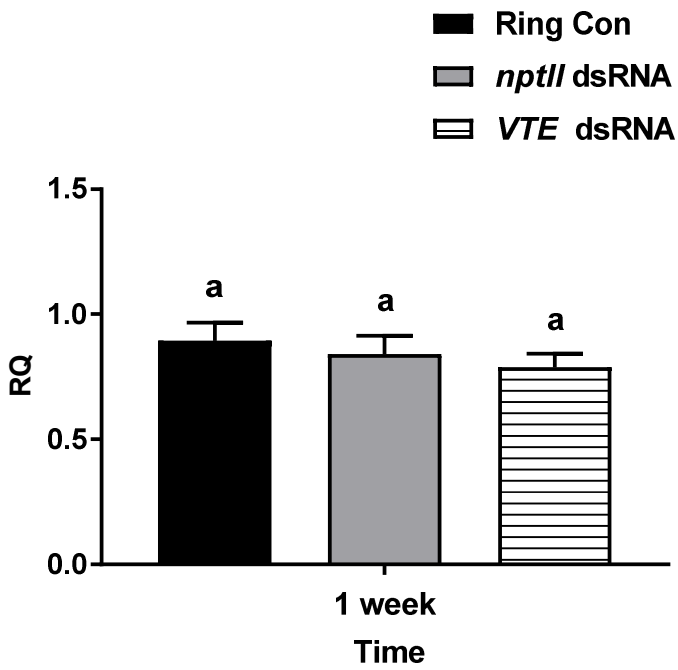



**Figure 3.13.** Expression of *V-ATPase subunit A* mRNA in *Aethina tumida* after feeding for 24 hr on agar diet containing 57 ng/mg of *V-ATPase subunit A* dsRNA (*VTE*), samples were removed 1 week after feeding. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for sucrose soaked control sample (Suc Con). All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

### 3.2.9 In *vivo* stability of dsRNA in the presence *Aethina tumida* larvae and frass

The secretion of extracellular ribonucleases by larvae was investigated by the addition of dsRNA to a solution after the removal of feeding larvae that had been immersed in sterile water for period of 8 hr. Figure 3.14 shows that complete degradation of the dsRNA under these conditions occurs and this is also observed when frass was added to dsRNA containing solutions. By contrast, dsRNA remained intact when wandering (non-feeding) larvae were incubated in dsRNA solutions. These results indicate that extracellular nucleases are secreted as part of the digestive process in the guts of feeding larvae.

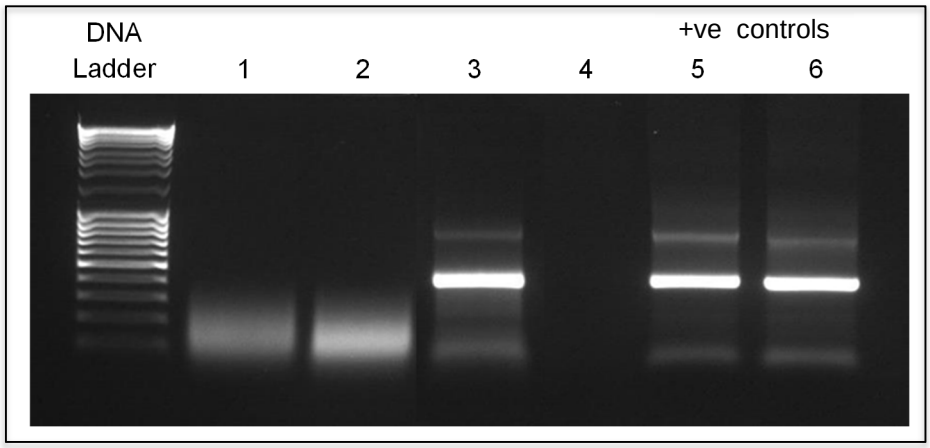



**Figure 3.14.** Stability of dsRNA in the presence of *Aethina tumida* larvae and frass. All samples were incubated for a period of 8 hr and analysed by agarose gel electrophoresis.10 kb DNA ladder; lane 1, dsRNA added after the removal of 7 day old (feeding) larvae; lane 2, feeding larvae incubated with dsRNA; lane 3, wandering larvae incubated with dsRNA; lane 4, larval frass incubated with dsRNA, lane 5 and lane 6, positive (+ve) controls denoting dsRNA added to 50 % sucrose solution and dsRNA alone, respectively.

### 3.2.10 *In vitro* stability of dsRNA in *Aethina tumida* larval gut extracts

The stability of dsRNA was assessed *in vitro* by incubating dsRNA in gut extracts for 0 to 60 min. Analysis of these samples showed that dsRNA degradation commenced within an incubation period of 5 min and degradation of the dsRNA was complete after 60 min (Figure 3.15).

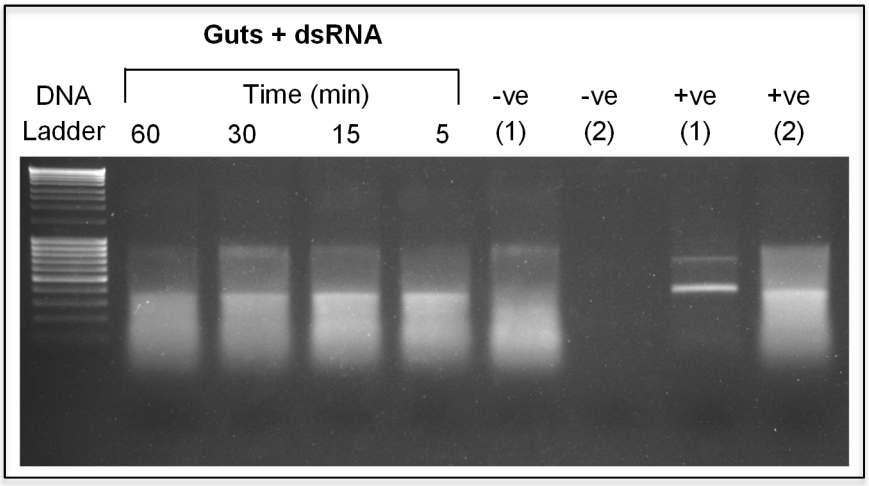



**Figure 3.15.** *In vitro* stability of dsRNA in *Aethina tumida* larval gut extracts. Samples were taken at indicated time points after incubation of 500 ng dsRNA with gut extract (approx. 1/10 of a larval gut; final volume 20 µl) and analysed by agarose gel electrophoresis, 20 µl was loaded in all lanes. Negative (-ve) controls are (1) gut sample alone, and (2) Ringers solution. Positive (+ve) controls are (1) dsRNA re-suspended in Ringers solution and (2) dsRNA and gut extract at 0 hr.

### 3.2.11 CLUSTALW analysis of *Aethina tumida* and *Apis mellifera Laccase 2* and *V-ATPase subunit A* mRNA

Partial sequences of *A. tumida* and *A. mellifera Laccase 2* (Genbank: FJ470292) and *V-ATPase subunit A* (GenBank: XM 006567414) were aligned to assess potential for cross-species RNAi effects. Comparisons of *A. tumida* and *A. mellifera Laccase 2* and *V-ATPase subunit A* mRNAs revealed the presence of conserved regions, however coverage was limited to, at most, a 15 bp region (Figure 3.16). The likelihood of introduced *A. tumida* dsRNA eliciting an RNAi response within the honey bee *A. mellifera* is low given the absence of 20-25 nt stretches of homology.

### 3.2.12 Effect of injected *Aethina tumida* dsRNA on phenotype and gene expression in *Apis mellifera*

To investigate whether *A. tumida* target dsRNAs caused mortality and/or down-regulation of *A. mellifera Laccase 2* and *V-ATPase subunit A* mRNAs, adult honey bees were injected with 50 ng of target dsRNAs. Survival for both controls and dsRNA treated *A. mellifera* was 100 % after 10 days. As shown in Figure 3.17, qPCR analysis confirmed that mRNA levels were not down-regulated in either *Laccase 2* injected pharate adults or *V-ATPase subunit A* injected adult honey bees, as compared to controls. Considerable variation in expression levels across different replicates was notable in these experiments and may, in part, be attributable to slight differences in the developmental stage of the bees that were used in the assays.

**(A)**

```
A. tumida       CGTGGAACCCAATATTACGATGGTGTGCCATTCGTTACACAATGTCCCATCCAACAAGGA 60
A. mellifera    AGGGGCTCTCAATATTACGACGGCGTACCATTCGTGACACAGTGCCCGATCCAAGAGGGT 240
                 * **  * *********** ** ** ******** ***** ** ** ****** * **

A. tumida       AACACCTTCAGGTACCAGTGGGTAGCCGGTAACGCCGGAACGCACTTTTGGCATGCCCAC 120
A. mellifera    AGCACCTTCAGGTACCAATGGACTGCTGGAAACGAAGGTACGCACTTCTGGCACGCCCAC 300
                * ***************  ***   ** ** ****  ** ******** ***** ******

A. tumida       ACCGGTCTGCAAAAGATGGACGGTTTGTATGGCAGCATCGTCATCCGTCAACCACCTTCC 180
A. mellifera    ACAGGATTGCAGAAAATGGACGGTCTGTACGGAAGCATAGTGATACGTCAACCGCCTAGC 360
                ** **  **** ** ********* **** ** ***** ** ** ******** ***  *

A. tumida       AAAGACCCCAACAGCAACTTGTACGACTACGATCTGACAACACACGTTATGCTGCTTTCC 240
A. mellifera    AAAGATCCTAACAGCAATCTTTACGACTACGATCTCACTACCCATGTCGTTCTAATCAGC 320
                ***** ** ********  * ************** ** ** ** **  * **  *   *

A. tumida       GATTGGATGCACGAAGATGCTGCCGAAAGATTCCCAGGAAGATTGGCTGTAAACACCGGT 300
A. mellifera    GATTGGTTCCATGAGAACGCGGCTGAACGTTTCCCCGGCCGGCTGGCGGTTAACACTGGC 380
                ****** * ** **  * ** ** *** * ***** **  *  **** ** ***** **

A. tumida       C------------------------------------------------------------ 301
A. mellifera    CAAGCGCCTGAAAGCGTGTTGATAAACGGGAAAGGCCAATTCAGGGATCCCAACACCGGT 360
                *
```

**(B)**

```
A. tumida       ------------------------------------------------------------GG 2
A. mellifera    AGAAATAATTCGTTTAGAAGGTGATATGGCTACTATACAGGTATATGAAGAAACTAGTGG 600
                                                                          **

A. tumida       TGTAACAGTTGGTGATCCGGTGTTGCGTACCGGTAAACCCTTGTCCGTCGAATTGGGACC 62
A. mellifera    TGTAACTGTGGGTGATCCAGTTTTACGTACTGGAAAGCCATTATCTGTAGAACTTGGACC 660
                ****** ** ******** ** ** ***** ** ** ** ** ** ** *** * *****

A. tumida       TGGTATTATGGGTTCAATTTTCGACGGTATCCAACGTCCGTTGAAAGACATCAACGATTT 122
A. mellifera    TGGTATTCTTGGCAGTATCTTTGATGGTATTCAAAGACCATTGAAAGATATCAATGAGCT 720
                ******* * **    ** ** ** ***** *** * ** ******** ***** **  *

A. tumida       GACCCAGAGCATTTACATTCCCAAGGGTGTGAACGTGCCCGCCCTTTCGAGGACGGCCAA 182
A. mellifera    TACAAACTCTATTTACATCCCAAAGGGTATTAATGTACCAGCATTATCAAGAACTGCTGC 780
                 **  *    ******** ** ****** * ** ** ** **  * ** ** ** **
A. tumida       ATGGGAATTCAATCCGTGGAACATCAAAT-TGGGAGCTCACTTAACGGGAGGTGACATCT 241
A. mellifera    TTGGGAATTTAATCCATCTAATATTAAAAATGGAAGC-CACATCACTGGTGGAGATTTAT 839
                 ******** ***** *  ** ** ***  *** *** *** * ** ** ** **  * *

A. tumida       ACGGTATCGTCCACGAAAACACCCTGGTGAAACACAAAATCGTCCTGCCACCTAAAGCCA 301
A. mellifera    TTGGTGTAGTCTATGAGAATACATTAGTGAAACATAAAATGATTTTACCTCCAAAAAGTA 899
                  *** * *** * ** ** **  * ******** *****  *  * ** ** ***   *

A. tumida       AGGG-------------------------------------------------------- 305
A. mellifera    AGGGAACTGTGACTTATATTGCACCTGCTGGCAATTATACAGTATCTGATGTTATTCTGG
```

**Figure 3.16.** CLUSTALW alignment of (A) *Apis mellifera Laccase 2* (GenBank: FJ470292)) and *Aethina tumida Laccase 2* mRNA; (B) *A. mellifera V-ATPase subunit A* (GenBank: XM 006567414) and *A. tumida V-ATPase subunit A* mRNA.

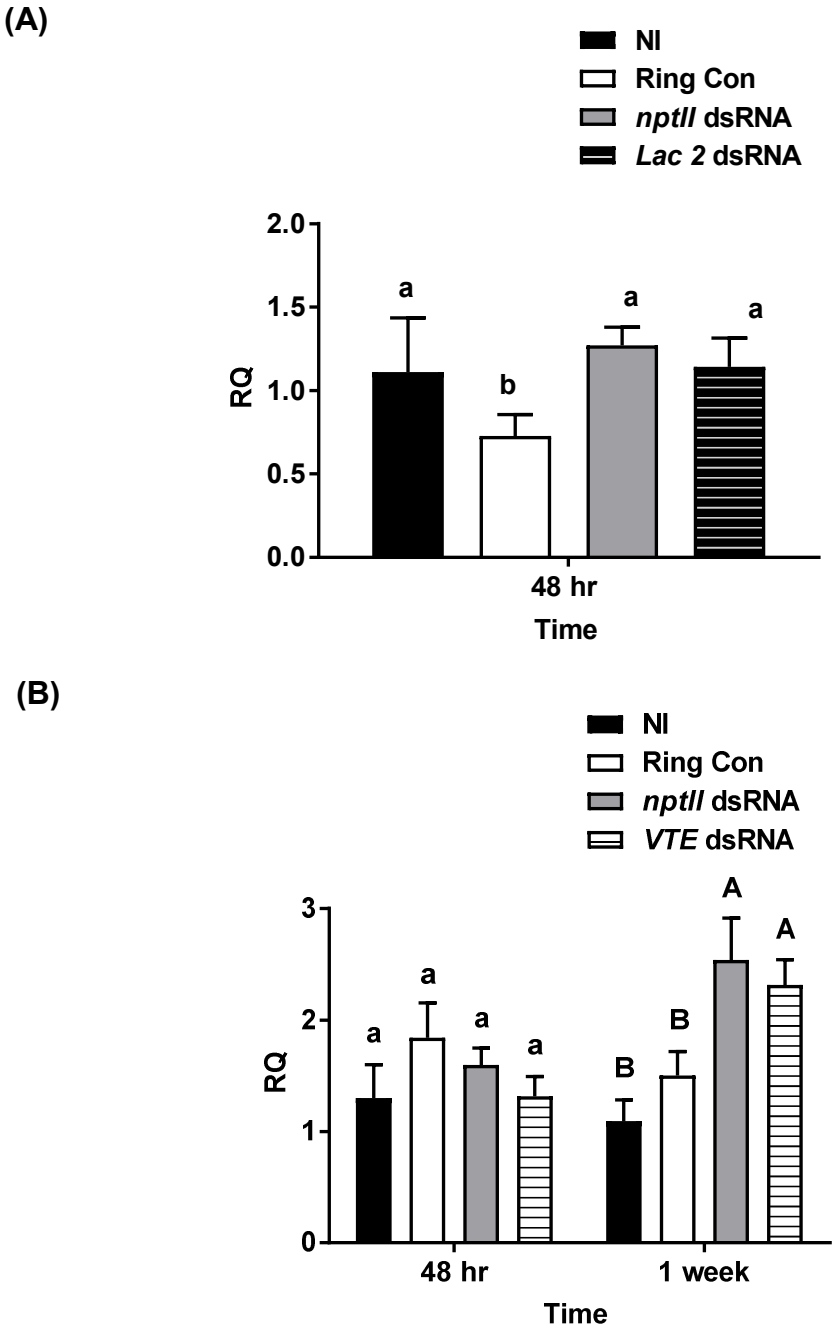



**Figure 3.17.** Relative expression of: (A) *Laccase 2* mRNA in 2 day old *Apis mellifera* pharate adults 48 hr after injection with 50 ng of *Aethina tumida Laccase* 2 dsRNA (*Lac 2*); (B) *V-ATPase subunit A* mRNA in *A. mellifera* 48 hr and 1 week post-injection with 50 ng of *A. tumida V-ATPase subunit A* dsRNA (*VTE*). Expression levels are normalised to Elongation factor-1 (EF-1) mRNA. Controls are non-injected (NI); Ringers (Ring Con) and *nptII* dsRNA injected. Expression levels are normalised to *GAPDH* mRNA. RQ set to 1 for NI. All error bars represent the ±SD of the mean, as determined from three independent replicates (n=5 insect per replicate), each with three technical replicates. Bars topped with the same letter are not statistically different at $P < 0.05$.

## 3.3 Discussion

*Aethina tumida*, a scavenger and predator of the European honey bee, has already spread from Africa to US and Australia, and more recently certain parts of Italy, highlighting the potential for it to become established in wider Europe and the UK. Current pest control measures are challenged by the need for target specificity and high efficacy. RNAi, able to cause the destruction of target specific mRNAs, offers possibilities for the development of a new approach to combat this economically significant pest without jeopardising the health of honey bee populations. Here we report significant dose-dependent mortality of *A. tumida* following the injections of 2-12.5 ng doses of dsRNAs targeting *Laccase 2* and *V-ATPase subunit A* mRNAs. Analysis of relative mRNA levels by qPCR confirmed target gene knock-down and significantly enhanced levels of gene suppression over time demonstrated that the RNAi effect was persistent and systemic.

Laccases are a group of multi-copper enzymes present in plants, fungi, bacteria and insects (Hoegger et al., 2006). In many insects, two types of laccase genes have been identified, namely *Laccase 1* and *Laccase 2* (Arakane et al. 2005; Dittmer et al., 2004). *Laccase 2*, a phenoloxidase gene, is expressed in the insect epidermis and has been shown, using RNAi, to be essential for normal beetle cuticle tanning (Arakane et al. 2005). Injections of dsRNA encoding *Laccase 2* into prepupal *T. castaneum* resulted in dose and time-dependant mortality. Delivery of 200 ng of dsRNA per prepupa inhibited tanning in adults and resulted in severe developmental abnormalities and mortality. When the dose was reduced to 2 ng per prepupa, this resulted in more normal looking adults, although a degree of malformation was observed and the tanning process was delayed by several days. This is comparable to the results presented in this study where *A. tumida* injected with 500 ng of *Laccase 2* dsRNA showed an albino type appearance, a distinct lack of melanisation and failure to emerge. A further reduction in dose to 12.5 ng and 10 ng, resulted in a similar phenotype and the adult that emerged was distinctly malformed, struggled to walk and died in a premature manner. The observed phenotype was confirmed to be a consequence of down-regulation of *Laccase 2* and, in addition qPCR analysis provided evidence for an increase in levels of gene suppression with time, indicative of transmission and persistence of the silencing signal.

V-type ATPases are highly conserved membrane bound proton pumps responsible for multiple processes including the acidification of organelles (e.g. secretory vesicles, lysosomes) and the maintenance of membrane potential. This enzyme is present in almost all epithelial tissues of insects and plays a vital role in nutrient uptake and ion balance in the insect digestive tract making it an ideal target for RNAi (Wieczorek et al., 2000; Wieczorek et al., 2009). V-type ATPases are heteromultimeric proteins comprised of a membrane bound protein conducting complex and a peripheral catalytic domain; sub-unit A is one of 8 peripheral domain sub-units that are located on the apical membrane surface. *V-ATPase subunit A* was used as a target gene in the breakthrough RNAi study where it was shown that orally delivered dsRNAs were highly efficacious towards *D. v. virgifera*, *D. undecimpunctata howardi* and *L. decemlineata* larvae (Baum et al., 2007). Here we show that *A. tumida* larvae have a strong RNAi response to *V-ATPase subunit A*, as delivery of as little as 2 ng of *V-ATPase subunit A* dsRNA elicited a lethal phenotype. This phenotype was observed several days prior to pupal metamorphism when *V-ATPase subunit A* transcript levels are at their highest during the life-cycle of *A. tumida*. Gene expression analysis revealed that larvae injected with *V-ATPase subunit A* dsRNA exhibited significantly enhanced levels of gene suppression from 48 hr to 1 week post-injection. Surprisingly, injection of control *nptII* dsRNA into feeding larvae seemed to result in a significant down-regulation of *V-ATPase subunit A* 1 week post-injection, compared to non-injected, Ringers control, with *V-ATPase subunit A* dsRNA injected insects being further down regulated compared to control groups. It is possible that this was a consequence of a slight difference in the developmental stage of the control *nptII* larvae as *V-ATPase subunit A* gene expression varies during the transition from wandering to pupal phase (Figure 3.4). Regardless, these data, as for *Laccase 2,* indicated that the silencing signal was amplified in *A. tumida* after injection with dsRNA.

Attempts to induce RNAi effects via oral delivery produced highly variable results. A factor that may influence RNAi efficiency is the development stage at which the insect is fed dsRNA (Huvenne and Smagghe, 2010). Araujo et al. (2006) reported that transcript levels of nitrophorin 2 from the saliva glands in *Rhodnius prolixus* were down-regulated in the 2$^{nd}$ instar relative to controls, however no effect was observed in 4$^{th}$ instar larvae after feeding on artificial diets containing dsRNA. Our results show

that injections of *V-ATPase subunit A* dsRNAs resulted in mortality and target gene suppression in both feeding and wandering stage larvae suggesting that life stage is not a limiting factor in eliciting an RNAi response within *A. tumida*.

It is clear that *A. tumida* are highly susceptible to dsRNA delivered via injection, but larvae fed on diets or "soaked" in solutions containing dsRNA showed no consistent evidence for RNAi effects. The complete lack of phenotype or gene suppression observed for feeding experiments with *Laccase 2* dsRNA were not unsurprising given the requirement for an RNAi effect upon delivery of the silencing signal from the gut to the epidermal tissue, where this gene is expressed. By contrast, 50 % mortality and deformities in surviving adults that had been soaked as feeding stage larvae in *V-ATPase subunit A* dsRNA containing solutions was indicative of an RNAi effect. Nonetheless these results could not be validated by qPCR analysis in a repeat experiment. We speculated that the lack of correlation between 50 % mortality compared to no effects on gene expression for *A. tumida* larvae fed on dsRNAs may be a consequence of differing amounts of dsRNA ingested and/or degradation within the gut of *A. tumida*, preventing sufficient uptake of dsRNAs by epithelial cells to induce an RNAi response. It is also possible that the lack of down-regulation observed in *V-ATPase subunit A* dsRNA fed larvae could be a consequence of qPCR analysis being carried out on non-affected larvae, where dsRNA was degraded before cellular uptake, as only 50 % mortality was recorded in the feeding bioassays. These results contrast with Baum et al. 2007 who reported that coating synthetic diets with target specific *V-ATPase subunit A* dsRNAs provided respective $LD_{50s}$ of 1.82 and 5.2 ng/cm$^2$ for *D. v. virgifera* and *L. decemlimeata* larvae. However, even in this study no effects on survival or growth were observed when larvae of the cotton boll weevil (*Anthonomus grandis*) were fed on diets coated with dsRNA, leading the authors to suggest that not all coleopteran larvae may be sensitive to orally delivered dsRNA. As for *A. tumida*, the desert locust (*Schistocerca gregaria*) and the migratory locust (*Locusta migratoria*) have been shown to be highly sensitive to dsRNA when delivered via injection, but oral delivery of dsRNA has proved unsuccessful (Luo et al., 2012; Wynant et al., 2014).

We speculated that the lack of consistent effects for *A. tumida* larvae fed on dsRNAs may be a consequence of dsRNA degradation within the gut of *A. tumida* preventing sufficient uptake of dsRNAs by epithelial cells to induce an RNAi response. Initial

experiments showing increased dsRNA degradation with time when feeding larvae were soaked in sucrose solutions was indicative of ribonuclease activity, either in the gut or larval regurgitant. *In vitro* studies also provided evidence that dsRNA was prone to degradation with complete digestion occurring within an hour of incubation with gut tissue, although it is noted that these homogenised extracts would contain intracellular and extracellular ribonucleases. Degradation of dsRNAs by extracellular ribonucleases was illustrated by the instability of dsRNA that had been added to solutions in which larvae had been soaked, and by degradation following the addition of frass to dsRNA containing solutions. By contrast, no degradation was observed when wandering (i.e. not feeding) stage larvae were incubated in dsRNA solutions providing further evidence that extracellular ribonucleases are secreted during digestion in the larval gut. Similarly Allen and Walker (2012) who found that RNAi could be induced by injection but not feeding in the hemipteran plant bug (*Lygus lineolaris*) hypothesised that dsRNA degradation prevented uptake of dsRNA into cells and demonstrated that saliva rapidly digested dsRNA. There is also direct evidence for the expression of dsRNA-degrading enzymes in the digestive juice of larvae of the lepidopteran *Bombyx mori* (Arimatsu et al., 2007). More recently Wynant et al. 2014 identified four candidate double stranded ribonucleases (dsRNase) that are expressed in the gut of the locust *Schistocerca gregaria* and subsequently provided evidence for the involvement of *Sg-dsRNAses 2* in the degradation of dsRNA. We provide further evidence here to illustrate that the protection of dsRNA from degradation by RNAses plays a key role in determining the successful application of RNAi for insect pest control.

The exploitation of RNAi as a strategy for the control of insect pests requires careful selection of target genes in order to achieve specific and effective silencing. Generally a specific segment of mRNA not shared amongst insects is targeted to elicit the RNAi effect in the selected pest. When dsRNAs are introduced into a cell they are cleaved into short fragments of approximately 20-25 nt in length and bind with high specificity to endogenous mRNA, disrupting the expression of the targeted protein product. Baum et al. 2007 reported that *D. v. virgifera V-ATPase subunit A* dsRNAs produced an effective oral RNAi effect in *D. v. virgifera* larvae but also (when fed at higher concentrations) in *L. decemlineata*. The nucleotide sequence identities between *D. v. virgifera* and *L. decemlineata* were 83 % for *V-ATPase subunit A* and 3 identical

regions of 20-29 nucleotides can be identified in the published sequence alignment providing an explanation for non-target effects. In this study the alignment of *A. mellifera* and *A. tumida Laccase 2* and *V-ATPase subunit A* mRNAs indicated at most conserved regions of 15 bp and sequence identities were 74 % and 68 %, respectively. Honey bees are known to be highly susceptible to RNAi (Aronstein et al., 2011; Amdam et al., 2003). We hypothesised that the absence of identical regions of more than 20 nucleotides in *A. mellifera* and *A. tumida Laccase 2* and *V-ATPase subunit A* sequences would ensure that RNAi effects would be specific to *A. tumida*. In agreement we demonstrated that injections of 50 ng of dsRNAs into honey bees had no effect on survival and did not induce suppression of either of the target genes.

From the data presented it is clear that *A. tumida* has a robust and systemic RNAi response to injected, but not ingested dsRNAs, targeting the genes *Laccase 2* and *V-ATPase subunit A.* An absence of effects on survival and gene expression in honey bees injected with *A. tumida* dsRNAs was consistent with target specificity predicted on the basis of sequence alignments of orthologous genes. Whilst oral delivery of *V-ATPase subunit A* dsRNA resulted in increased *A. tumida* larval mortality and malformed survivors, these results could not be verified by qPCR analysis. Evidence for degradation of ingested dsRNAs by extracellular ribonucleases in the guts of feeding larvae is thought to explain, at least in part, the lack of consistency in feeding experiments. The development and implementation of RNAi based pesticides holds great potential for new target specific and environmentally benign applications. However, to translate this approach into a viable control strategy for target specific control of *A. tumida* in apiculture a further research to develop a suitable method to induce an oral RNAi response is required.

# CHAPTER 4 | DEVELOPMENT OF NOVEL FUSION PROTEINS AS A CONTROL METHOD FOR *AETHINA TUMIDA*

## 4.1 Introduction

The funnel web spider toxin Hv1a, which targets insect calcium channels, has been shown to be highly potent towards many different insect pests after injection, including Coleoptera, making it an ideal candidate for development as a novel bioinsecticide (Fletcher et al., 1997; Tedford et al., 2004; Bloomquist, 2003; Mukherjee et al., 2006). However, the lack of oral activity of Hv1a clearly limits its potential for commercial application. Linking Hv1a to the carrier protein GNA has shown that following ingestion GNA facilitates transport of the Hv1a peptide across the gut epithelium into the circulatory whereby the toxin can reach the site of action in the CNS when fed to lepidopteran *M. brassicae* larvae (Fitches et al., 2012). Additionally, the authors provided evidence for GNA binding to the nerve cord of *M. brassicae* following injection and feeding of fluorescently labelled fusion protein, suggesting GNA can act as an anchor binding to the nerve cord, increasing the local concentration of the Hv1a peptide dramatically enhancing the activity of the peptide (Fitches et al., 2012). Hv1a contains three conserved disulphide bonds that form an inhibitory cystine knot that is vital for toxin activity. As such *P. pastoris,* capable of correctly forming disulphide cross links was used to generate four fusion proteins, whereby the Hv1a or modified Hv1a (K>Q modification to remove potential KEX2 cleavage site) (discussed in Chapter 2 section 2.4.1 iii) coding sequence was linked to the N- or C- terminus of GNA.

In this chapter the biological activity of GNA/Hv1a, Hv1a/GNA fusion proteins, and K>Q variants towards *A. tumida* is demonstrated via injection and oral delivery bioassays. Injection bioassay results indicated that all four fusion proteins were similarly toxic towards *A. tumida* wandering larvae and that the insecticidal activity of Hv1a was enhanced when linked to the carrier protein GNA. Oral delivery of fusion proteins to 5 day old *A. tumida* led to a dose dependant decline in survival, however the $LC_{50}$ for GNA/Hv1a was approx. 2 times lower than the other fusion protein variants. *In vitro* stability assays were carried out to confirm that the enhanced toxicity of GNA/Hv1a towards *A. tumida* larvae was a consequence of prolonged stability in the presence and the gut extract of feeding larvae. Consequently GNA/Hv1a was selected for applied bioassays whereby bee brood treated with the fusion protein, was shown to result in significant levels of mortality. Finally, the toxicity of fusion protein

variants towards honey bee workers and larvae was assessed by injection and feeding assays where no detrimental effects on survival were recorded. To our knowledge this is the first report of a fusion protein based biopesticide showing potential use as a novel control method for *A*. *tumida*.

## 4.2 Constructs of recombinant GNA and Pro-Hv1a toxin; GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants

A version of the GNA and Hv1a coding sequence in which codons were optimised for expression in *P. pastoris* was synthesised commercially. The mature toxin Hv1a and/or mature GNA carrier protein sequences were subsequently ligated into pGAPZαB vector in frame with the α-factor secretory signal. Hv1a or Hv1a (K>Q) toxin was linked to the N- or C-terminal of mature GNA nucleotide sequence. The inclusion of the 34$^{th}$ amino acid sequence K>Q modification, was altered by point mutation from a lysine to glutamine residual to remove a KEX2 cleavage site (Pyati et al., 2014). The expression constructs for production of recombinant GNA and Pro-Hv1a toxin and their fusion proteins were introduced in Chapter 2 sections 2.4.1 (i), (ii) and (iii). The diagrammatic representation of recombinant GNA and Pro-Hv1a toxin; GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants, predicted DNA and deduced amino acid sequences are shown in Figures 4.1, 4.2, 4.3 & 4.4.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
+1FR:  ·M··R··F··P··S··I··F··T··A··V··L··F··A··A··S··S··A··L··A··A··P··V··N··T··T·
DNA:   ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT
+1FR:  ·T··E··D··E··T··A··Q··I··P··A··E··A··V··I··G··Y··S··D··L··E··G··D··F··D··V·
DNA:   GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT
+1FR:  ·A··V··L··P··F··S··N··S··T··N··N··G··L··L··F··I··N··T··T··I··A··S··I··A··A·
DNA:   AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCAGAAGATACTAGAGCTGATCTTCAAGGT
+1FR:  ·K··E··E··G··V··S··L··E··K··R··E··A··E··A··A··A··E··D··T··R··A··D··L··Q··G·
DNA:   GGTGAAGCTGCTGAAAAGGTTTTTAGAAGATCTCCAACTTGTATTCCATCTGGTCAACCATGTCCATACAACGAA
+1FR:  ·G··E··A··A··E··K··V··F··R··R··S··P··T··C··I··P··S··G··Q··P··C··P··Y··N··E·
DNA:   AACTGTTGTTCTCAATCTTGTACTTTTAAGGAAAACGAAAACGGTAATACTGTTCAAAGATGTGATGCGGCCGCC
+1FR:  ·N··C··C··S··Q··S··C··T··F··K··E··N··E··N··G··N··T··V··Q··R··C··D··A··A··A·
DNA:   GTCGACCATCATCATCATCATTGA
+1FR:  ·V··D··H··H··H··H··H··*·
```

**(B)**

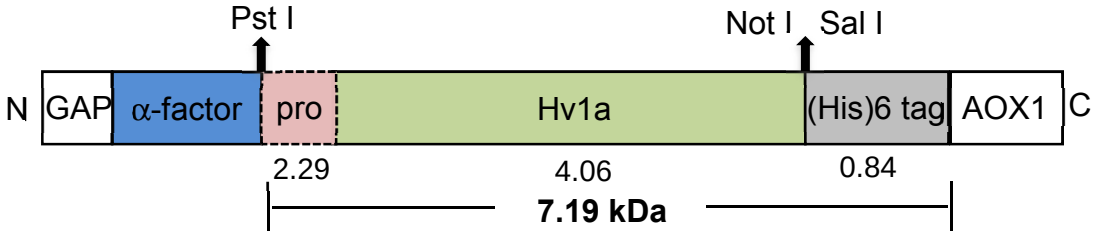



**Figure 4.1.** Expression construct in pGAPZαB encoding for pro-Hv1a. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. The pro-region of Hv1a toxin is indicated in pink. The Hv1a toxin and C-terminal histidine tag and stop codon are depicted in green and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
+1FR:  ·M··R··F··P··S··I··F··T··A··V··L··F··A··A··S··S··A··L··A··A··P··V··N··T··T·
DNA:   ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT
+1FR:  ·T··E··D··E··T··A··Q··I··P··A··E··A··V··I··G··Y··S··D··L··E··G··D··F··D··V·
DNA:   GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT
+1FR:  ·A··V··L··P··F··S··N··S··T··N··N··G··L··L··F··I··N··T··T··I··A··S··I··A··A·
DNA:   AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCAGCGGCCGCCGACAATATTTTGTACTCC
+1FR:  ·K··E··E··G··V··S··L··E··K··R··E··A··E··A··A··A··A··A··A··D··N··I··L··Y··S·
DNA:   GGTGAGACTCTCTCTACAGGGGAATTTCTCAACTACGGAAGTTTCGTTTTTATCATGCAAGAGGACTGCAATCTG
+1FR:  ·G··E··T··L··S··T··G··E··F··L··N··Y··G··S··F··V··F··I··M··Q··E··D··C··N··L·
DNA:   GTCTTGTACGACGTGGACAAGCCAATCTGGGCAACAAACACAGGTGGTCTCTCCCGTAGCTGCTTCCTCAGCATG
+1FR:  ·V··L··Y··D··V··D··K··P··I··W··A··T··N··T··G··G··L··S··R··S··C··F··L··S··M·
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**(B)**

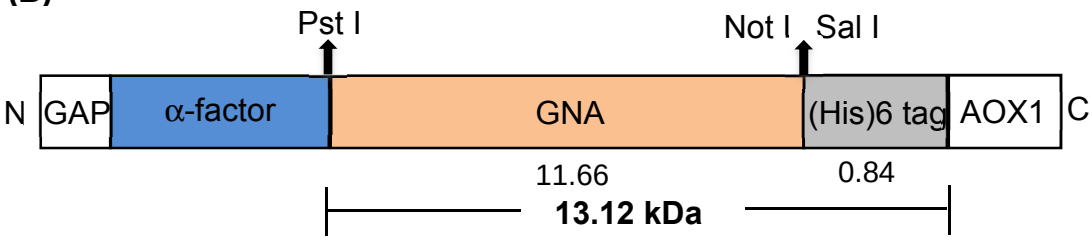


**Figure 4.2.** Expression construct in pGAPZαB encoding for GNA. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. The GNA and C-terminal histidine tag and stop codon are depicted in orange and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
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+1FR:  ·L··S··M··Q··T··D··G··N··L··V··V··Y··N··P··S··N··K··P··I··W··A··S··N··T··G·
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**(B)**

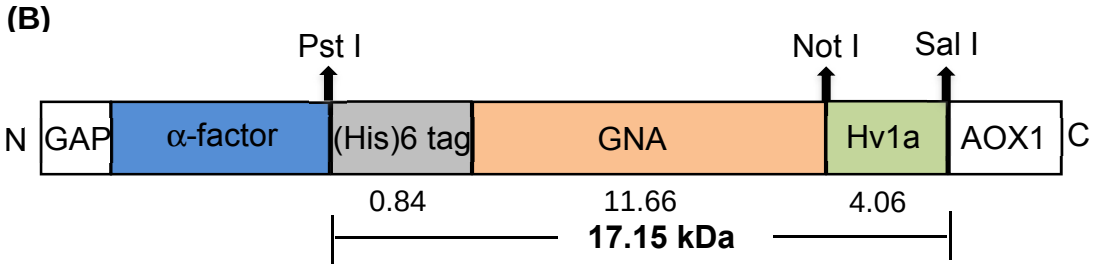


**Figure 4.3.** GNA/Hv1a or GNA/Hv1a (K>Q) expression construct in pGAPZαB. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. GNA, Hv1a and N-terminal histidine tag and stop codon are depicted in orange, green and grey, respectively. The amino acid underlined and in red in the mature toxin sequence indicates the KEX2 cleavage site, the amino acid in red is altered to a glutamine (Q) in GNA/Hv1a(K>Q). The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
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DNA:   GGTGAAGCTGCTGAAAAGGTTTTTAGAAGATCTCCAACTTGTATTCCATCTGGTCAACCATGTCCATACAACGAA
+1FR:  ·G··E··A··A··E··K··V··F··R··R··S··P··T··C··I··P··S··G··Q··P··C··P··Y··N··E·
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+1FR:  ·N··C··C··S··Q··S··C··T··F··K··E··N··E··N··G··N··T··V··K··R··C··D··A··A··A·
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+1FR:  ·D··N··I··L··Y··S··G··E··T··L··S··T··G··E··F··L··N··Y··G··S··F··V··F··I··M·
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**(B)**

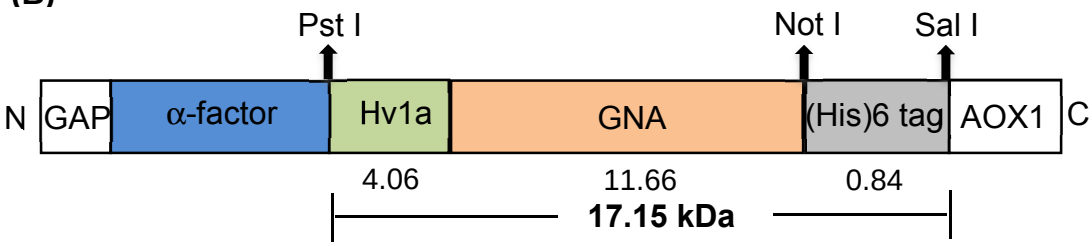


**Figure 4.4.** Hv1a/GNA and Hv1a(K>Q) /GNA expression construct in pGAPZαB. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. Hv1a, GNA, and C-terminal histidine tag and stop codon are depicted in green, orange and grey, respectively. The amino acid underlined and in red in the mature toxin sequence indicates the KEX2 cleavage site, the amino acid in red is altered to a glutamine (Q) in GNA/Hv1a(K>Q). The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

## 4.3 Expression and purification of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants

Sequence confirmed plasmid DNA encoding fusion proteins were linearised with *Bln*I and transformed into SMD1168H (protease deficient strain) of *P. pastoris*, using Zeocin anti-biotic for selection. Positive transformants were inoculated in 10 ml YPG cultures with Zeocin and grown for 48 hr at 30°C. Culture supernatants (20 µl) were analysed for expression of fusion proteins by Western blotting, using anti-GNA antibodies.

For each construct the best expressing clone was selected for large-scale protein production. Fermentation was carried out in a 5 L bioreactor under controlled environmental conditions (Chapter 2, section 2.4.5). The pGAPZ alpha factor secretory signal simplifies the purification process as it directs the secretion of expressed proteins out of the yeast cells and into the growth media, so that proteins can be purified directly from the fermented culture supernatant. Supernatant was obtained via centrifugation, prior to purification the resultant supernatant was passed through a series of filters and subsequently purified by nickel affinity chromatography (Chapter 2, section 2.4.6). Eluted peaks containing target proteins were diluted 50:50 with deionised water and subsequently de-salted by dialysis and freeze-dried. Purified recombinant pro-Hv1a were separated using Tris-Tricine gel (15 % acrylamide) and analysed by western blotting using anti-His antibodies (Figure 4.5 A & B). In the Tris-Tricine gel depicted in Figure 4.5 A, recombinant pro-Hv1a gave a major protein band at approx. 14.4 kDa, which was greater than the predicted mass of 7.19 kDa. As shown in Figure 4.5 B, the 14.4 kDa band was immunoreactive with the anti-His antibodies possible suggesting that recombinant pro-Hv1a was expressed in dimeric form (Yang, 2014). As pro-Hv1a migrates as a smear rather than a tight band, due to the presence of disulfide bridges, quantification was carried out at Durham University using a BCA protein assay with known amounts of BSA standards. The proportion of fusion protein in lyophilised samples was estimated semi-quantitatively by comparison with known amounts of recombinant GNA standards on SDS-PAGE gels (Figure 4.6 A & B). Separation of purified GNA/Hv1a and GNA/Hv1a(K>Q) by SDS-PAGE indicated that both fusion proteins ran as tight double bands at predicted molecular weight of approx. 17 kDa (Figure 4.6 A). The lower protein band is a cleavage product consisting of mostly intact fusion protein, with a small amount of Hv1a cleaved off the C-terminus

of the toxin (Fitches pers com, 2018). In contrast, separation of purified Hv1a/GNA and Hv1a(K>Q)/GNA by SDS-PAGE showed the presence of a protein corresponding to the predicted molecular weight of approx. 17 kDa, with an additional lower protein mass of 14 kDa corresponding to recombinant GNA, indicating a degree of cleavage of the fusion protein. (Figure 4.6 B). Additionally, the presence of contaminating higher Mw proteins were observed when large amounts of Hv1a/GNA and Hv1a(K>Q)/GNA were analysed by SDS-PAGE. Fermentation was carried out at both Durham University and Fera Science Ltd, except for Pro-Hv1a which was produced at Durham University with an expression level of 80 mg/L. The following expression level were based on fermentation carried out at Fera Science Ltd: GNA/Hv1a was approx. 28 mg/L; GNA/Hv1a (K>Q) was approx. 34 mg/L; Hv1a/GNA was approx. 50 mg/L and Hv1a(K>Q)/GNA was approx. 100 mg/L culture supernatant, respectively, estimated by semi-quantitative SDS-PAGE.

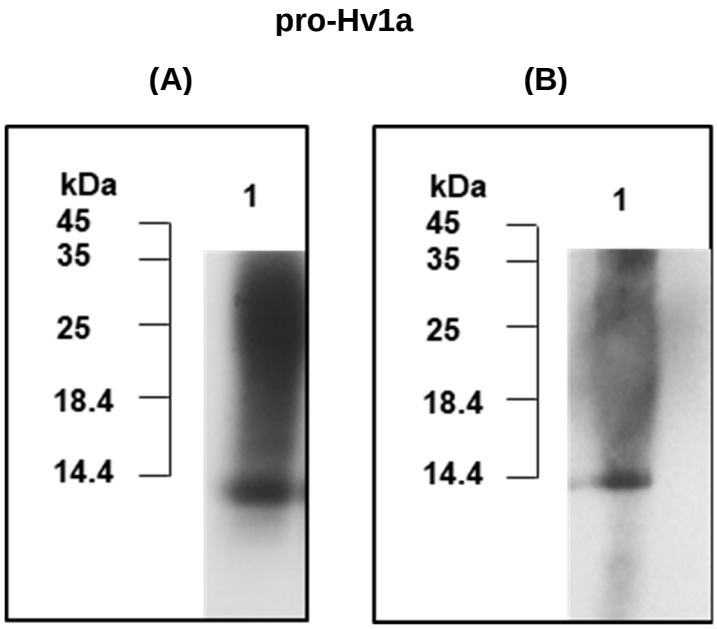


**Figure 4.5.** (A) Separation of pro-Hv1a by Tris-Tricine gel electrophoresis: lanes 1 shows 37 µg of lyophilised pro-Hv1a after purification by nickel affinity chromatography. (B) Western blotting analysis of sample of pro-Hv1a using anti-His antibodies: lane 1 shows 10 µg of lyophilised pro-Hv1a after purification by nickel affinity chromatography.

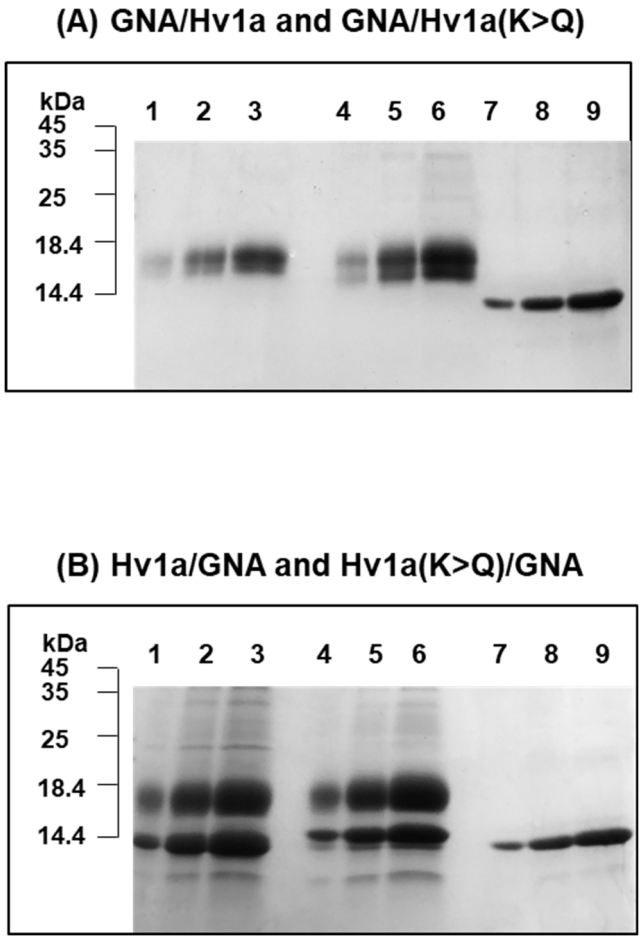


**Figure 4.6.** SDS-PAGE analysis of purified fusion protein: (A) GNA/Hv1a and GNA/Hv1a(K>Q): lanes 1 to 3 and 4 to 6 show 5, 10 & 25 µg purified GNA/Hv1a and GNA/Hv1a(K>Q), respectively and (B) Hv1a/GNA and Hv1aGNA(K>Q): lanes 1 to 3 and 4 to 6 show 5, 10 & 25 µg purified Hv1a/GNA and Hv1a(K>Q)/GNA, respectively. Lanes 7 to 9 show respectively 0.5, 2 & 5 µg GNA standard.

## 4.4 Toxicity GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants against *Aethina tumida* wandering larvae after injection into the haemolymph

Wandering *A. tumida* larvae (n=10 per treatment; average weight 17.46 mg) were injected with doses ranging from 0.25 -2 µg of recombinant pro-Hv1a, 7.5 -17.5 µg of recombinant GNA, 0.3 -2 µg GNA/Hvla, Hv1a/GNA or K>Q variants, with 20 mM sodium phosphate (pH 7.4) (SP) buffer solution serving as a negative control. Survival was monitored daily for 7 days. Figure 4.7 shows the injections results of pro-Hv1a, GNA, GNA/Hv1a, Hv1aGNA or K>Q variants and $LD_{50}$s for each treatment are depicted in Table 4.1.

A decline in survival was observed 6 days after injection of 1 and 2 µg of Hv1a, with 70 % mortality being recorded on day 7 ($P<0.01$; Mantel-Cox log-rank test; $LD_{50}$ 0.77 µg/µl). By contrast, a more rapid response was observed in larvae injected with comparable doses of GNA/Hv1a, GNA/Hv1a(K>Q), Hv1a/GNA or Hv1a (K>Q)/GNA with significant decreases in survival observed 2 to 3 days post injection, with 100 % mortality recorded ($P<0.0001$; Mantel-Cox log-rank test). A further reduction in doses of GNA/Hv1a and GNA/Hv1a(K>Q) to 0.5-0.3 µg resulted in a respective 70-100 %, 10-0 % and 0 % mortality being recorded. By comparison the injection of Hv1a/GNA and Hv1a(K>Q)/GNA resulted in higher levels of larval mortality with comparable doses achieving 80-100 %, 70 % and 60-70 % mortality, respectively. The $LD_{50}$'s for GNA/Hv1a and GNA/Hv1a(K>Q) were a similar 0.44 and 0.47 µg/µl, whilst Hv1a/GNA and Hv1a(K>Q)/GNA $LD_{50}$'s were slightly lower, at a respective 0.33 and 0.25 µg/µl. The results of the injection bioassay showed that the toxicity of Hv1a was greatly enhanced when linked to the carrier protein GNA. Injection of 1 and 2 µg of the fusion proteins (equivalent to 0.26 and 0.52 µg/µl of Hv1a) resulted in 100 % mortality after 2 to 3 days, whereas mortality in the corresponding Hv1a treatment, which was approx. 4 times the dose of the toxin compared to fusion proteins, was delayed with 70 % mortality being recorded after 7 days. Subsequent injection of GNA at 17.5 µg resulted in a significant reduction in survival, with 100 % mortality being recorded after 3 days ($P<0.0001$; Mantel-Cox log-rank test). A reduction in dose to 15, 12.5 and 10 µg lead to a dose dependant decrease in survival with 50 %, 40 % and 10 % mortality being recorded, respectively ($LD_{50}$ 13.76 µg/µl). These results indicate that GNA was toxic to *A. tumida* larvae but doses required to induce significant mortality were some 20 times greater than fusion protein treatments.

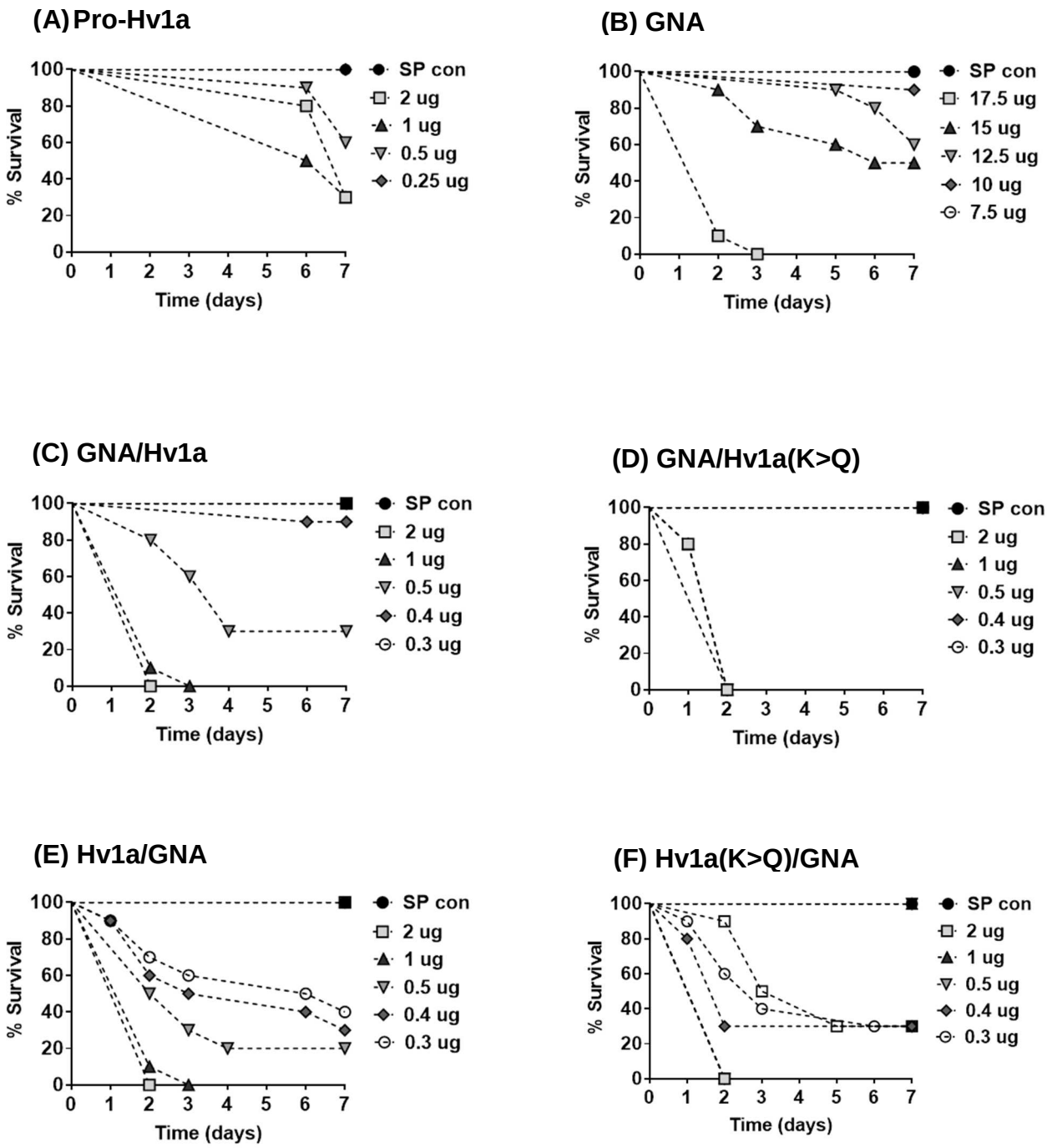


**Figure 4.7.** Survival of *Aethina tumida* wandering larvae injected with different doses of: (A) pro-Hv1a; (B) GNA; (C) GNA/Hv1a; (D) GNA/Hv1a(K>Q); (E) Hv1a/GNA and (F) Hv1a(K>Q)/GNA. Proteins in all cases were re-suspended in sodium phosphate (SP) buffer. Injection volume was 1 µl. SP served as negative control (SP Con). Mortality was monitored daily for 7 days. N=10 per treatment.

**Table 4.1.** *Aethina tumida* wandering larvae injection $LD_{50}$s (day 7 post injection), for recombinant pro-Hv1a, GNA, GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants.

| Treatment | $LD_{50}$ (µg/ul) | $LD_{50}$ (µg/g insect) |
|---|---|---|
| **pro-Hv1a** | 0.77 | 43.53 |
| **GNA** | 13.76 | 788.09 |
| **GNA/Hv1a** | 0.44 | 25.20 |
| **GNA/Hv1a(K>Q)** | 0.47 | 26.92 |
| **Hv1a/GNA** | 0.33 | 18.90 |
| **Hv1a(K>Q)/GNA** | 0.25 | 14.32 |

### 4.5 Stability of Hv1a/GNA in components of the artificial diet

Hv1a/GNA was selected, prior to conducting feeding bioassays, to assess stability in *A. tumida* larval artificial diet. The diet consists of 50 % (v/w) aqueous honey solution (1 ml) and 2 g of crushed bee pollen. Hv1aGNA was added at a final concentration of 5000 ppm to aqueous honey solution, bee pollen or both components of the artificial diet, and samples were taken immediately or after 3 days incubated at 20°C. Pollen and artificial diet samples were re-suspended in 1 x PBS to achieve a final fusion protein concentration of 100 ng/µl thereafter the samples were centrifuged at 13 000 rpm for 5 min and the supernatants analysed by western blotting, using anti-GNA antibodies. As shown in Figure 4.8, no evidence of cleavage or degradation was observed for Hv1a/GNA incubated in the separate components of the diet or in the combined pollen and honey artificial diet after 3 days.

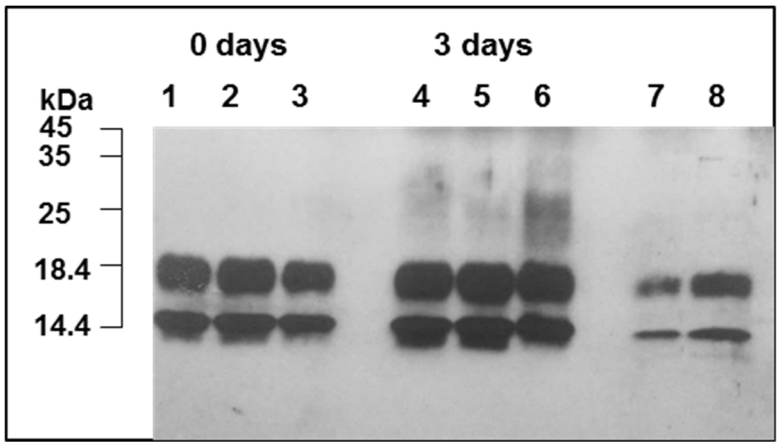


**Figure 4.8.** Western analysis (anti-GNA antibodies) of Hv1a/GNA stability in *Aethina tumida* artificial diet (and separate components thereof) at time 0 and after 3 days incubated at 20°C. Lane 1 & 4 shows honey and pollen; lane 2 & 5 pollen alone and lane 3 & 6 honey alone. 100 ng was loaded for all samples. Standards are 50 and 100 ng of Hv1a/GNA. Mw standards (kDa) based on Ponceau S staining are indicated.

## 4.6 Detection of ingested recombinant GNA in feeding *Aethina tumida* larvae after delivery

To investigate if recombinant GNA was resistant to proteolytic degradation within the gut of *A. tumida* larvae and subsequently also able to cross the gut epithelium, haemolymph and gut samples were extracted from insects fed on artificial diet containing recombinant GNA. Larvae (n=10 per treatment) were allowed to feed *ad libitum* on artificial diet containing GNA at 5000 ppm for 24 hr. All samples were analysed via western blotting, using anti-GNA antibodies.

Analysis of diet containing recombinant GNA showed the presence of an immunoreactive protein of similar mass to standard GNA (Figure 4.9). Homogenised gut samples showed the presence of recombinant GNA after 24 hr, with no corresponding immunoreactive band being detected in the control samples (Figure 4.9). Antibody cross-reactivity with a high Mw gut protein of approx. 35 kDa was observed in all samples, however these were distinguishable from recombinant GNA. Transport of recombinant GNA into the haemolymph was evident from the presence of a prominent immunoreactive band corresponding to the size of recombinant GNA,

which was absent in the negative control haemolymph. For both haemolymph and gut samples excess of 100 ng of recombinant GNA was detected.

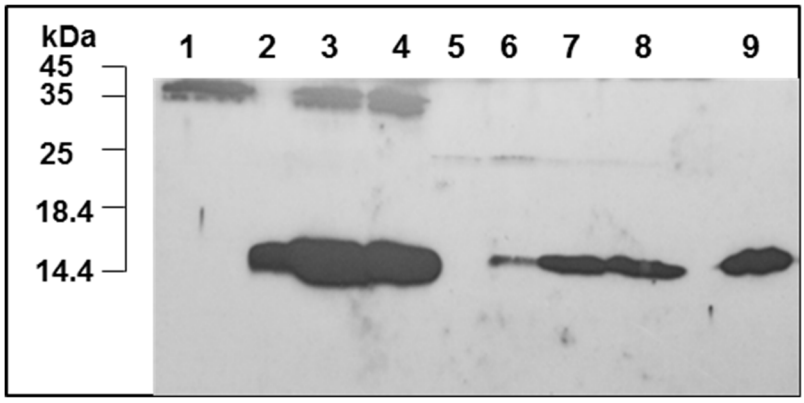


**Figure 4.9.** Western analysis (anti-GNA antibodies) of 7 day old *Aethina tumida* larvae haemolymph and guts after feeding on artificial diet containing 5000 ppm recombinant GNA: lanes 1 & 2 show haemolymph (10 µl) of control and GNA fed larvae respectively; lane 3 & 4 are replicate gut samples of GNA fed larvae (n=5), approx. 40 µg total protein loaded; lane 6 to 8 represents a loading of 10, 25 and 50 ng of artificial diet containing GNA and lane 9 represents 100 ng recombinant GNA standard. Mw standards (kDa) based on Ponceau S staining are indicated.

## 4.7 Oral delivery of recombinant pro-Hv1a and GNA; GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to *Aethina tumida* larvae

### 4.7.1 Oral delivery of recombinant GNA and GNA/Hv1a incorporated into artificial diet

Seven day old larvae (n=20 per treatment) were placed onto 900 mg of artificial diet containing recombinant GNA and GNA/Hv1a at a concentration of 5000 ppm. Negative control larvae were fed on artificial diet containing SP buffer. Larvae were allowed to feed for 7 days, with fresh diet being provided after 3 days, and survival was monitored daily. As shown in Figure 4.10, SP and GNA mortality was 5 % after 7 days. By comparison, 40 % mortality was recorded 3 days after feeding on GNA/Hv1a, and further increased to 100 % after 6 days; survival was significantly different to the control SP buffer and GNA treatments ($P<0.0001$; Mantel-Cox log-rank test). Although this assay was repeated at 3000 and 2000 ppm GNA/Hv1a, no mortality was recorded over a feeding period of 7 days.

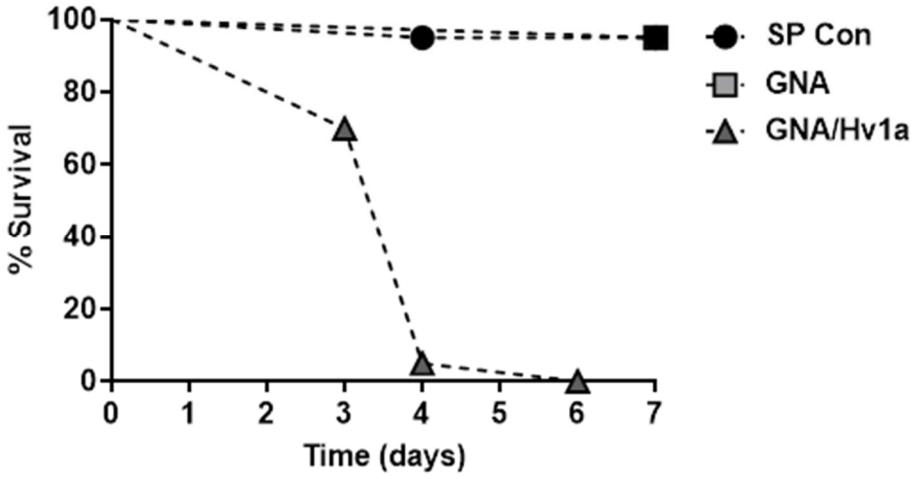


**Figure 4.10.** Survival of 7 day old *Aethina tumida* larvae fed on artificial diet containing 5000 ppm of recombinant GNA and GNA/Hv1a. Sodium phosphate buffer served as negative control (SP Con). Larvae were provided with fresh diet after 3 days and mortality was monitored daily for 7 days. with N=20 per treatment**.**

### 4.7.2 Development of a drinking bioassay to orally deliver recombinant pro-Hv1a and GNA; GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to 5 day old *Aethina tumida* larvae

Failure to achieve consistent mortality results in artificial diet studies led to the development of an alternative orally delivery method. Preliminary tests were conducted to assess the viability of delivering fusion proteins in a SP/sucrose solution (13 % v/v). To this end 5 day old larvae (average weight 11.56 mg) were supplied with SP/sucrose solution containing 2 µl of red food dye whilst a control treatment was maintained on artificial diet (Figure 4.11 A & B). No SP/sucrose solution remained in the microcentrifuge lids after a feeding period of 24 h and it was clear from the presence of dye in the fore gut of A. *tumida* larvae that consumption had occurred. The SP solution with dye was replenished twice more (every 24 hr) with the presence of dye in the fore gut becoming more apparent (Figure 4.11 C & D). Although it was evident that the larvae had consumed the SP/sucrose, it was impossible to determine the amount consumed compared to the amount being displaced by the larvae entering the solution. Furthermore, there was a marked difference in development of larvae reared on the sucrose solution and artificial diet (Figure 4.11 E & F). As such SP/sucrose solution fed larvae were then placed onto artificial diet for a further 4 days. Larvae fed on SP/sucrose solution on average weighed 17.42 mg (n=10), whereas larvae feed on artificial diet alone achieved an average weight of 19.1 mg (n=10). The difference in mean weight between the two diets was not significant (non-paired t-test $P>0.05$) and no mortality was recorded during the 7 day bioassay period.

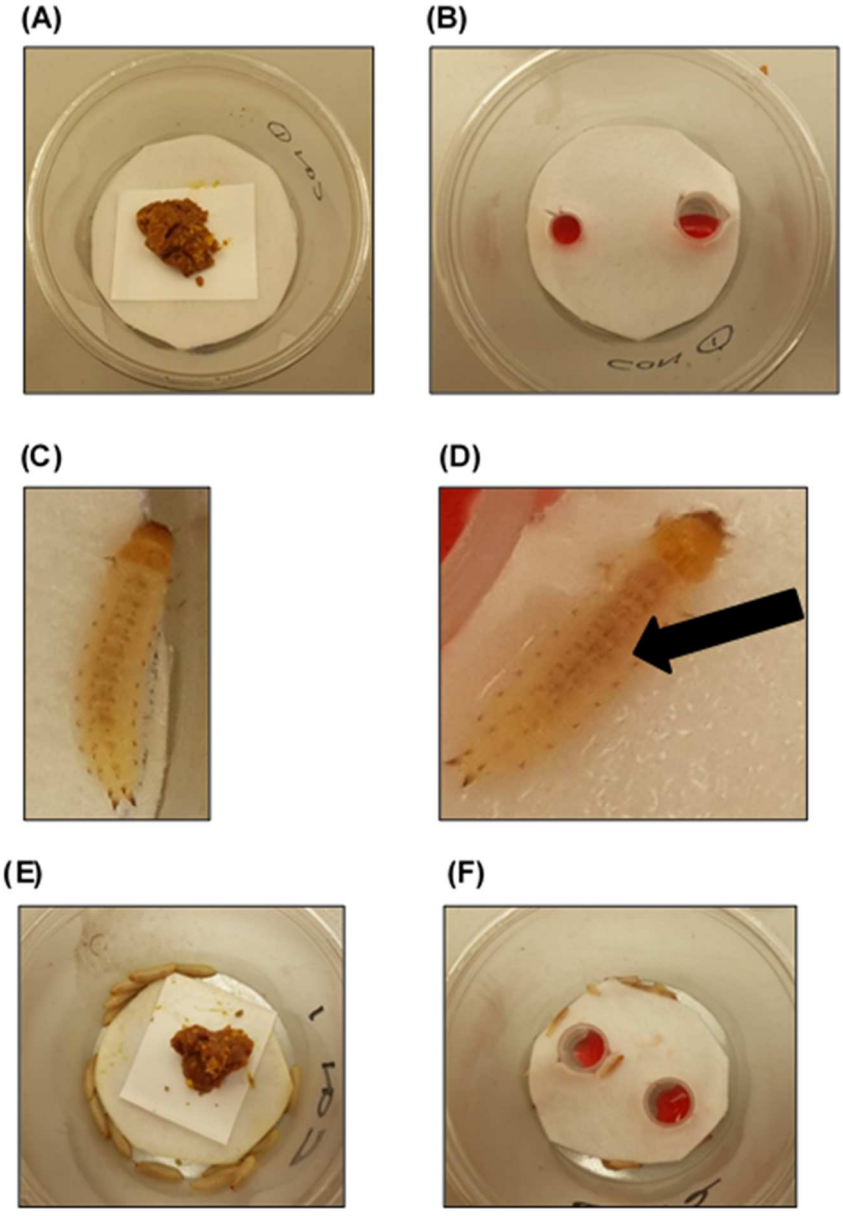


**Figure 4.11.** Diets used in the development feeding bioassay: (A) artificial diet comprising of 50 % (v/w) aqueous honey solution (1 ml) and 25 g of crushed bee pollen and (B) SP/sucrose solution (13 % v/w) with 2 µl of red food dye. (C) Larvae fed on SP and sucrose solution. (D) Larvae fed on SP and sucrose solution containing red dye, arrow indicates the presence of dye in the fore gut. (E) and (F) show larvae 72 hr post feeding indicating the size difference between larvae fed on (C) artificial diet and (D) SP/sucrose solution.

### 4.7.3 Oral delivery GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to *Aethina tumida* feeding larvae via a drinking bioassay

Five day old larvae (n= 20 per treatment) were transferred into plastic pots and supplied with SP/sucrose solution (13 % v/w) containing recombinant GNA (5 mg/ml), Pro-Hv1a (1.25 mg/ml), GNA/Hv1a, Hv1a/GNA or K>Q variants at final concentrations of 0.312 - 5 mg/ml. Negative control larvae were fed on SP/sucrose solution without recombinant proteins. The diet was changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial diet until they entered the wandering stage (i.e. approx. 4 days post liquid feed).

Figure 4.12 shows the feeding bioassay results of pro-Hv1a, GNA, GNA/Hv1a, Hv1aGNA or K>Q variants and $LD_{50}$s for each treatment are depicted in Table 4.2. As shown in Figure 4.12 A-D no mortality was recorded in pro-Hv1a or SP control group whereas 25 % mortality was recorded for larvae fed on GNA at 5 mg/ml ($P<0.05$; Mantel-Cox log-rank test). Two days after feeding on GNA/Hv1a(K>Q) or GNA/Hv1a at 5 and 2.5 mg/ml larvae exhibited impaired mobility and a “writhing” phenotype, this coincided with 30 % and 50 % mortality being recorded in the 5 mg/ml GNA/Hv1a(K>Q) and GNA/Hv1a treatment, respectively. Thereafter 100 % mortality was recorded 4 to 7 days post feeding on GNA/Hv1a at concentration of 5, 2.5 or 1.25 mg/ml ($P<0.0001$ Mantel-Cox log-rank test). A further reduction in dose to 0.625 mg/ml resulted in 75 % mortality being recorded after 7 days, which was significantly different from SP and GNA controls ($P<0.0001$; $P<0.01$; Mantel-Cox log-rank test). The lowest dose of 0.312 mg/ml GNA/Hv1a caused a non-significant 15 % mortality ($LC_{50}$ 0.52 mg/ml). GNA/Hv1a(K>Q) at a dose of 5 mg/ml induced a comparable level of mortality to GNA/Hv1a, with 100 % mortality observed 5 days post feeding ($P<0.0001$; Mantel-Cox log-rank test). A reduction in dose to 2.5 and 1.25 mg/ml resulted in a respective 60 and 50 % mortality at day 7, which was significantly different from SP and GNA control ($P<0.01$; $P<0.05$; Mantel-Cox log-rank test). The lowest dose of 0.312 mg/ml GNA/Hv1a(K>Q) caused a non-significant 10 % mortality. The $LC_{50}$ for GNA/Hv1a (K>Q) was 1.18 mg/ml, which was approx. 2 times higher than the $LC_{50}$ for GNA/Hv1a.

Oral delivery of Hv1a/GNA and Hv1a(K>Q)/GNA at 5 or 25 mg/ml resulted in a significant 90-95 % and 75-85 % mortality being recorded after 7 days ($P<0.0001$; Mantel-Cox log-rank test). A reduction in dose of Hv1a/GNA or Hv1a(K>Q)/GNA to concentrations of 1.25, 0.625 and 0.312 mg/ml resulted in 65-70 %, 15-30 % and 0-

20 % mortality, which was only significant for the 1.25 mg/ml dose in comparison to the SP and GNA control treatments ($P<0.001$; $p<0.01$; Mantel-Cox log-rank test). The day 7 $LC_{50}$s for Hv1a/GNA or Hv1a(K>Q)/GNA were 1.14 or 0.89 mg/ml, respectively which was approx. 2 and 1.7 times greater than GNA/Hv1a.

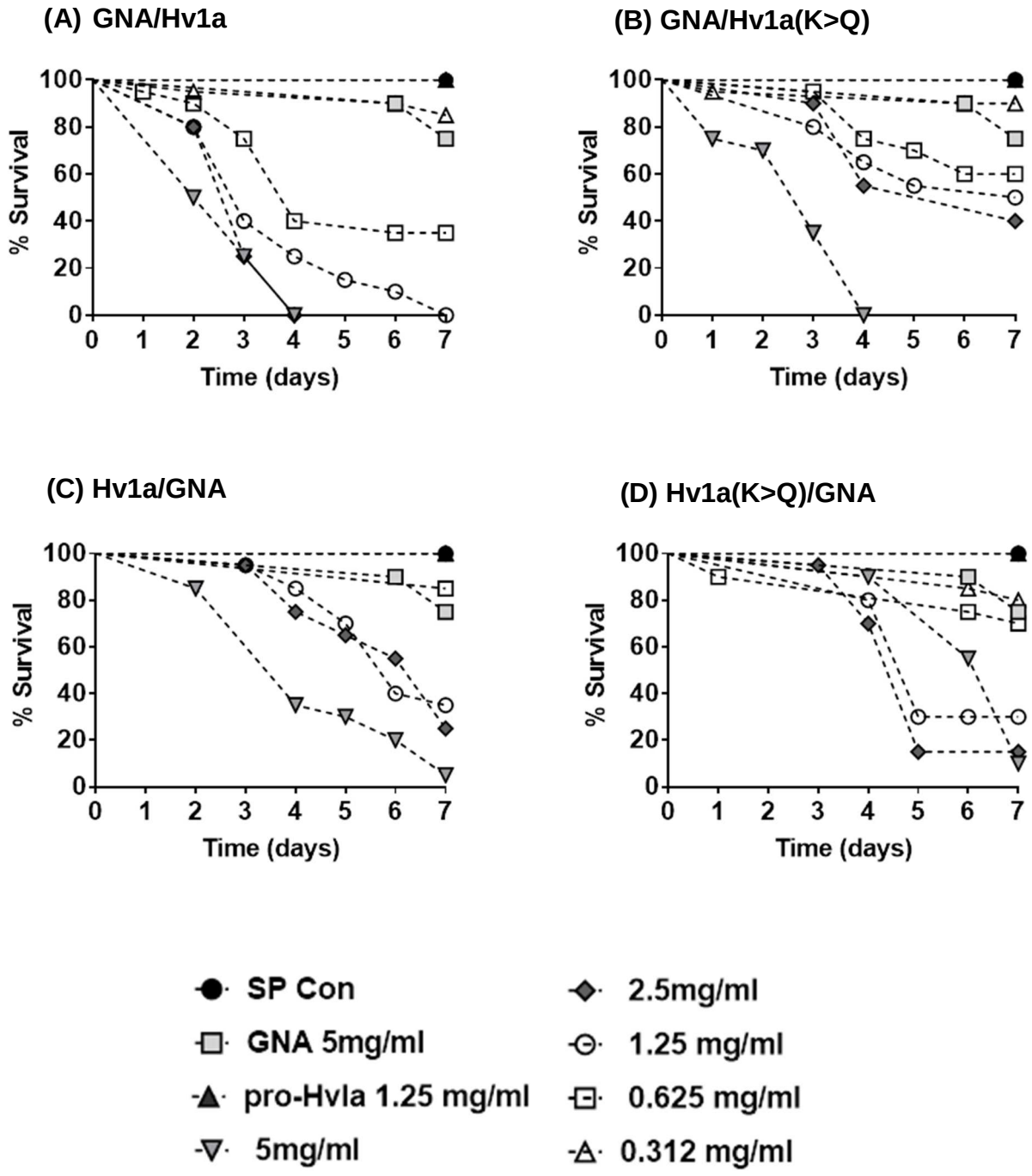


**Figure 4.12.** Survival of 5 day old *Aethina tumida* larvae fed on 5 mg/ml of GNA, 1.25 mg/ml pro-Hv1a and 0.312 to 5 mg/ml of (A) GNA/Hv1a, (B) GNA/Hv1a(K>Q), (C) Hv1a/GNA and (D) Hv1a(K>Q)/GNA. Sodium phosphate buffer served as negative control (SP Con). Solutions were changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial diet until they entered the wandering stage. N= 20 per treatment.

**Table 4.2.** $LC_{50}$ at day 7 following oral delivery, via a drinking assay, of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants against feeding *Aethina tumida* larvae.

| Treatment | $LC_{50}$ (mg/ml) |
|---|---|
| **GNA/Hv1a** | 0.52 |
| **GNA/Hv1a(K>Q)** | 1.18 |
| **Hv1a/GNA** | 1.14 |
| **Hv1a(K>Q)/GNA** | 0.89 |

### 4.7.4 Stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants within the gut of feeding *Aethina tumida* larvae

The presence of GNA/Hv1a, Hv1a/GNA and K>Q variants in haemolymph (n=20 per treatment) and gut samples extracted from larvae (n=5 per treatment and replicate) were analysed by western blotting using anti-GNA antibodies. Larvae were fed for 24 hr on SP/sucrose solutions containing fusion proteins at a concentration of 2.5 mg/ml. Analysis of all gut samples confirmed that the fusion proteins were cleaved after 24 hr, which was evident by the presence of a single immunoreactive band corresponding to the size of recombinant GNA, and absent in the control samples (Figure 4.13). Western analysis showed evidence for transport of GNA carrier across the gut epithelium, since GNA was present in all haemolymph samples 24 hr after feeding. The presence of immunoreactive gut proteins of a high Mw of approx. 30 and 25 kDa was observed Figure 4.13 C and D respectively, however these were distinguishable from the intact fusion protein standard.

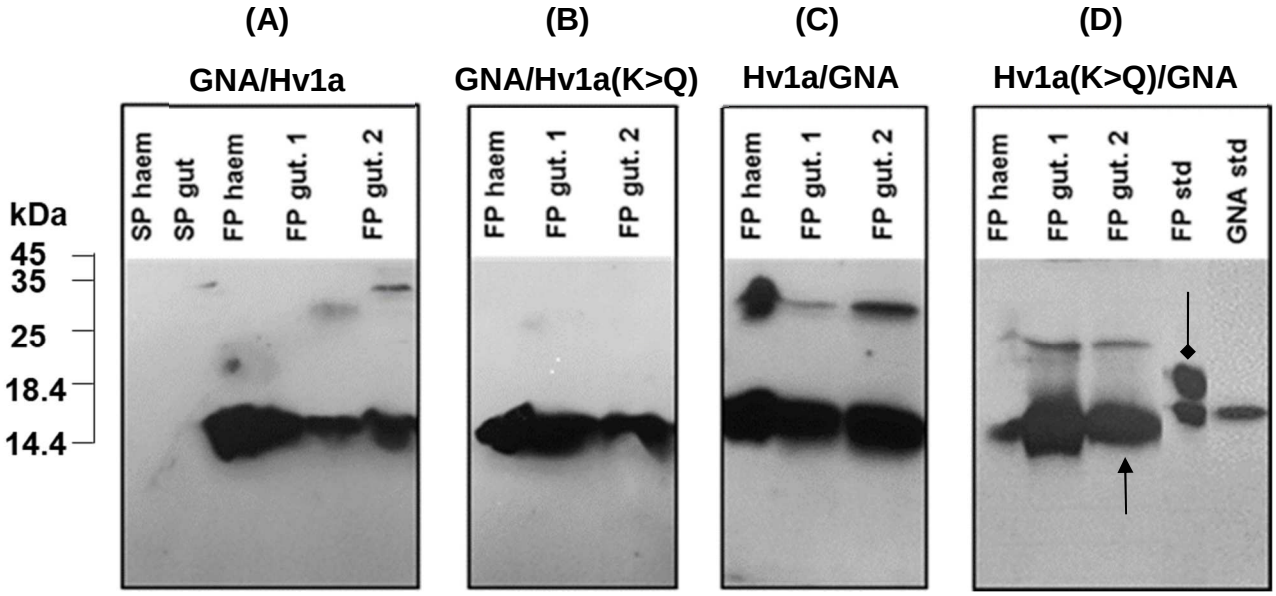


**Figure 4.13.** Western analysis (anti-GNA antibodies) of 5 day old *Aethina tumida* larvae haemolymph and gut samples after feeding on sodium phosphate buffer (SP)/sucrose solution (13 % v/w) containing: (A) GNA/Hv1a; (B) GNA/Hv1a(K>Q); (C) Hv1a/GNA or (D) Hv1a/(K>Q)/GNA at a concentration of 2.5 mg/ml. For SP and fusion protein (FP) haemolymph and gut samples (two replicates depicted as R1 and R2) 10 µl and 10 µg total protein was loaded, respectively. In Figure D FP and GNA standard represent 100 ng of Hv1a/GNA and recombinant GNA. Cleaved GNA and intact fusion protein is indicated by an arrow head and diamond head, respectively.

### 4.7.5 *In vivo* stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants in sucrose solutions containing *Aethina tumida* larvae

The stability of fusion proteins in the presence of secreted larval extracellular digestive proteases and frass was investigated by incubating larvae (n=10 per treatment) in 100 µl of SP/sucrose solution (13 % v/w) containing 2.5 mg/ml of GNA/Hv1a, Hv1a/GNA or K>Q variants, with 5 µl aliquots taken at the following time points: 0,1, 2, 4, 6, 8 and 24 hr. Samples were analysed for the presence of fusion proteins by western blotting, using anti-GNA antibodies.

As shown in Figure 4.14, GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants show varying levels of susceptibility to proteolytic degradation over the 24 hr period. Removal of the Hv1a peptide is indicated by a reduction in the molecular mass of the GNA-immunoreactive protein that corresponds to the mass of GNA alone. Hv1a/GNA was highly susceptible to proteolytic degradation, with very little intact fusion protein being detected after 1 hr, and complete removal of the Hv1a toxin after 2 hr. For Hv1a(K>Q)/GNA, degradation of intact fusion protein commences within 1 hr, when approximately 1/5$^{th}$ of incubated intact fusion protein remains, and after 6 hr complete removal of the Hv1a peptide leaving only cleaved GNA is evident. Analysis of GNA/Hv1a(K>Q) showed less susceptibility to proteolytic degradation after 1 and 2 hr, with approximately half the amount of intact fusion protein being detected. Thereafter GNA/Hv1a(K>Q) was rapidly degraded with very little intact fusion protein being detected after 4 and 6 hr respectively, with complete removal of the Hv1a peptide observed after 8 hr. In contrast GNA/Hv1a showed very little evidence of proteolysis, with the fusion protein remaining intact for 8 hr.

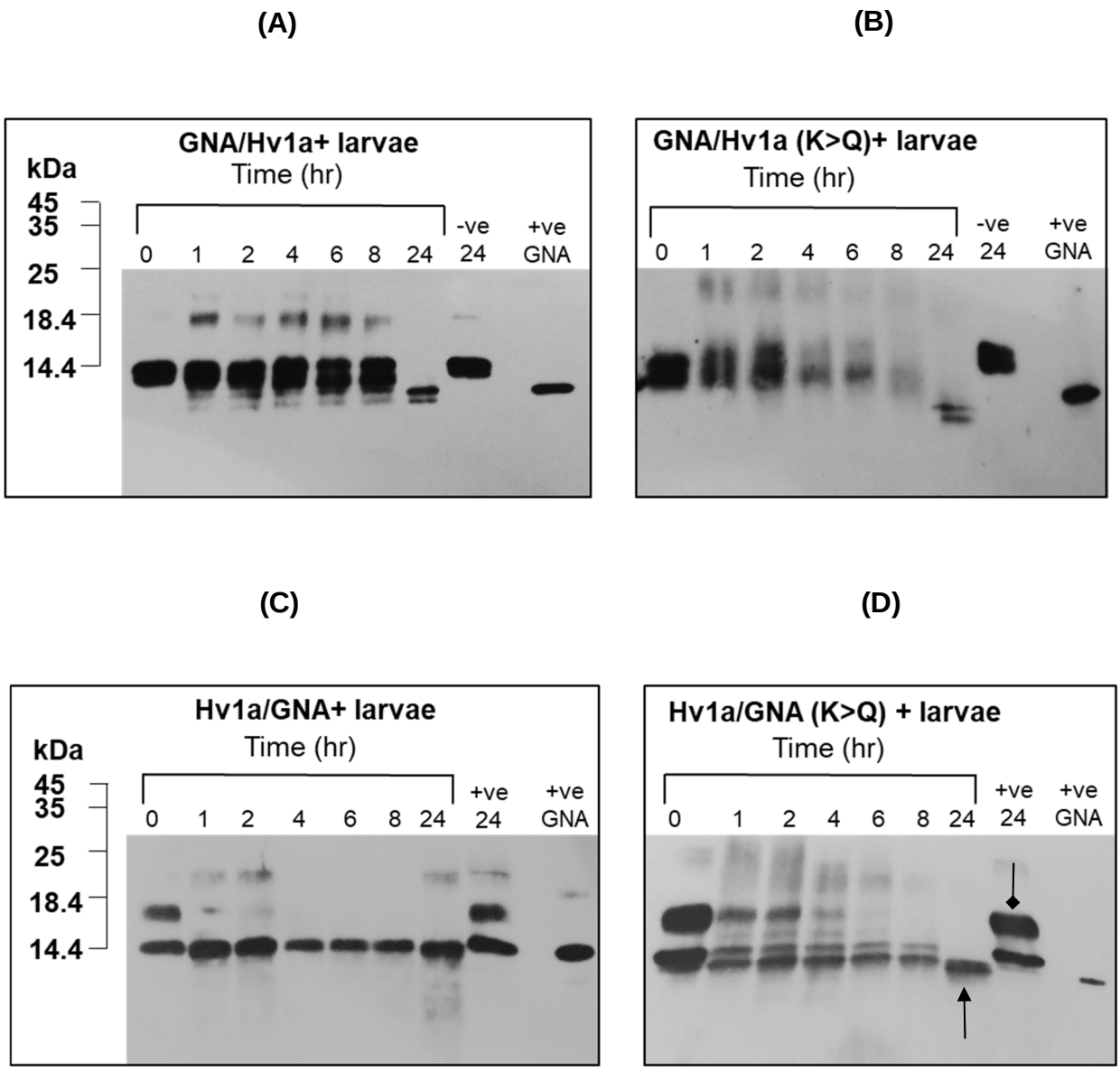


**Figure 4.14.** Western analysis (anti-GNA antibodies) of fusion protein containing solutions in which 5 day old *Aethina tumida* larvae had been immersed for different periods of time: (A) GNA/Hv1a; (B) GNA/Hv1a(K>Q); (C) Hv1a/GNA or (D) Hv1a/(K>Q)/GNA at a concentration of 2.5 mg/ml. For each time point 300 ng of each fusion protein (FP) was loaded. +ve 24 denotes FP incubated for 24 hr with no larvae present and +ve GNA represents 100 ng recombinant GNA standard. Cleaved GNA and intact fusion protein is indicated by an arrow head and diamond head, respectively.

### 4.7.6 *In vitro* stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants in feeding *Aethina tumida* gut extracts

The stability of GNA/Hv1a, Hv1a/GNA or K>Q variants were assessed *in vitro* by incubating the fusion proteins in gut extracts for 0,1, 2, 4, 6, 8 and 24 hr. Samples were analysed for the presence of fusion proteins by western blotting, using anti-GNA antibodies (Figure 4.15).

Hv1a(K>Q)/GNA was highly susceptible to proteolytic degradation when incubated in *A. tumida* gut extracts, with > 2% of intact fusion protein detected after an incubation period of 1 hr, and complete cleavage of the Hv1a peptide evidenced by the presence of a single immunoreactive band corresponding to the Mw of recombinant GNA apparent after 2 hr. For Hv1a/GNA partial degradation was evident after 1 hr as approx. 50 ng of intact fusion protein was detected in comparison to the 0 hr sample. After 2 hr there was a further reduction in amount of intact fusion protein, with approx. half the amount being detected in comparison to the 1 hr sample. No further degradation was detected after 4 and 6 hr. However, after 8 hr the amount of intact Hv1a/GNA was further reduced (to approx. 15 ng) and after 24 hr only cleaved GNA was detected. Analysis of GNA/Hv1a and GNA/Hv1a(K>Q) showed less susceptibility to degradation with some intact fusion protein detected at 24 hr. Partial proteolysis of both GNA/Hv1a and GNA/Hv1a(K>Q) was observed after 1 hr and continued over the 24 hr period with increasing levels of cleaved GNA and decreasing levels of intact fusion protein detected, in comparison to the control samples. GNA/Hv1a(K>Q) was degraded after 6 hr, with approx. 10 ng of intact fusion protein remaining, which further decreased to approx. 5 ng after 8 and 24 hr incubation periods, respectively. GNA/Hv1a was the most stable of all variants tested; after 6 and 8 hr approx. 50 ng of intact fusion protein was detected. After 24 hr approx. 5 times more GNA/Hv1a was detected in comparison to GNA/Hv1a(K>Q).

The results of these *in vitro* assays suggest that GNA/Hv1a was 2 times more toxic to *A. tumida* as compared to the other fusion proteins due to enhanced stability in the presence of extracellular gut proteases enabling a greater amount of the Hv1a toxin to be delivered to the haemolymph where it can access ion channels within the CNS.

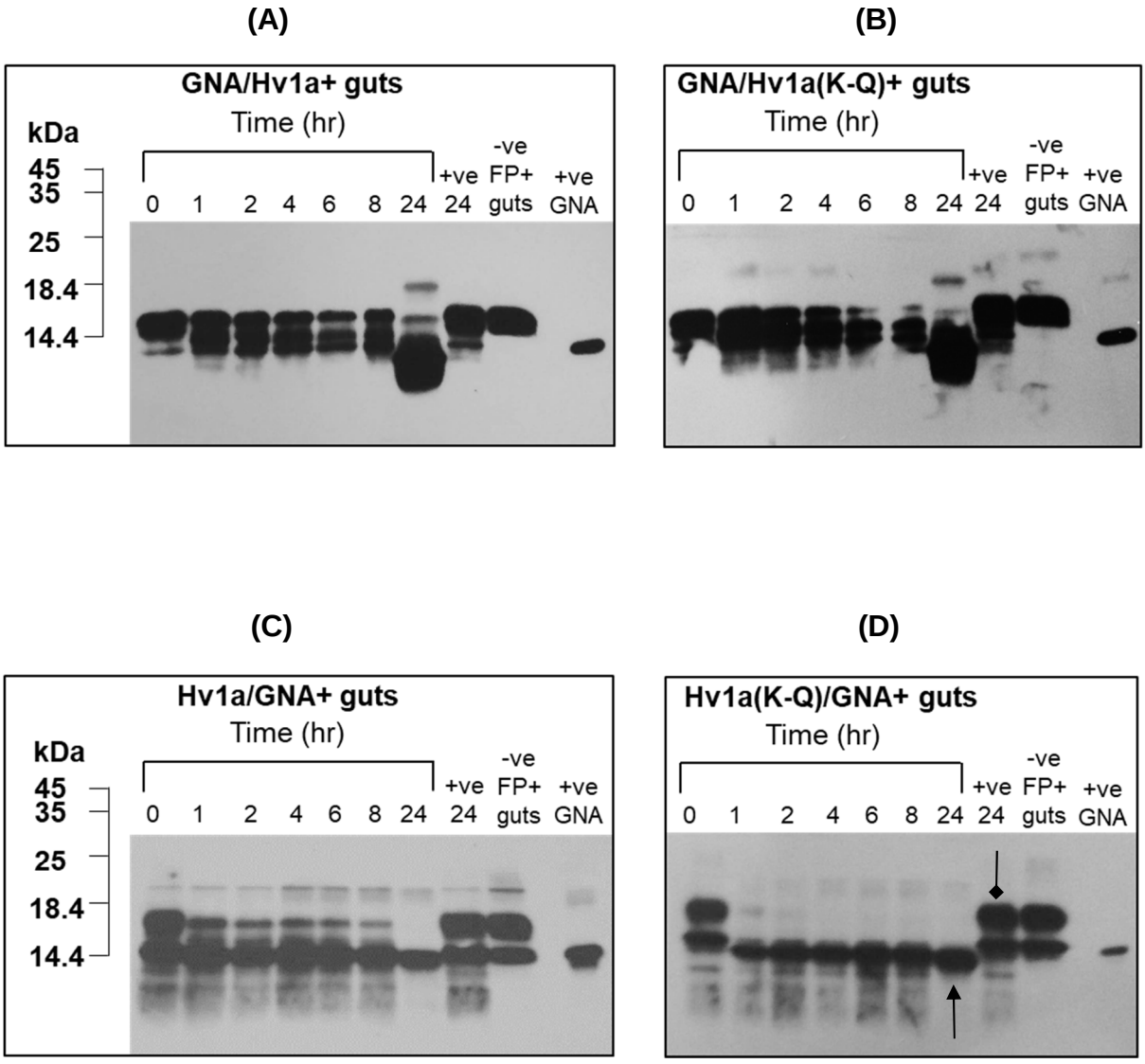


**Figure 4.15.** Western analysis (anti-GNA antibodies) of 5 day old *Aethina tumida* larvae gut extracts incubated with: (A) GNA/Hv1a; (B) GNA/Hv1a(K>Q); (C) Hv1a/GNA or (D) Hv1a/GNA(K>Q). Samples were taken at indicated time points after incubation of 75 µg of the respective fusion proteins (FP) with 40 µl gut extract (equivalent to two larval guts). For each time point 300 ng of each FP was loaded. +ve 24 denotes FP incubated for 24 hr without gut extract, -ve FP +guts refers to a boiled gut sample incubated with FP for 24 hr and +ve GNA represents 100 ng recombinant GNA standard. Cleaved GNA and intact fusion protein is indicated by an arrow head and diamond head, respectively.

### 4.7.7 Treatment of bee brood and egg slides with GNA/Hv1a, to assess oral toxicity to *Aethina tumida* feeding larvae

GNA/Hv1a, shown to be approx. 2 times more toxic to *A. tumida* larvae than GNA/Hv1a(K>Q), Hv1a/GNA or Hv1a(K>Q)/GNA, shows the greatest promise with respect to potential application of a fusion protein based product in the field. As such GNA/Hv1a was selected to assess the oral toxicity towards *A. tumida* larvae in an "applied" experiment.

Two separate bioassays were set-up whereby bee brood (two replicates; SP control n=370 and 290 eggs; GNA/Hv1a n=340 and 350 [+/- 10]), or an egg slide and bee brood were treated with GNA/Hv1a at 5 mg/ml (one replicate; SP control n=210 eggs; GNA/Hv1a n=360 eggs). Negative controls consisted of brood sprayed with a SP solution without recombinant protein. The brood was sprayed every 24 hr for 72 hr and thereafter larvae were supplied with brood *ad libitum* until they entered the wandering stage and survival was assessed. The egg and bee brood assay were conducted as stated above, except egg slides were split open to expose the eggs and subsequently sprayed with 250 µl of GNA/Hv1a at a final concentration of 5 mg/ml or SP solution as a negative control.

Four days post larval emergence a significant difference in feeding damage to the bee brood was observed between GNA/Hv1a treatment in comparison to the control group (Figure 4.16). Reduced feeding in the GNA/Hv1a treatment coincided with the appearance of dead larvae on and surrounding the bee brood. Figure 4.17 shows the survival of wandering larvae after either the bee brood or the egg slide and bee brood were treated with GNA/Hv1a at a final concentration of 5 mg/ml as compared to the control (SP sprayed) treatment. For the bee brood assay control survival until the wandering larvae stage ranged between 90.3-96.5 %, whereas a significant reduction in survival was observed in the GNA/Hv1a treatment with 43.7-55.2 % mortality recorded ($P$ <0.0001; Chi square test). Similarly, in the egg and bee brood assay a significant reduction in survival was recorded in the GNA/Hv1a treatment with 49.5 % mortality observed ($P$ <0.0001; Chi square test) as compared to 88 % survival in the control treatment.

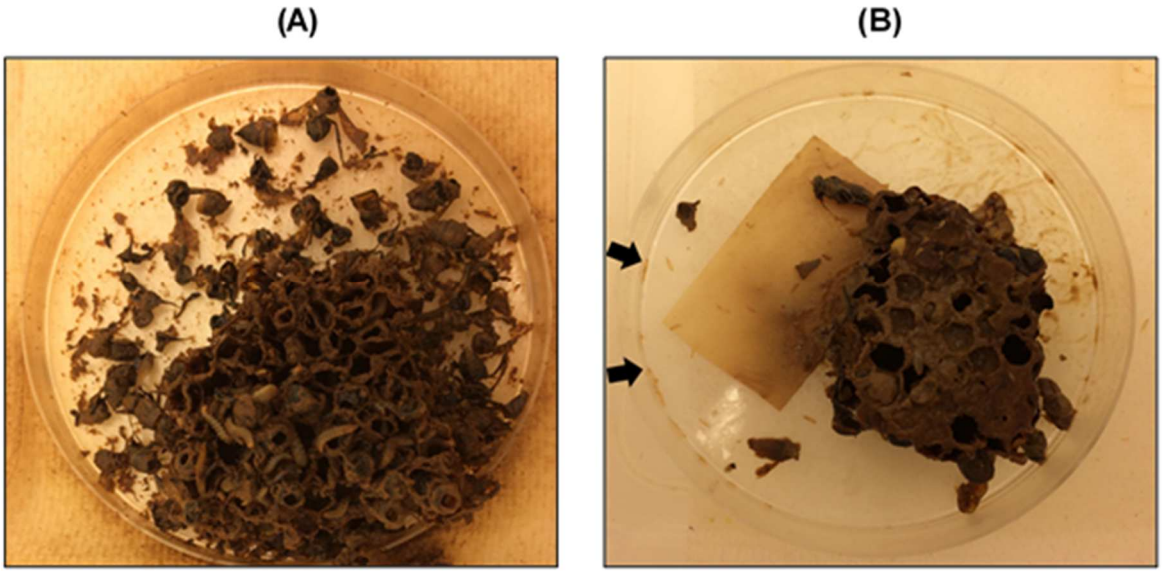


**Figure 4.16.** Damage to bee brood caused by feeding *Aethina tumida* larvae in (A) control treatment; brood sprayed with sodium phosphate solution and (B) brood sprayed with GNA/Hv1a (1 ml of 5 mg/ml GNA/Hv1a solution every 24 hours for 72 hours). Arrows indicate dead larvae.

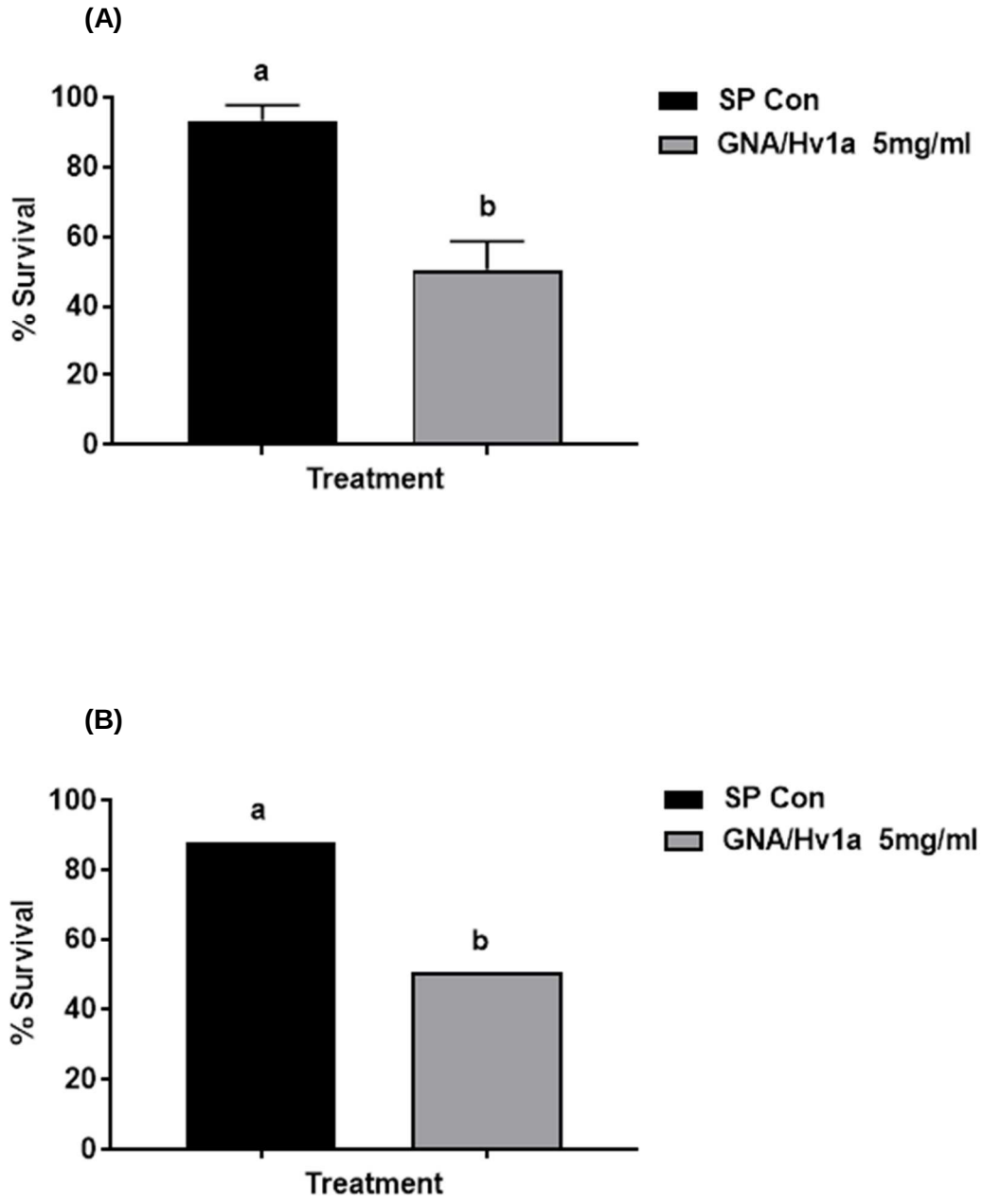


**Figure 4.17.** Survival of *Aethina tumida* at the wandering larvae stage after: (A) bee brood or (B) eggs and bee brood were treated with GNA/Hv1a at a final concentration of 5 mg/ml or sodium phosphate (SP Con) control solution. The brood was sprayed with solution every 24 hr for 72 hr and thereafter larvae were supplied with brood *ad libitum* until they entered the wandering stage and survival was assessed Error bars represent the ±SD of the mean, as determined from 2 independent replicates. Bars topped with different letter are statistically different at $P < 0.05$.

### 4.7.8 Western blotting analysis to confirm ingestion of GNA/Hv1a by *Aethina tumida* feeding larvae

During the bioassays alive, dead as well as larvae displaying the "writhing" phenotype, thought to be due to impaired mobility, were collected for western analysis to assess if GNA/Hv1a had been ingested. As shown in Figure 4.18 a single immunoreactive corresponding to the size of recombinant GNA was detected in the samples displaying the "writhing" phenotype, whereas for dead larvae a small amount of intact fusion protein and GNA was detected confirming ingestion of GNA/Hv1a. Additionally, an immunoreactive smear was observed in both lane 1 and 2, possible suggesting the binding of GNA/Hv1a to endogenous gut proteins. In contrast for the live larval sample no intact fusion protein or cleaved GNA was detected. Additionally, higher Mw proteins were detected in the control GNA/Hv1a and GNA samples in lane 4 and 5, respectively. It is that these higher Mw proteins represent a dimeric form of GNA, as a consequence of the standard samples not being completely denatured after boiling.

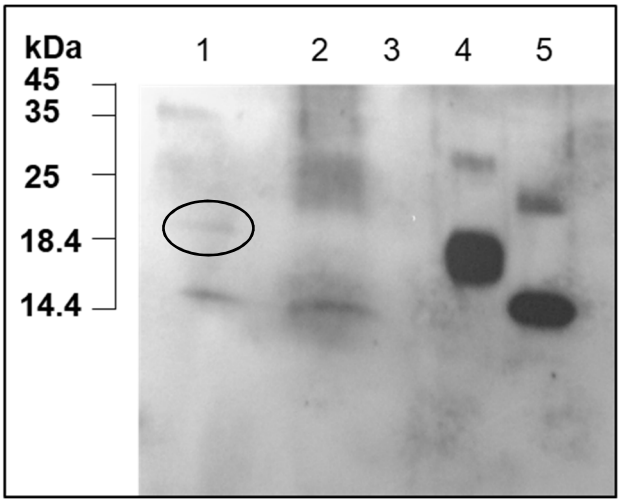


**Figure 4.18.** Western analysis (anti-GNA antibodies) of *Aethina tumida* larvae fed on bee brood treated with GNA/Hv1a at a final concentration of 5 mg/ml. Lane 1 protein extracts (40 µg total protein loaded in each lane) prepared from dead larvae; lane 2 larvae displaying the "writhing" phenotype and lane 3 live larvae. The circle in lane 1 indicates intact GNA/Hv1a. Lanes 4 and 5 represent 100 ng of GNA/Hv1a and recombinant GNA, respectively.

## 4.8 Oral delivery of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to *Aethina tumida* adults

### 4.8.1 Oral delivery of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to *Aethina tumida* adults via a drinking bioassay

Approx. 1 week old adults (averaging weight 16.11 mg) were supplied with recombinant GNA at 5 mg/ml, pro-Hv1a at 1.25 mg/ml and a range (0.625-5 mg/ml) of concentrations of GNA/Hv1a, Hv1a/GNA or K>Q variants (Figure 4.19). Negative control adults were fed on SP/sucrose solution without recombinant proteins. The diet was changed every 48 hr for 6 days and thereafter adults were supplied 50 % (v/v) honey solution until day 14.

**Figure 4.19.** An example of the adult *Aethina tumida* feeding bioassay. *Aethina tumida* adults were supplied with SP/sucrose solution (13 % v/w) containing either recombinant GNA at 5 mg/ml, pro-Hv1a at 1.25 mg/ml and a range (0.625-5 mg/ml) of concentrations of GNA/Hv1a, Hv1a/GNA or K>Q variants. Solutions were changed every 48 hr for 6 days and thereafter adults were supplied 50 % (v/v) honey solution until day 14.

No mortality was recorded for GNA/Hv1a(K>Q) or Hv1a(K>Q)/GNA treatments over the course of the bioassay period (results not shown). As shown in Figure 4.20 no mortality was recorded in pro-Hv1a or GNA control groups after 14 days, with 5 % mortality recorded for SP treated adults ($P>0.05$; Mantel-Cox log-rank test). Adults fed on Hv1a/GNA at 5 mg/ml showed a rapid decline in survival with 65 % mortality recorded after 2 days rising to 95 % after 6 days ($P<0.0001$; Mantel-Cox log-rank test). A further reduction in dose to 2.5 mg/ml resulted in 40 % mortality after 14 days, which was significantly different from the control group ($P<0.01$; Mantel-Cox log-rank test). No mortality was recorded in the 1.25 or 0.625 mg/ml treatment ($LC_{50}$ Hv1a/GNA day 14, 2.52 mg/ml). The onset of mortality in the 5 mg/ml GNA/Hv1a treatment was slightly delayed, as compared to Hv1a/GNA, with 25 % mortality recorded after 4 days, increasing to 55 % after 6 days and 90 % after 14 days ($P<0.0001$; Mantel-Cox log-rank test). A further reduction in dose to 2.5 and 1.25 mg/ml resulted in a dose dependant decline in survival with 60 and 30 % mortality being recorded after 14 days ($P<0.0001$; $P<0.05$; Mantel-Cox log-rank test). The lowest dose of GNA/Hv1a at 0.625 mg/ml failed to induce mortality with 100 % survival recorded ($LC_{50}$ GNA/Hv1a day 14, 2.02 mg/ml).

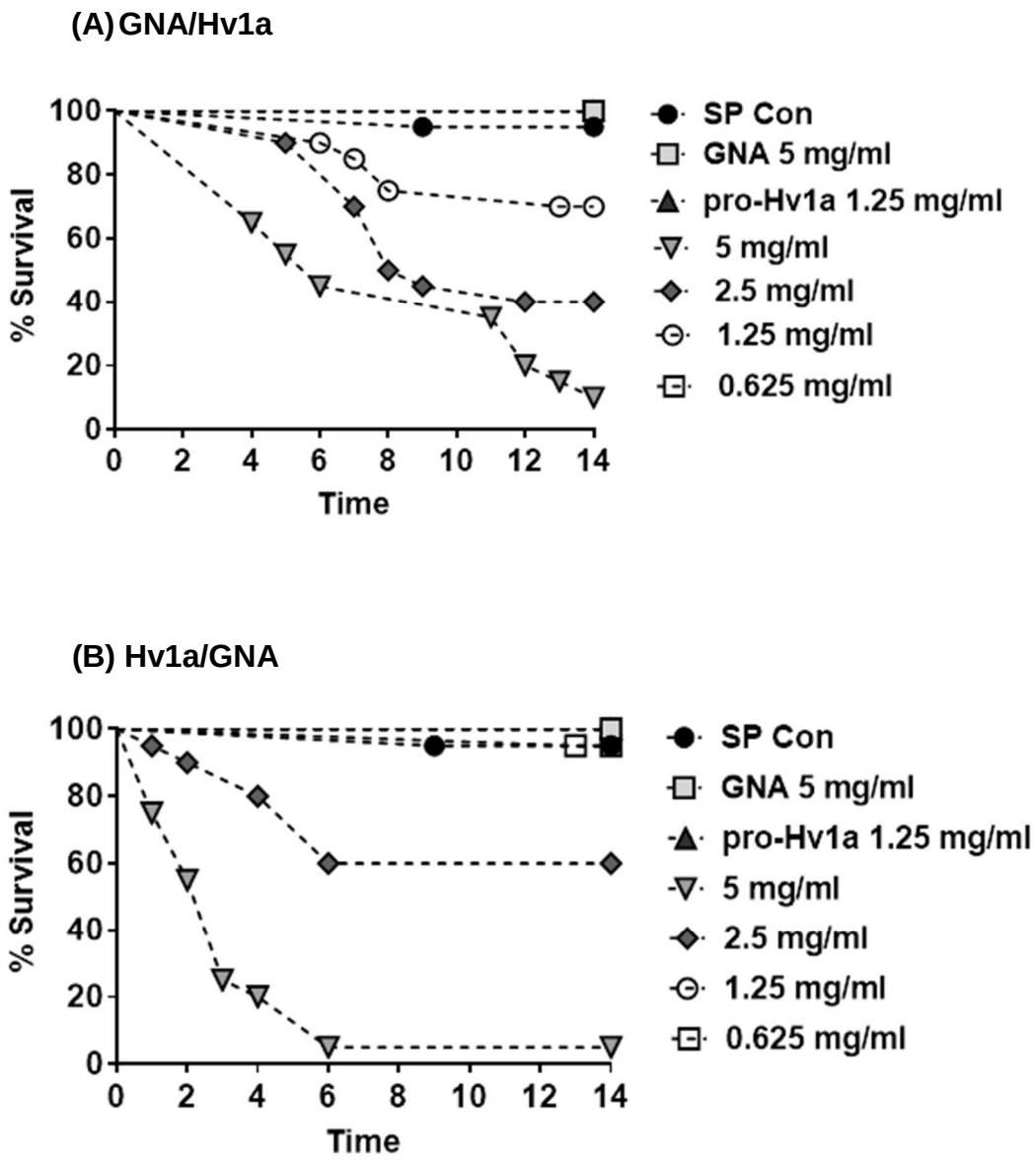


**Figure 4.20.** Survival of *Aethina tumida* adults (approx. 1 week old at day 0) fed on 5 mg/ml of recombinant GNA, 1.25 mg/ml pro-Hv1a and 0.625 to 5 mg/ml of: (A) GNA/Hv1a and (B) Hv1a/GNA. Sodium phosphate (SP Con) buffer served as negative control. Solutions were changed every 48 hr for 6 days and thereafter adults were supplied 50 % (v/v) honey solution until day 14. N= 20 per treatment.

### 4.8.2 Stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants within the gut of *Aethina tumida* adults

The stability of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants was assessed, as previously described for larvae (section 4.7.4) in adult gut samples by western analysis. Analysis of GNA/Hv1a, Hv1a/GNA and K>Q fusion protein variants in the gut samples confirmed that the Hv1a toxin was cleaved from GNA after 24 hr, which was evident by the presence of a single immunoreactive band corresponding to the size of recombinant GNA, which was absent in the control samples. Figure 4.21 shows results for GNA/Hv1a as the western blot results for all the fusion protein variants were virtually identical. We speculated that GNA/Hv1a and Hv1a/GNA are stable for a longer period in the gut of *A. tumida* adults as 90-95 % mortality was observed at a concentration of 5 mg/ml whereas no mortality was recorded for the K>Q variants at the corresponding dose.

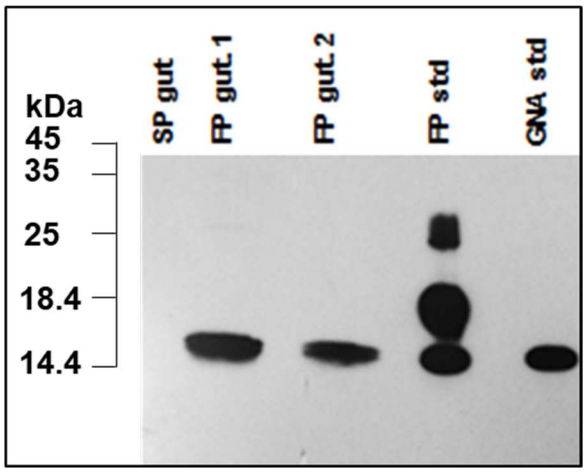


**Figure 4.21.** Western analysis (anti-GNA antibodies) of approx. 1 week old *Aethina tumida* adults guts after feeding on sodium phosphate (SP)/sucrose solution (13 % v/w) containing GNA/Hv1a, at a concentration of 2.5 mg/ml. SP and fusion protein (FP) haemolymph and gut samples (two replicates depicted as R1 and R2) 10 µl and 10 µg total protein was loaded, respectively. Standards represent 100 ng of GNA/Hv1a (FP std) and recombinant GNA (GNA std).

## 4.9 Toxicity of recombinant pro-Hv1a toxin, recombinant GNA and GNA/Hv1a fusion protein to *Apis mellifera*

### 4.9.1 *Apis mellifera* adult injection bioassays

Newly emerged *A. mellifera* workers (n=20), supplied from Fera Science Ltd Home Apiary, were injected with 10 µg pro-Hv1a and 40 µg of recombinant GNA or GNA/Hv1a, with SP solution serving as a negative control. As shown in Figure 4.22 A no mortality was recorded over the 7 day bioassay period in the SP and Hv1a treatment, with 5 % mortality being recorded in the GNA treatment. No significant mortality was recorded in the GNA/Hv1a, with 90 % survival being recorded by the end of the 7 day bioassay period (P>0.05; Mantel-Cox log-rank test).

### 4.9.2 *Apis mellifera* larval feeding bioassays

An acute toxicity assay was performed on larval honey bees (n=20 per treatment). A single oral dose of 100 µg/larva of recombinant GNA or GNA/Hv1a, with SP solution serving as a negative control was administered to 4 day old larva. Larvae were monitored for 7 days after ingestion of a single acute dose of recombinant GNA or GNA/Hv1a. As shown in Figure 4.22 B, 5 % mortality was recorded after 7 days in the control treatment. Mortality at day 7 was a non-significant 25 % and 15 % respectively for the GNA and GNA/Hv1a treatments (P>0.05; Mantel-Cox log-rank test)

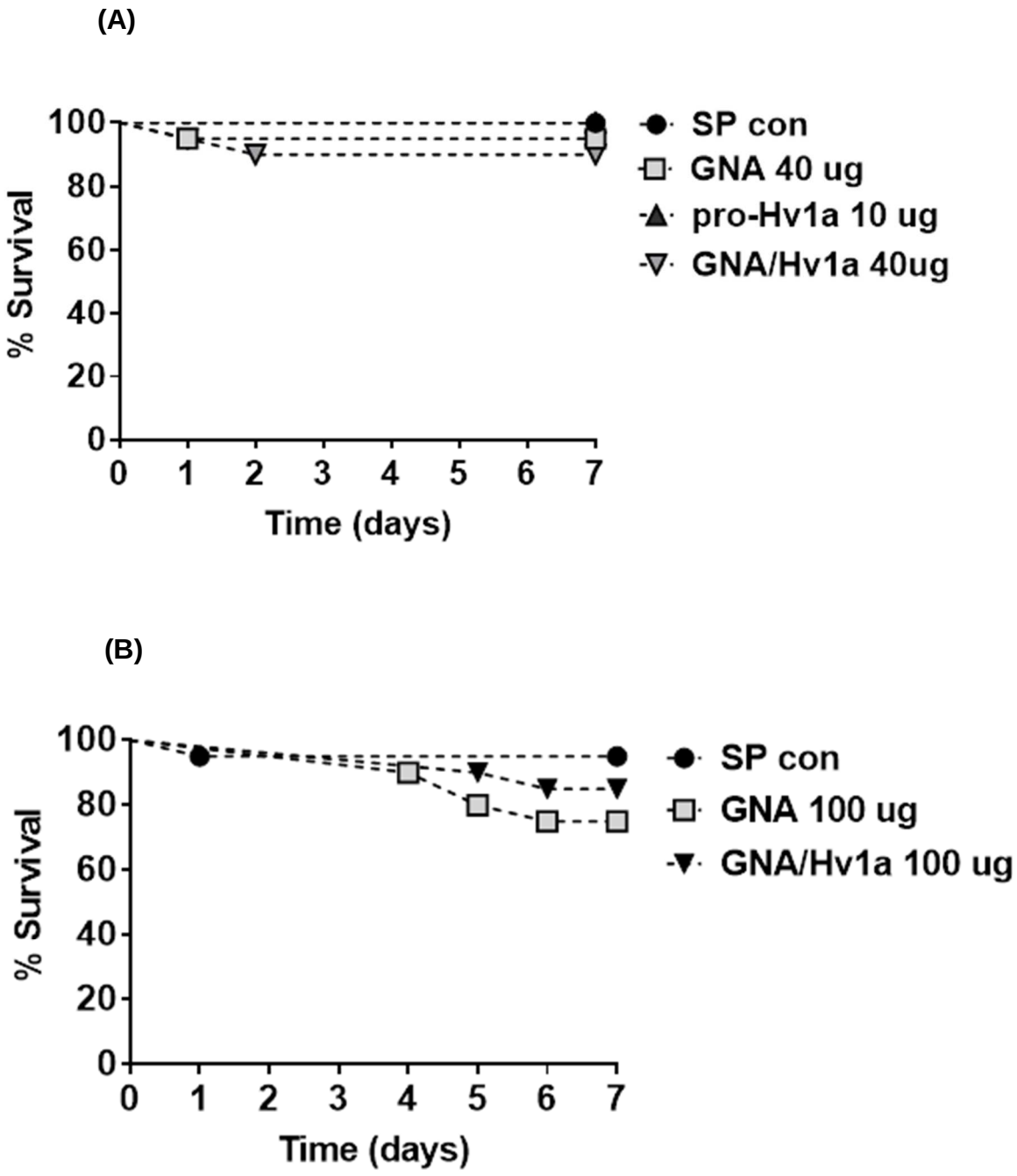


**Figure 4.22.** Survival of *Apis mellifera*: (A) adults injected with 10 µg Hv1a, 40 µg of GNA or GNA/Hv1a. Controls included sodium phosphate buffer (SP) injected bees. (B) larvae fed on a single dose of 100 µg GNA and GNA/Hv1a. Controls included sodium phosphate buffer (SP) fed larvae. Mortality was monitored daily for 7 days. N=20 per treatment.

## 4.10 Discussion

Current pest control measures for *A. tumida* are largely ineffective suffering variability in levels of control. The in-hive organophosphate designed to control adult beetles is routinely used in conjunction with GardStar7 (40 % permethrin), a soil treatment product aimed to control “wandering” larvae (i.e. in search of a suitable site for pupation) that leave the hive and enter the soil to complete their life-cycle (Delaplane, 1998; Hood and Miller, 2003). Organophosphates are highly toxic to bees, wildlife and humans (Carson, 1962) and hence all hive honey combs must be removed prior to treatment. Additionally, the continued use of pyrethroids such as permethrin can give rise to resistance, and upon contact, is deleterious to honey bees (De Guzman et al., 2011). Thus, the need for target specificity and efficacious alternative control strategies are urgently required. The data presented in this chapter shows that fusion protein technology offers enormous potential for the development of a novel biopesticide to combat this economically significant pest without jeopardising the health of honey bee populations.

Both the recombinant pro-Hv1a toxin alone and Hv1a containing fusion proteins show insecticidal activity after injection into *A. tumida* wandering larvae with the expected symptoms of paralysis and mortality, indicating correct folding and processing of the toxin. Furthermore, it strongly suggests that the Hv1a toxin targets the $Ca_v$ channels in *A. tumida*. Injection of recombinant pro-Hv1a resulted in a $LD_{50}$ of 43.53 µg/g insect or 11 nmoles/g insect against *A. tumida* larvae, which is typically higher than values reported for recombinant Hv1a. For example, the $ED_{50}$ reported for *H. armigera* is 3 nmole/g and the $PD_{50}$ for *H. armigera* is 0.25 nmole/g, which is a respective 2-fold and 25-fold lower than the $LD_{50}$ of our recombinant toxin pro-Hv1a (Atkinson et al., 1998; Bloomquist et al., 2003). Fitches et al. (2012) suggested that the differences in the toxicity of Hv1a towards different species may be a consequence of the differences in the ability of the toxin to disrupt the ion channel function or the different sources of toxin (i.e. synthetic, recombinant or native toxin). Trung et al. (2006) reported that injection of GNA at 20 µg per *L. oleracea* larva (approx. 500 µg per g insect) had no effect on survival, which was 95 % over 6 days. Surprisingly, in our hands injection of GNA into non-feeding wandering larvae elicited a $LD_{50}$ after 7 days of 788.09 µg/g insect. If feeding larvae were injected with GNA one could hypothesis that this result was a consequence of the retrograde transport of GNA from

the haemolymph to gut, causing morphological changes in the gut epithelium thereby interfering with nutrient uptake and absorption (Powell et al.,1998; Du et al. 2000; Fitches et al., 2001; Sadeghi et al. 2008; Yang et al., 2014). The $LD_{50}$ values for GNA/Hv1a and GNA/Hv1a(K>Q) were a comparable 25.20 and 26.92 µg/g insect (equivalent to 6.3 and 6.7 µg/g insect of Hv1a), whilst Hv1a/GNA and Hv1a(K>Q)/GNA $LD_{50}$'s were slightly lower at 18.90 and 14.32 µg/g insect (equivalent to 4.7 and 3.58 µg/g insect of Hv1a), respectively. With respects to Hv1a/GNA the $LD_{50}$ was approx. 2.6-fold lower than the value reported for *M. brassicae* larvae, whereby injection of 50 µg/g insect resulted in 45 % mortality (Fitches et al., 2012). In contrast, Hv1a and GNA alone were approx. 6 to 12-fold and 30 to 50-fold less toxic, respectively, by injection towards *A. tumida* wandering larvae as compared to the fusion protein variants. Fitches et al. (2012) provided direct evidence for GNA localisation to CNS by injecting FITC-labelled GNA and subsequently showed binding of GNA to the nerve cord of *M. brassicae*, suggesting that GNA may assist in localising Hv1a to the CNS of exposed insects. These injection results are in agreement with Fitches et al. (2012), showing that the fusion of Hv1a to the carrier protein GNA can enhance biological activity of the recombinant toxin.

Incorporation of GNA/Hv1a into artificial diet comprising of pollen and honey solution at 5000 ppm resulted in 100 % mortality after 6 days, however when this assay was repeated at 3000 and 2000 ppm, no mortality was recorded over the course of the bioassay. Consequently, the fusion proteins were incorporated into a sterile SP/sucrose solution with the hope of reducing the proteolytic activity within the gut of *A. tumida* exposed to a sugar solution as compared to the protein rich artificial diet.

The drinking feeding assay provided clear evidence of a dose dependent decline in survival after oral delivery of GNA/Hv1a, Hv1a/GNA and K>Q variants towards *A. tumida* feeding larvae. In these assays no effects on survival were observed after *A. tumida* larvae fed on the toxin alone. Our results are in agreement with Fitches et al. (2012) who also observed no mortality or reduction in growth of fifth stadium lepidopteran *M. brassicae* larvae fed daily for four days on droplets containing 9.6 µg Hv1a. This outcome was not surprising due to the absence of a delivery system such as GNA to direct the toxin to the CNS. Oral delivery of GNA to *A. tumida* larvae had no effect on survival and growth, whereas marginal effects on growth from GNA have been documented in several previous assays against lepidopteran larvae, potentially

suggesting that coleopterans are less sensitive to the effects of GNA (Fitches et al., 1997; Fitches et al., 2001; Fitches et al., 2012; Yang et al., 2014). In contrast, the fusion of Hv1a to the N-or C-terminus of GNA with and without the K>Q modification led to significant levels of mortality, in comparison to the control treatments. The $LC_{50}$ value after 7 days for GNA/Hv1a was 0.52 mg/ml which was 1.7-fold lower than that of Hv1a(K>Q)/GNA ($LC_{50}$ 0.89 mg/ml); 2.2-folder lower than for Hv1a/GNA ($LC_{50}$ 1.14 mg/ml) and 2.3-fold lower than for GNA/Hv1a(K>Q) ($LC_{50}$ 1.18 mg/ml). These results suggest that the biological activity of all fusion protein variants was greatly enhanced when delivered in a solution as compared to artificial diet, as significant levels of mortality were recorded at much lower doses than the 5 mg/ml required to induce mortality in diet assays. This is likely to be a consequence of reduced proteolytic activity within the gut of *A. tumida* exposed to a sugar solution as compared to the protein rich pollen based artificial diet resulting in increased stability of the intact fusion protein and hence increased delivery of toxin to the haemolymph.

The results obtained from the oral delivery bioassays indicated that GNA/Hv1a was the most effective fusion protein against *A. tumida* larvae as compared to the other fusion protein variants, as 100 % mortality was observed at a concentration of 1.25 mg/ml as compared to 50-70 % mortality for GNA/Hv1a(K>Q), Hv1a/GNA or Hv1a(K>Q)/GNA at the equivalent dose. We hypothesised that increased oral toxicity of GNA/Hv1a was a consequence of enhanced resistance to proteolysis as compared GNA/Hv1a(K>Q), Hv1a/GNA or Hv1a(K>Q)/GNA in the gut of *A. tumida*. As such western blotting experiments to determine transport of intact fusion protein 24 hr after oral delivery were carried out. The results showed only the presence of cleaved GNA in both haemolymph and gut samples for all fusion protein variants. This was in contrast with previous reports for Hv1a/GNA by Fitches et al. (2012), where intact fusion protein was detected in *M. brassicae* haemolymph 48 hr post feeding on diet containing Hv1a/GNA. Consequently, stability assays were carried out to determine the length of time the fusion proteins remained intact *in vitro* in the gut and *in vivo* in the presence of feeding *A. tumida* larvae. A significant amount of intact GNA/Hv1a was detected in the presence of *A. tumida* larvae after 8 hr whereas degradation of Hv1a/GNA was completed after 2 hr and 4 hr for both Hv1a(K>Q)/GNA and GNA/Hv1a(K>Q). Enhanced stability of GNA/Hv1a was further illustrated by incubation with gut extracts, whereby greater amounts of intact fusion protein was

detected after 24 hr as compared to all other fusion protein variants. The results of these assays provide clear evidence for enhanced stability of GNA/Hv1a, which we hypothesize resulted in increased delivery of the Hv1a toxin to the haemolymph; thereby achieving an $LC_{50}$ approx. two times lower than the other fusion protein variants. Injection bioassays conducted by Pyati et al. (2014) showed that the Hv1a toxin incorporating the K>Q amino acid change had the same toxicity towards *M. brassicae* in comparison to the unmodified version. The ICK motif of the Hv1a toxin is made up of a ring formed by two disulfide bridge, with a third disulfide bridge that penetrates the ring to create the pseudo-knot (Saez et al., 2010). It is the third disulfide bridge and the formation of the cysteine knot that provides the Hv1a toxin with exceptional stability (Tedford et al., 2001; Herzig and King, 2015). The third disulfide bridge is formed on the $36^{th}$ amino acid and the K>Q amino acid change, from a lysine to a glutamine residue, is on the $34^{th}$ amino acid (Fletcher et al., 1997; Pyati et al. (2014). Tedford et al., (2001) showed that $Lys^{34}$ is critical for the stabilisation of the β-hairpin structure, which is vital for the insecticidal activity of Hv1a toxin. Alteration of a lysine to a glutamine could decrease the hydrophobicity index of the cysteine side-chain thiol groups ultimately decreasing the stability of the third disulfide bridge (Herzig and King, 2015), therefore reducing the oral toxicity of both the K>Q fusion protein variants against *A. tumida* larvae. Furthermore, it is hypothesised that Hv1a/GNA cleavage occurs between the C-terminus of the toxin and the N-terminus of GNA (Fitches et al., 2012). The C-terminus of Hv1a peptide (residue 33-36), without the K>Q amino acid change, includes the sequence VKRC, which is similar to the signal sequence EKRE present in the α-factor signal expression vector that is cleaved by the KEX2 gene product between R and E (Fitches et al., 2012). It is possible that the enhanced stability of GNA/Hv1a compared to Hv1a/GNA could be a consequence of this potential cleavage site being more exposed when Hv1a is linked to the N-terminus of GNA.

Owing to enhanced efficacy and stability of GNA/Hv1a as compared to Hv1a/GNA and K>Q variants, this variant was selected to carry out further “applied” experiments; whereby bee brood, or eggs and bee brood, were sprayed with fusion protein and larval survival subsequently assessed after a period of 14 days. As compared to controls, reduced feeding and mortality was observed in the fusion protein treatment four days after larval emergence (Figure 4.16). Subsequently, a significant 45-56 %

reduction in survival of wandering larvae (as compared to 88-96 % for controls) was observed for the GNA/Hv1a treatment. Levels of mortality observed in the brood assays were not as high as that recorded in the drinking assays although this is not surprising as emerging larvae in treated brood were not as directly exposed to the fusion protein as in the drinking assays. In addition, as suggested by results from earlier artificial diet bioassays, ingested GNA/Hv1a was likely to be more prone to gut proteolysis as the emerging larvae were also feeding on protein rich brood. Incomplete spray coverage of the brood may also have allowed a proportion of larvae to avoid ingestion of the fusion protein altogether.

Oral delivery of GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants to adults met with variable results. No mortality was observed in the K>Q variant treatments and this could be a consequence of the destabilisation of the third disulphide bridge, as discussed above. Significant levels of mortality were however recorded in the GNA/Hv1a and Hv1a/GNA treatments with $LC_{50}$ values of 2.02 mg/ml and 2.52 mg/ml. Adult $LC_{50}$s were 3.9-fold and 2.2-fold higher than those obtained for feeding larvae. Whilst difficult to determine directly, the delayed onset of adult mortality (e.g. 90 % adult mortality after 14 days of feeding as compared to 100 % larval mortality just 5 days after feeding on 5 mg/ml fusion protein) suggests that the beetles may have ingested less fusion protein than the larvae. It is also possible that adults *A. tumida* have a more alkaline gut environment than the larvae. Herzig and King (2005) have demonstrated that Hv1a rapidly degrades in highly alkaline guts of pH >8, therefore compromising the stability of Hv1a and in turn its effectiveness. Further research into the gut pH of *A*. *tumida* adults is required to determine if an alkaline gut environment was responsible for the variable and reduced mortality recorded in the drinking bioassay.

Nakasu et al. (2014) has demonstrated that Hv1a/GNA has negligible effects on honey bee larvae and adults following injection and ingestion of the fusion protein. The author hypothesised that the lack of toxicity of the Hv1a toxin towards *A. mellifera* workers and larvae could be a consequence of ineffective binding of the toxin to the target site of action in the CNS. It was also suggested that the lack of toxicity of Hv1a/GNA following injection could suggest that the binding sites in the $Ca_V$ channels of honey bees are significantly different to lepidopteran larvae, which typically show 90-100 % reduction in survival after injection with comparable doses (Nakasu et al.,

2014). In contrast ω-ACTX-Hv2a has been shown to block $Ca_v$ channels of honey bees and it is thought that the limited sequence homology to Hv1a could account for the difference in toxicity towards honey bees (Wang et al., 2001; Nakasu et al., 2014). To confirm Nakasu et al. (2014) results, GNA/Hv1a was selected to carry out injection bioassays against honey bee workers and feeding assays against honey bee larvae, as this fusion protein shows the greatest potential for the control of *A. tumida* in apiculture. Injection of GNA/Hv1a resulted in 10 % mortality after 7 days, which was similar to the results reported by Nakasu et al. (2014), where mortality of honey bees injected with Hv1a/GNA was 17 % 48 hr post injection. Conversely to Nakasu et al. (2014) who reported 57 % mortality for honey bees injected with 20 µg GNA, we did not observe mortality despite the higher dose of 40 µg being administered. However, our results are in line with those reported for lepidopteran (*L. oleracea*) larvae where injection doses of 20 µg GNA had a no significant effects on survival (Trung et al., 2006).

The development and implementation of fusion protein based pesticides holds great potential for new target specific and environmentally benign applications for control of *A. tumida* in apiculture. Insecticides are often formulated with emulsifiers, stabilisers, surfactants or other adjuvants that require dissolution in organic solvents and it has been demonstrated that Hv1a is stable long-term in organic solvents which is vital for the development of a next generation biopesticide (Foy and Pritchard, 1996; Herzig and King; 2005). Future research needs to focus on the formulation of GNA/Hv1a to enhance its stability in the environment.

# CHAPTER 5 | IDENTIFICATION AND CHARACTERISATION OF DIGESTIVE PROTEASES FROM *AETHINA TUMIDA*

## 5.1 Introduction

The biochemistry of digestion in insects is varied according to whether they are phytophagous, carnivorous, omnivorous or parasitic (Gillott, 1980). The alimentary canal of insects is divided into three regions: foregut, midgut and hindgut. The foregut and hindgut are endodermal in origin and are lined with a cuticle. The midgut lacks a cuticle lining but produces a permeable peritrophic membrane which functions to protect the epithelium lining from mechanical damage by moving food and at the same time allows for nutrient absorption by being porous in nature (Hopkins and Harper, 2001). Insects use several types of enzymes secreted by the midgut epithelial cells to digest a wide range of food diets, with carbohydrases and proteases breaking down the molecules into absorbable elements in the midgut (Terra, 1990). Proteases, also referred to as peptidases, are hydrolytic enzymes present in both the digestive system and haemolymph of insects that play a vital role in food digestion, polyphenoloxidase activation, liberation of amino acids for growth, toxin activation/detoxification and inflammation processes (Neurath, 1984). With regards to digestion, proteases are responsible for breaking peptide bonds, enabling the degradation and subsequent absorption of dietary proteins. Insect proteases are classified according to their mechanism of catalysis for activity as serine proteases, cysteine proteases, asparatic proteases and metalloproteinases (Bode and Huber, 1992). Several insect studies have provided evidence for possible selection and adaptation of proteases in response to the ingested protein content (Jongsma and Bolter 1997; Bown et al. 2004; Moon et al. 2004). The insect gut pH is not very variable, but is adapted to suit different methods of feeding, and different diets they feed on, which in turn determines the type of digestive enzymes present in the midgut, as they function optimally at different pH values (Applebaum, 1985; Dow, 1986; Terra, 1990; McGhie et al., 1995). For instance, Lepidoptera have an alkaline midgut pH and typically possess serine proteases like trypsin, chymotrypsin and elastase which perform optimally at neutral to high pH. In contrast some coleopterans possess an acidic midgut environment ranging from pH 3.5-5.5 and instead rely predominantly on cysteine or aspartic proteases which function efficiently at an acidic pH (Schuler et al., 1998; Hilder and Boulter, 1999).

Proteases inhibitors (PI) are widely distributed in microorganism, animals and plants. PIs can be divided into four main types, serine, cysteine, aspartic or metallo-proteases, bases on the digestive protease they inhibit. All inhibitors have a reactive site which acts on the active site of the target enzyme (Broadway, 1995). PIs act by reducing the activity of digestive enzymes, preventing ingested proteins from being reduced to free amino acids and preventing utilisation of the ingested protein, resulting in amino acid deficiency which affects growth, development and survival (Gatehouse et al., 1993). The role of plant PIs as a defence mechanism against insect attack was first demonstrated by Mickel and Standish (1947). Consequently, numerous studies have been carried out using PIs incorporated into artificial diet and expressed *in planta* to evaluate the ability of PIs to inhibit enzyme activity in the gut of certain coleopteran and lepidopteran pest insects (Lipke et al., 1954; Applebaum et al., 1964; Green and Ryan, 1972; Hilder et al., 1987; Gatehouse et al., 1997; Schuler et al., 1998; Hilder and Boulter, 1999).

In this chapter the gut proteolytic enzymes of feeding *A. tumida* larvae were characterised with the use of three PIs (trypsin-specific inhibitor, trypsin- and chymotrypsin-specific inhibitor and cysteine proteases inhibitor) by inhibiting enzyme activity within gut extracts. The dominant digestive protease was identified as trypsin. Consequently, a trypsin inhibitor was incubated together with *A. tumida* gut extracts and GNA/Hv1a and Hv1/GNA to determine if this protease was responsible for degradation of the intact fusion protein (Chapter 4) and whether the stability of GNA/Hv1a and Hv1a/GNA could be enhanced over an assay period of 24 hr.

## 5.2 Profile of *Aethina tumida* gut extracts

Crude *A. tumida* feeding and wandering larval gut extracts (Figure 5.1) were analysed by SDS-PAGE and Native-PAGE using 12.5 % acrylamide gels. SDS-PAGE results (Figure 5.1A) show a range of proteins for both wandering and feeding *A. tumida* larvae. Two dominant proteins of approx. 100 and 250 kDa were observed in wandering and feeding larval samples. Other obviously dominant protein bands of 15, 22 and 55 kDa are evident in wandering larvae. Several minor proteins of approx. 20, 22, 30, 32, 55 and 70 kDa were observed in feeding larvae. Native-PAGE results (Figure 5.1B) showed four dominant proteins in wandering *A. tumida* larvae at 20, 60, 150 and 250 kDa and 2 minor proteins at 10 and 25 kDa. Two dominant corresponding proteins of approx. 150 and 250 kDa, and 3 minor proteins (approx. masses of 35, 60 and 130 kDa) were observed in feeding *A. tumida* larva.

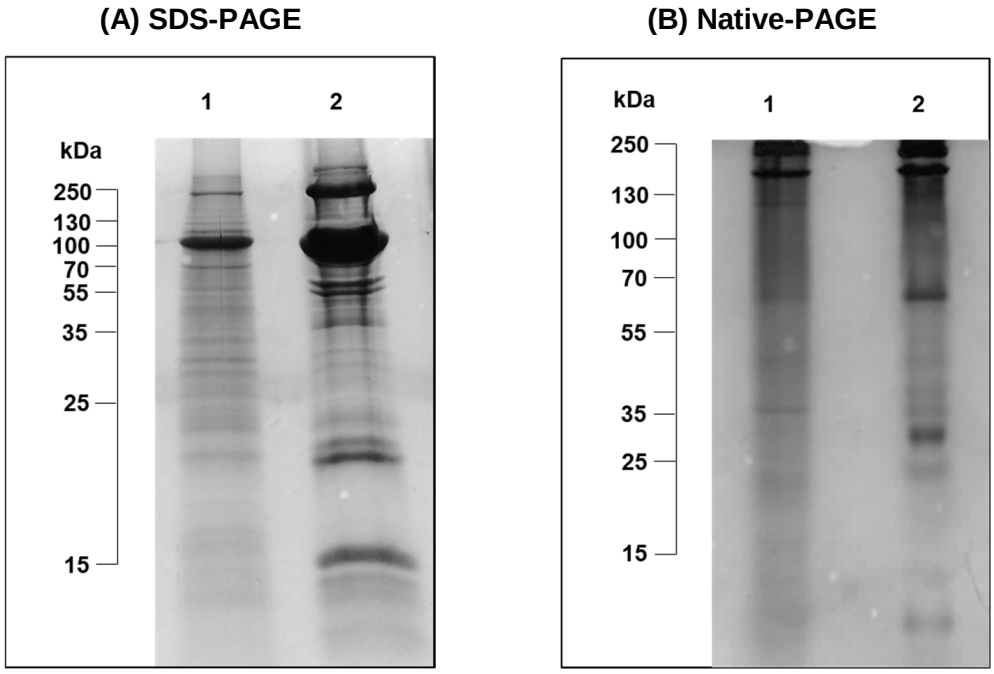


**Figure 5.1.** Protein profile of *Aethina tumida* larvae gut extract, determined by: (A) SDS-PAGE and (B) Native-PAGE. A total of 10 µg of gut extract was loaded and protein bands were visualised by staining with Coomassie. Lane 1 represents feeding larvae gut extracts and lane 2 shows wandering larvae.

## 5.3 Identification of feeding *Aethina tumida* digestive proteases using electrophoretic zymography

For the detection of protease activity bands, the protein substrate casein was used. After separating feeding *A. tumida* larval gut proteins on SDS-PAGE and renaturing proteins by removing SDS from the gel, proteases activity bands were stained by incubating the gels in casein substrate. In preliminary studies a total of four (1 to 4) protease bands were identified in feeding larval samples but were absent in wandering (non-feeding) larval samples (Figure 5.2). The digestive proteases ranged from approx. 15 to 200 kDa and could be classified into three groups according to their apparent molecular mass. Activity band 1 was considered high molecular-mass proteases (HMP) at approx. 200 kDa, activity band 2 was considered medium molecular-mass proteases (MMP) at approx. 35 kDa, whereas activity band 3 and 4 were classified as low-molecular-mass proteases (LMP) ranging from approx. 15 to 20 kDa.

To characterise the digestive proteases within the gut of feeding *A. tumida* larvae three protease inhibitors were use: SKTI, SBBI and trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) to inhibit trypsin, chymotrypsin, and trypsin and cysteine proteases, respectively. No inhibition of any of the protease bands was observed using 50 µM E-64 proteases inhibitor, despite being used at 5 times the manufactures recommended concentration (Figure 5.3). By contrast, protease bands 1, 2 and 4 in Figure 5.4 decreased in activity (reduced or absence of clearance) with the addition of 100 µM of SKTI and SBBI. An increase of SKTI and SBBI concentrations to 250 µM and 500 µM led to complete inhibition of protease bands 1, 2 and 4 as compared to the controls, suggesting they are trypsin-like proteases that are sensitive to both SKTI and SBBI. No inhibition of activity band 3 was observed with the addition of either inhibitor at the highest concentration of 500 µM, suggesting it could be either be a different class of proteases or chymotrypsin/trypsin-like proteases that was insensitive towards SKTI and SBBI. Overall the results of the zymogram suggest that trypsin seems to be the dominant class of proteases in the gut of feeding *A. tumida* larvae.

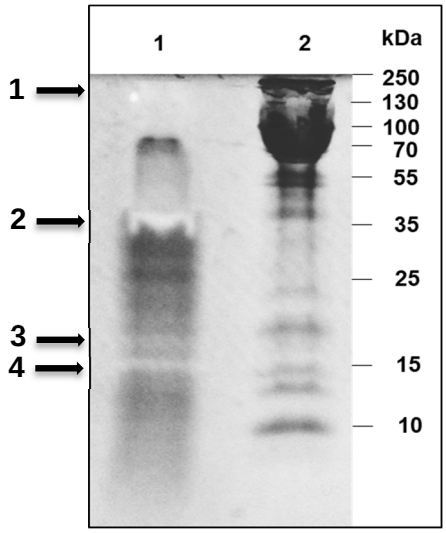


**Figure 5.2.** Casein substrate-based protease activity profile in the gut extracts of *Aethina tumida* feeding and wandering larvae. A total of 15 µg of gut extract was loaded and activity (clearance of protein) were visualised by incubating in casein and staining with Coomassie. Lane 1 represents feeding larval and lane 2 wandering larval gut extracts. Protease activity bands are indicated 1-4.

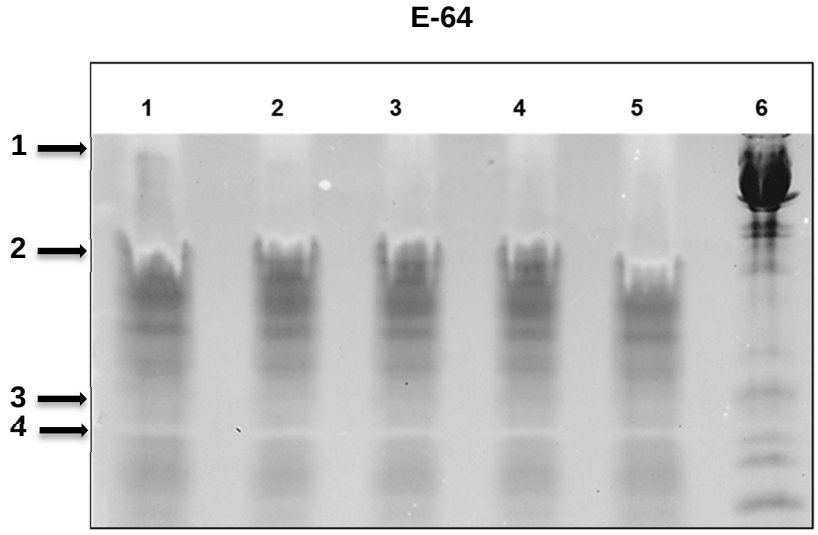


**Figure 5.3.** Casein substrate-based protease activity band profile of *Aethina tumida* feeding and wandering larvae incubated with E-64. A total of 15 µg of gut extract was treated with E-64 and protease activity visualised by incubating in casein and staining with Coomassie. Lanes 1 & 5 show feeding larval gut extracts and lane 6 shows wandering larvae without protease inhibitor. Lanes 2, 3 & 4 show feeding larvae gut extract with protease inhibitor at final concentrations of 10 µM, 25 µM and 50 µM, respectively. Protease activity bands are indicated 1-4.

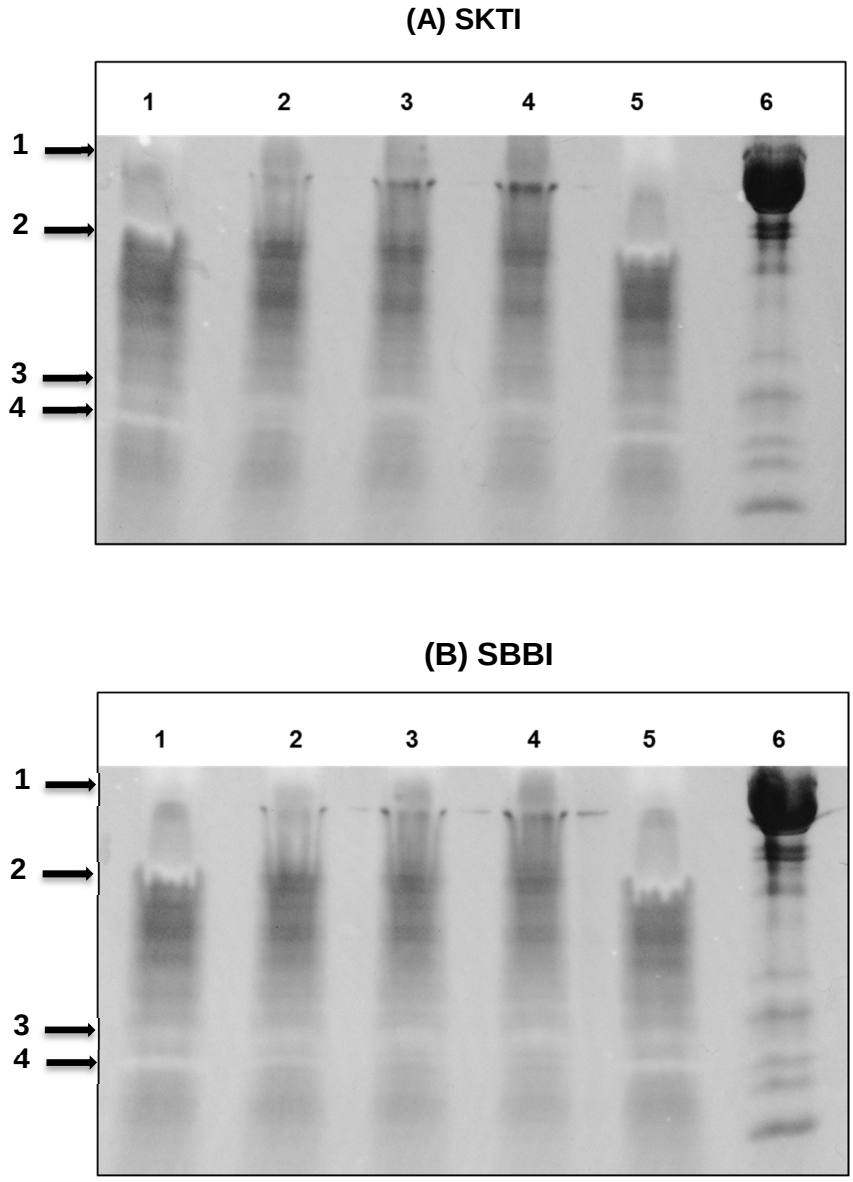


**Figure 5.4.** Casein substrate-based protease activity band profile of *Aethina tumida* feeding and wandering larvae incubated with (A) SKTI or (B) SBBI. A total of 15 µg of gut extract was treated with SKTI or SBBI and protease activity visualised by incubating in casein and staining with Coomassie. Lanes 1 & 5 show feeding larval gut extracts and lane 6 shows wandering larvae without protease inhibitor. Lanes 2, 3 & 4 show feeding larvae gut extract with protease inhibitor at final concentrations of 100 µM, 250 µM and 500 µM, respectively. Protease activity bands are indicated 1-4.

### 5.4 Analysis of Hv1a amino acid sequence using Peptide cutter

Analysis of the mature Hv1a sequence using PeptideCutter (http://web.expasy.org/peptide_cutter) indicated that it contained three cleavage sites for trypsin. Figure 5.5 shows the positions of the cleavage sites located towards the C-terminal of the sequence at amino acid 25, 34 and 35.

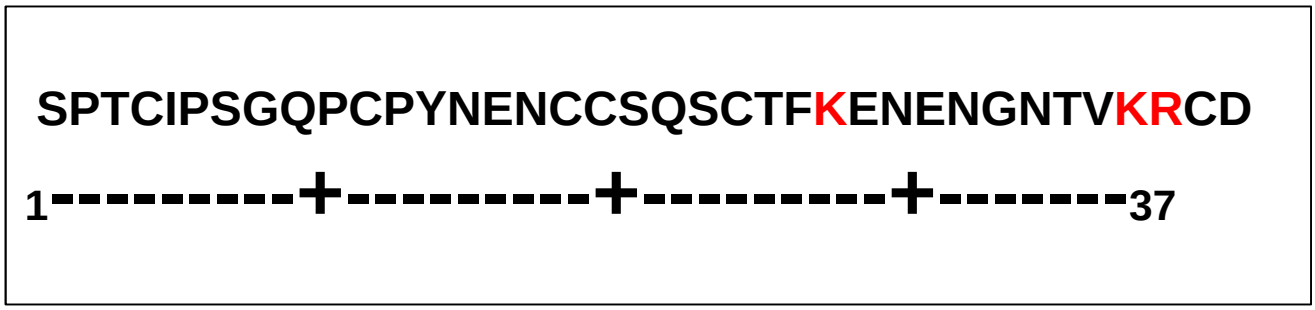


**Figure 5.5.** Mature Hv1a sequence showing the location of three trypsin cleavage sites indicated in red as predicted by PeptideCutter (http://web.expasy.org/peptide_cutter).

### 5.5 *In vitro* stability of GNA/Hv1a and Hv1a/GNA fusion proteins in feeding *Aethina tumida* larvae gut extracts with the addition of SKTI

The stability of GNA/Hv1a and Hv1a/GNA was assessed *in vitro* by incubating the equivalent of two larval guts (40 µl) with 75 µg of GNA/Hv1a or Hv1a/GNA. Protein content was estimated using Coomassie Plus (Bradford) Assay Kit using Bovine serum albumin as standards and for every 15 µg of gut extract present 500 µM SKTI was added. Samples were analysed for the presence of fusion proteins by western blotting, using anti-GNA antibodies. As shown in Figure 5.6 both GNA/Hv1a and Hv1a/GNA remain fully intact when incubated in the presence of SKTI at a concentration of 500 µM for 24 hr. Previous analysis (Chapter 4; Figure 4.14) showed that no intact GNA/Hv1a or Hv1a/GNA was detectable after incubation with gut extracts in the absence of the trypsin inhibitor under comparable conditions. Taken together these results strongly suggest that trypsin was responsible for the degradation of both GNA/Hv1a and Hv1a/GNA in the gut of *A. tumida* larvae.

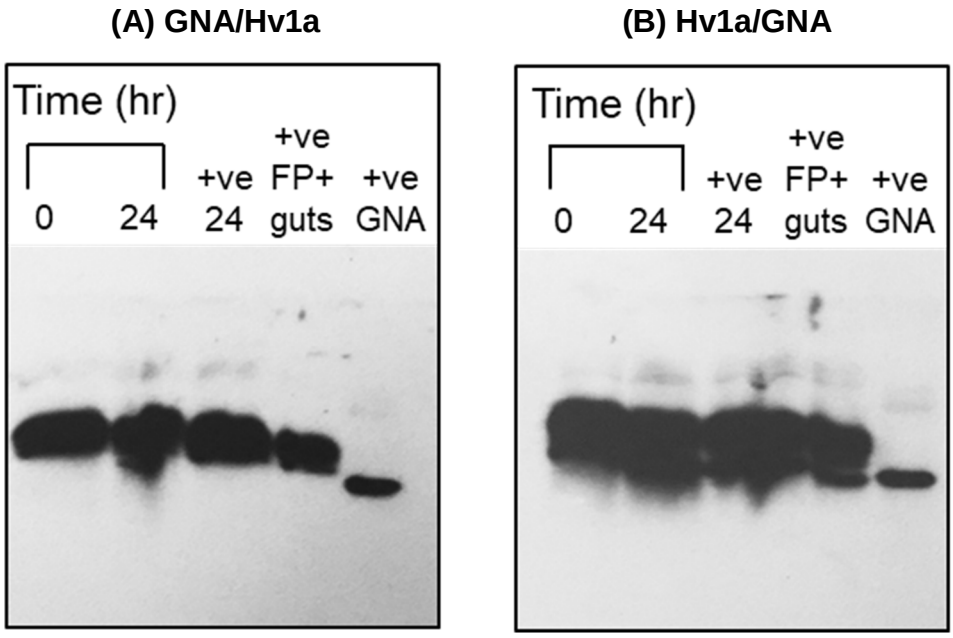


**Figure 5.6.** Western analysis (anti-GNA antibodies) of 5 day old *Aethina tumida* larval gut extracts incubated with SKTI and: (A) GNA/Hv1a or (B) Hv1a/GNA. Samples were taken at the indicated time points after incubation of 75 µg of the respective fusion proteins (FP) with 40 µl gut extract (equivalent of two larval guts). For each time point 300 ng of each FP was loaded. +ve 24 denotes FP incubated for 24 hr without gut extract, +ve FP +guts refers to a boiled gut sample incubated with FP and SKTI for 24 hr and +ve GNA represents 100 ng recombinant GNA standard.

## 5.6 Discussion

Insect pests have evolved highly efficient strategies to counteract the effects of PIs by up regulating the expression of proteases belonging to different classes (Terra and Cristofoletti 1996; Johnson and Rabosky 2000). It has been suggested that serine proteases were the basal digestion elements in primitive coleopterans, but that continuous exposure to diets rich in serine PIs has enabled diversification of catalytic mechanisms involved in protein proteolysis (Terra and Cristofoletti 1996; Johnson and Rabosky 2000). Serine proteases are known to be dominant in lepidopteran gut environments, contributing to 95 % of the total digestive activity, whereas coleopterans have a wider range of gut proteases, with serine, cysteine and metalloproteases being reported as the dominant protease types (Terra 1990; Terra and Ferreira 1994; Terra and Cristofoletti 1996; Mochizuki 1998; Johnson and Rabosky 2000; Castro-Guillen et al., 2012). For example, the alfafa weevil, *Hypera postica* (Elden, 1995; Wilhite et al., 2000); the black vine weevil, *Otirhynchu sulcatus* (Michaud et al., 1995) and the boll weevil, *Antonomus grandis* (Murdock et al., 1987), have slightly acidic midguts (approx. pH 6) and rely on cysteine proteases. In contrast other coleopterans such as *R. ferugineus* (Alarcon et al., 2002) and the citrus weevil, *Diaprepes abbreviates* (Yan et al., 1999) have alkaline midguts ranging between pH 9-10 and rely on serine proteases as the domain digestive enzymes. In other coleopterans several classes of proteases are responsible for proteolysis, for instance serine, cysteine and aspartyl proteases have been identified in the guts of the rice weevil, *Sitophilus oryzae* (Alfonso Rubi et la., 2003) and serine and cysteine proteases in the midgut of the weevil *Baris coerulescens* (Bondaé-Bottino et al., 1999) and the cabbage weevil, *Ceutorhynchus assimilis* (Girard et al., 1998); and serine and aspartyl proteases in a sugar beet weevil, *Aubeonymus mariaefranciscae* (Ortego et al., 1998). Furthermore, Bolter and Jongsma (1995) and Oppert et al. (2005) have shown that the coleopteran *L. decemlineata* and *T. castaneum*, who depend on cysteine proteases can express serine proteases when cysteine PIs are encountered, by-passing the attempts to inhibit its digestive processes. It is proposed that cysteine proteases could be used as an alternative way to cope with the presence of SPIs in the natural diet (Aguirre-Mancilla et al. 2014).

The zymography results reported here showed the presence of four activity bands, that were classified according to molecular masses into three separate groups: HMP (activity band 1; 200 kDa), MMP (activity band 2; 35 kDa) and LMP (activity band 3 & 4; 15-20 kDa). As previously mentioned that cysteine and serine proteases are two of the three dominant enzymes involved in protein digestion in coleopteran insects is well documented (Baker, 1982; Murdock et al., 1987; Terra and Ferreira, 1994; Terra and Cristofoletti, 1996; Oppert et al., 2003). Consequently, three proteases inhibitors, SKTI (trypsin-specific inhibitor) and SBBI (trypsin- and chymotrypsin-specific inhibitor); and E-64 (cysteine proteases inhibitor), were tested for their ability to inhibit proteolysis in *A. tumida* gut extracts. Of the four protease bands identified, none were inhibited with the addition of E-64. By contrast, the addition of 500 µM of SBBI and SKTI resulted 100 % reduction in activity of three out of the four activity bands. Although a single activity band was insensitive to SKTI and SBBI this does not rule out it being a chymotrypsin or trypsin-like proteases. The latter was evidenced by Chougule et al. (2008) whereby an activity band identified in *M. brassicae* gut extract showed no sensitivity towards SKTI or SBBI, however the addition of chymostatin (chymotrypsin inhibitor) caused complete inhibition of the activity band.

Different classes of enzymes are found in insect species with different gut pH. Insects with an alkaline midgut pH typically possess serine proteases like trypsin, chymotrypsin and elastase which perform optimally at neutral to high pH (Applebaum, 1985; Dow, 1986; Terra, 1990; McGhie et al., 1995). The gut pH of *A. tumida* has been determined to be approx. pH 7 and the results of the zymogram clearly showed that trypsin seems to be the dominant serine protease present in the gut of feeding *A. tumida* larvae, which is not surprising as serine proteases function optimally at neutral to alkaline pH. Our results agree with several studies where serine proteases have been reported to be the dominant enzyme class in the digestive system of several coleopterans. Thie & Houseman (1990), Oppert et al. (2006) and Hosseininaveh et al. (2007) have shown that serine proteinase activity was the prominent digestive enzyme in the gut of the yellow mealworm, *Tenebrio molitor*, larger black flour beetle, *Cynaeus angustus*, and khapra beetle, *Trogoderma granarium*, respectively. Other coleopterans such as *R. ferugineus* (Alarcon et al., 2002) and the citrus weevil, *Diaprepes abbreviates* (Yan et al., 1999) have also been shown to have an alkaline

midgut environment, also relying predominantly on serine proteases for protein digestion.

Herzig and King (2015) have shown that the Hv1a toxin is rapidly degraded at pH 9 or above, however it has been shown to be remarkably stable for 24 hr, with essentially no degradation, in buffers ranging between pH 1 to 7, suggesting that the pH within the gut of *A. tumida* feeding larvae is unlikely to contribute to the degradation of intact fusion protein.

Analysis of the Hv1a sequences using PeptideCutter confirmed the presence of three potential trypsin cleavage sites located towards the C-terminal of the sequence at amino acid 25, 34 and 35. To confirm trypsin was responsible for the proteolytic degradation of intact GNA/Hv1a and Hv1a/GNA, these fusion proteins were incubated in the presence of SKTI (trypsin-specific inhibitor) at a concentration of 500 µM for 24 hr. The results indicated that both fusion proteins remained fully intact when incubated with SKTI. In contrast, when incubated in the presence of gut proteins but in the absence of the trypsin inhibitor, no intact fusion protein only cleaved GNA was detectable over a similar 24 hr time period (Chapter 4; Figure 4.15). These results further demonstrate the role of trypsin in the proteolytic degradation of the Hv1a peptide and cleavage of the peptide from the carrier protein GNA.

The co-feeding of fusion proteins with PIs such as SKTI, holds enormous potential for hypothetically reducing proteolysis in the gut environment and thereby increasing levels of Hv1a toxin that can be delivered to the haemolymph of *A. tumida*. However, further research into this area is required to validate this assumption, particularly as it is known that insects such as *H. armigera* and *T. castaneum* larvae are capable of counteracting effects of PIs (Bown et al., 1998; Oppert et al., 2005) and *A. tumida* may well possess similar catalytic mechanism.

# CHAPTER 6 | EVALUATION OF SOYBEAN KUNITZ TRYPSIN INHIBITOR AS AN ALTERNATIVE CARRIER DOMAIN FOR INSECTICIDAL FUSION PROTEIN

## 6.1 Introduction

SKTI administered via artificial diet and transgenic plants, has been shown to have insecticidal effects against species belonging to the orders Coleoptera, Lepidoptera, Hemiptera and Orthoptera (Gatehouse et al., 1993; Johnson et al., 1995; Lee et al., 1999; McManus et al., 2005; Shukla et al., 2005). SKTI consists of 12 criss-crossing antiparallel β-strands proteins that are highly resistant to thermal and chemical denaturation, with two disulfide bridges involving Cys $^{39\text{-}86}$ and Cys $^{138\text{-}145}$ critical for the inhibitory function (Steiner et al., 1965; Lehle et al., 1994; Tetenbaum and Miller, 2001). SKTI's mechanism of action is thought to be attributed to the hypersecretion of digestive enzymes of the insect caused by their inhibition, which ultimately results in a decrease of essential amino acids available to the insect (Gatehouse et al., 1993). SKTI also affect water balance, the development of the insect and its enzymatic regulation (Boulter, 1993). Gatehouse et al. (1993) transformed tobacco plants with SKTI and demonstrated high growth inhibitory effects in *H. virescens* larvae. SKTI has also been transformed into rice plants and resulted in 40-60 % mortality of brown plant-hopper *Nilaparvata lugens*, (Lee et al., 1999).

The carrier protein GNA has been successfully used to transport insecticidal peptides into the circulatory system of an insect, allowing biologically active proteins to be converted into effective and orally active insecticides (Fitches et al., 2002; Fitches et al., 2004; Trung et al., 2006; Fitches et al., 2012; Yang et al., 2014). In chapter 4 (section 4.7.6) we demonstrated that GNA based fusion proteins were orally active towards *A. tumida*, however they were prone to cleavage in the gut after ingestion. Additionally, in Chapter 5 (section 5.5) we demonstrated that addition of SKTI prevented the cleavage of both GNA/Hv1a and Hv1a/GNA after 24 hr. Furthermore, Down et al. (1999) has shown evidence for transport of SKTI, whereby SKTI was detected in the hemolymph of *L. oleracea*, after oral administration of the protein in artificial diet. It was the findings in the afore-mentioned that formed the rational for evaluating SKTI potential as an alternative carrier protein to GNA for the delivery of Hv1a to the circulatory system of *A. tumida*. An initial construct was designed based on GNA/Hv1a, whereby the Hv1a coding sequence was linked to the C- terminus of SKTI nucleotide sequence by a 3-alanine linker region. Biological

activity of SKTI/Hv1a was assessed after injection into *A. tumida* larvae, with no effects on mortality being observed. It was speculated that the lack of insecticidal activity was attributed to the misfolding of the toxin during expression in the yeast cells. As such two additional fusion proteins were designed incorporating either a flexible (Gly-Gly-Gly-Gly-Ser motif) or rigid linker (Proline rich motif region) to improve protein folding and function (Sabourin et al., 2007). Inclusion of a flexible linker region did not result in a biologically active fusion protein. However, the incorporation of a rigid linker showed limited biological activity after injection into *A. tumida* larvae, at approx. 40 times the dose required to achieve similar levels of mortality after injection of GNA based fusion proteins.

## 6.2 Constructs encoding recombinant SKTI and fusion proteins SKTI/Hv1a, SKTI/Hv1a with Gly-Gly-Gly-Gly-Ser linker (EL) and SKTI/Hv1a with X2 proline rich domain (X2 PRD)

Codons were optimised for expression in *P. pastoris*, and the mature toxin Hv1a and/or mature SKTI carrier protein sequences subsequently ligated into pGAPZαB vector in frame with the α-factor secretory signal. The Hv1a toxin sequence was linked to the N- or C-terminal of mature SKTI nucleotide sequence. Three expression constructs were designed: Hv1a coding sequence was linked to the C- terminus of SKTI nucleotide sequence by a 3-alanine linker region (SKTI/Hv1a); flexible Gly-Gly-Gly-Gly-Ser linker (SKTI(EL)/Hv1a) (Sabourin et al. 2007) and rigid linker incorporating two repeats of a Proline rich motif (SKTI/Hv1a X2 PRD) (Bonning et al., 2014). A diagrammatic representation of sequenced expression constructs and deduced amino acid sequences are shown in Figures 6.1, 6.2, 6.3 & 6.4.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
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DNA:   ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT
+1FR:  ·T··E··D··E··T··A··Q··I··P··A··E··A··V··I··G··Y··S··D··L··E··G··D··F··D··V·
DNA:   GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT
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+1FR:  ·K··E··E··G··V··S··L··E··K··R··E··A··E··A··A··A··H··H··H··H··H··H··D··F··V·
DNA:   CTCGATAATGAAGGTAACCCTCTTGAAAATGGTGGCACATATTATATCTTGTCAGACATAACAGCATTTGGTGGA
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+1FR:  ·A··V··K··I··G··E··N··K··D··A··M··D··G··W··F··R··L··E··R··V··S··D··D··E··F·
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**(B)**

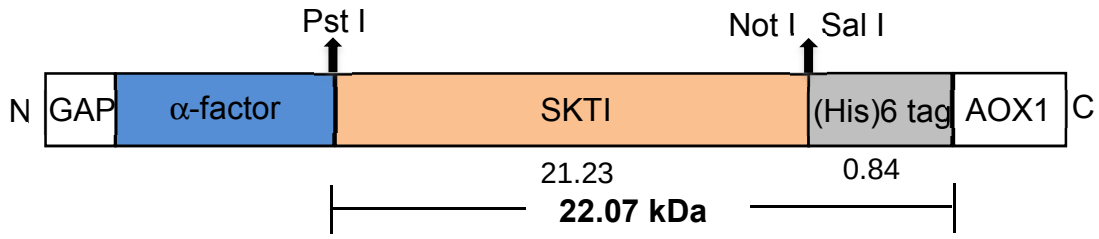


**Figure 6.1.** SKTI expression construct in pGAPZαB. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. The SKTI and C-terminal histidine tag and stop codon are depicted in orange and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
+1FR:  ·M··R··F··P··S··I··F··T··A··V··L··F··A··A··S··S··A··L··A··A··P··V··N··T··T·
DNA:   ACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTT
+1FR:  ·T··E··D··E··T··A··Q··I··P··A··E··A··V··I··G··Y··S··D··L··E··G··D··F··D··V·
DNA:   GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT
+1FR:  ·A··V··L··P··F··S··N··S··T··N··N··G··L··L··F··I··N··T··T··I··A··S··I··A··A·
DNA:   AAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTGCAGCACATCATCATCATCATCATGATTTCGTG
+1FR:  ·K··E··E··G··V··S··L··E··K··R··E··A··E··A··A··A··H··H··H··H··H··H··D··F··V·
DNA:   CTCGATAATGAAGGTAACCCTCTTGAAAATGGTGGCACATATTATATCTTGTCAGACATAACAGCATTTGGTGGA
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+1FR:  ·I··R··A··A··P··T··G··N··E··R··C··P··L··T··V··V··Q··S··R··N··E··L··D··K··G·
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DNA:   CATGATGATGGAACCAGGCGTTTGGTGGTGTCTAAGAACAAACCGTTAGTGGTTCAGTTTCAAAAACTTGATAAA
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DNA:   GATTGAGTCGAC
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**(B)**

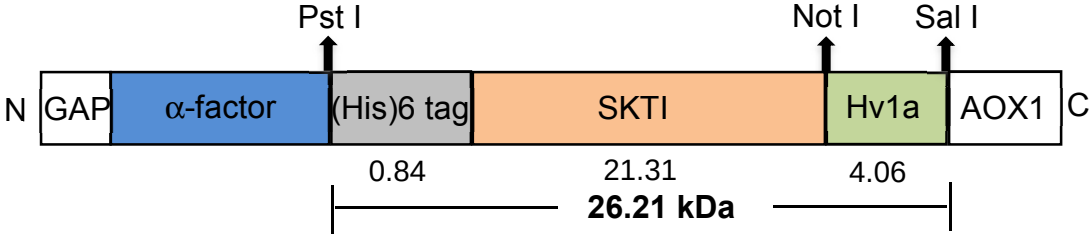


**Figure 6.2.** SKTI/Hv1a expression construct in pGAPZαB. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites. SKTI, Hv1a and N-terminal histidine tag and C-terminal stop codon are depicted in orange, green and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
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DNA:   GCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCT
+1FR:  ·A··V··L··P··F··S··N··S··T··N··N··G··L··L··F··I··N··T··T··I··A··S··I··A··A·
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**(B)**

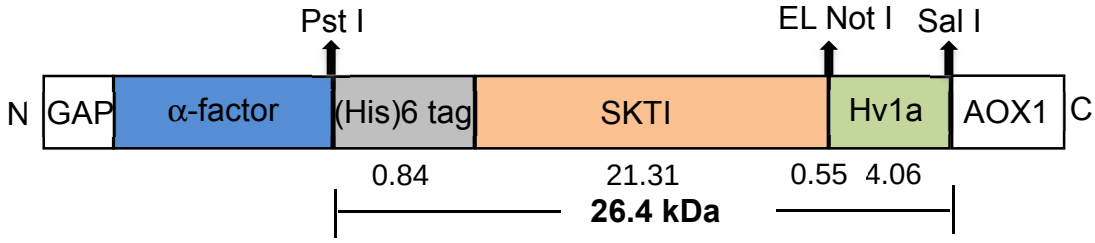


**Figure 6.3.** SKTI/Hv1a with Gly-Gly-Gly-Gly-Ser (EL) linker extension expression construct in pGAPZαB. (A) DNA, deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites and pink denotes the Gly-Gly-Gly-Gly-Ser extension linker. SKTI, Hv1a and N-terminal histidine tag and C-terminal stop codon are depicted in orange, green and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

**(A)**

```
DNA:   ATGAGATTTCCTTCAATTTTTACTGCTGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACA
+1FR:  ·M··R··F··P··S··I··F··T··A··V··L··F··A··A··S··S··A··L··A··A··P··V··N··T··T·
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+1FR:  ·I··G··T··I··I··S··S··P··Y··R··I··R··F··I··A··E··G··H··P··L··S··L··K··F··D·
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+1FR:  ·S··F··A··V··I··M··L··C··V··G··I··P··T··E··W··S··V··V··E··D··L··P··E··G··P·
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+1FR:  ·P··G··P··Q··P··P··P··P··P··P··S··P··T··P··V··G··G··A··A··G··G··D··D··A·
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+1FR:  ·A··A··G··S··P··T··C··I··P··S··G··Q··P··C··P··Y··N··E··N··C··C··S··Q··S··C·
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**(B)**

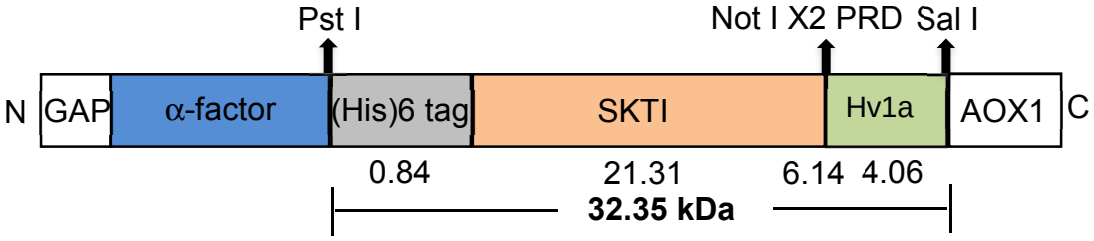



**Figure 6.4.** SKTI/Hv1a with X2 Proline rich motif (X2 PRD) linker extension expression construct in pGAPZαB. (A) DNA and deduced amino acid sequence and (B) diagrammatic representation. The α-factor prepro sequence is indicated in blue. Purple indicates *Pst I*, *Not I* and *Sal I* restriction sites and pink denotes the X2 PRD extension linker. SKTI, Hv1a and N-terminal histidine tag and C-terminal stop codon are depicted in orange, green and grey, respectively. The position of the pGAPZαB N-terminal GAP promotor sequence and C-terminal AOX1 transcription termination region are shown.

## 6.3 Expression and purification of SKTI/Hv1a, SKTI/Hv1a with Gly-Gly-Gly-Gly-Ser linker (EL) and SKTI/Hv1a with X2 proline rich domain (PRD)

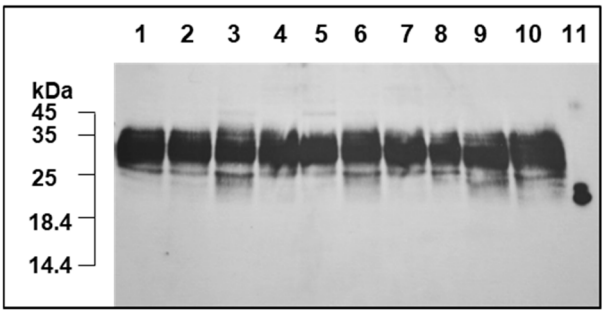
Sequence confirmed plasmid DNA encoding fusion proteins were linearised with *Bln*I and transformed in to SMD1168H (protease deficient strain) of *P. pastoris*, using Zeocin anti-biotic for selection. Positive transformants were inoculated in 10 ml YPG cultures with Zeocin and grown for 48 hr at 30°C. Culture supernatants (20 µl) were analysed for expression of fusion proteins by Western blotting, using anti-SKTI antibodies.

SKTI/Hv1a and SKTI(EL)/Hv1a expression screens showed the presence of a single immunoreactive band of approx. 30 kDa (Figure 6.5). The predicted mass for SKTI/Hv1a and SKTI(EL)/Hv1a is approx. 25.9 and 26.4 kDa, respectively. Results for expression screen analysis of SKTI/Hv1a X2 PRD are shown in Figure 6.5. The predicted mass of SKTI/Hv1a X2 PRD is approx. 32.35 kDa, however a single immunoreactive band of approx. 40 kDa was detected. The presence of immunoreactive bands detected at the incorrect Mw could suggest that glycosylation of the recombinant proteins had occurred, as is commonly observed for proteins expressed in *P. pastoris* (Macauley-Patrick et al., 2005). Alternatively, this could be due to the high number of cysteine residues making separation by SDS-PAGE less efficient. The presence of a single band for all constructs suggests minimal cleavage of the expressed fusion proteins. The best expressing clones for all recombinant proteins were selected for large-scale protein production by bench top fermentation. Fermentation was carried out in a 5 L bioreactor under controlled environmental conditions (Chapter 2, section 2.4.5). The pGAPZ alpha factor secretory signal simplifies the purification process as it directs the secretion of expressed proteins out of the yeast cells and into the growth media, so that proteins can be purified directly from the fermented culture supernatant. Supernatant was obtained via centrifugation and passed through a series of filters and subsequently purified by nickel affinity chromatography (Chapter 2, section 2.4.6). Eluted peaks containing target proteins were diluted 50:50 with deionised water and subsequently de-salted by dialysis and freeze-dried. The proportion of fusion protein in lyophilised samples was estimated semi-quantitatively using commercial SKTI standards on SDS-PAGE gels (Figure 6.6).

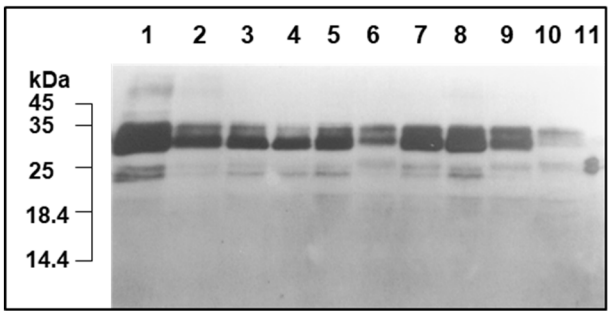
Separation of purified SKTI/Hv1a and SKTI(EL)/Hvla by SDS-PAGE showed the presence of a protein corresponding to the predicted molecular weight of approx. 27

kDa, with an additional protein of lower mass of 20 kDa corresponding to SKTI standards, indicating a degree of cleavage of the fusion protein (Figure 6.6 A & B). These results were in contrast to small scale cultures screens where a slightly larger immunoreactive protein of approx. 30 kDa was detected. Analysis of purified SKTI/Hv1a X2 PRD showed the presence of proteins of approx. 40 kDa in gels stained for total proteins (Figure 6.6 C), which was comparable to the molecular mass of the immunoreactive proteins in small scale cultures of clones transformed with SKTI/Hv1a X2 PRD, suggesting that glycosylation of the recombinant protein had occurred. Furthermore, separation of purified SKTI/Hv1a X2 PRD showed the presence of a lower protein mass of 20 kDa corresponding to SKTI standards, indicating a degree of cleavage of the fusion protein. The expression level of SKTI/Hv1a and SKTI(EL)/Hv1a was approx. 60 mg/L; and SKTI/Hv1a X2 PRD was approx. 80 mg/L respectively, estimated by semi-quantitative SDS-PAGE.

**(A) SKTI/Hv1a**




**(B) SKTI(EL)/Hv1a**




**(C) SKTI/Hv1a X2 PRD**

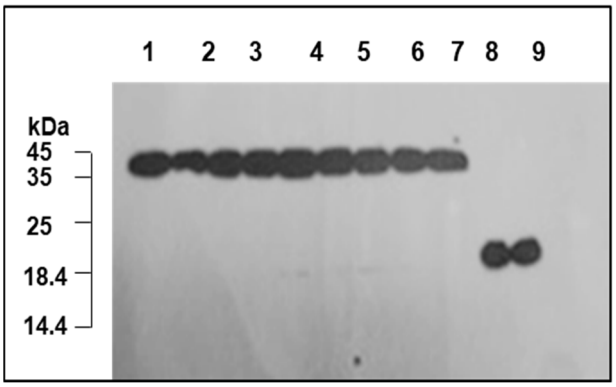
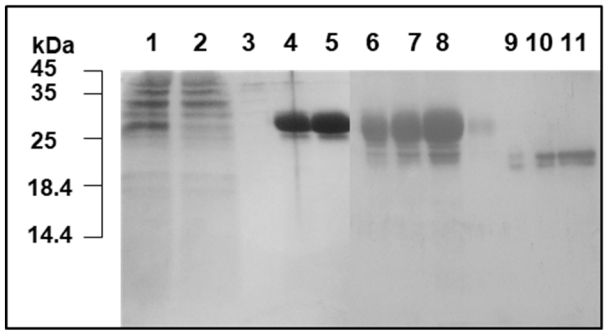
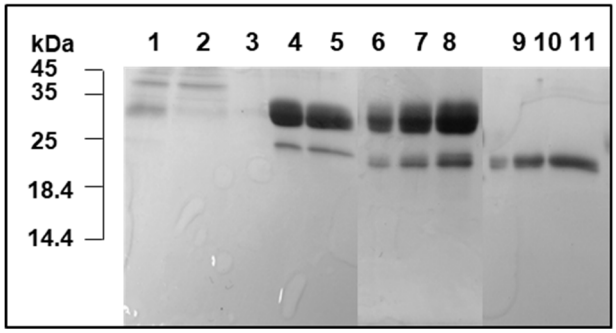



**Figure 6.5.** Western blot screening of culture supernatants collected from clones expressing recombinant SKTI/Hv1a, SKTI/Hv1a with Gly-Gly-Gly-Gly-Ser (EL) linker extension and SKTI/Hv1a with X2 Proline rich motif (X2 PRD) fusion proteins from shake-flask yeast cultures using anti-SKTI antibodies: (A) SKTI/Hv1a and (B) SKTI(EL)/Hv1a. Lanes 1 to 10 show SMD clones 1-10; lanes 11 show 25 ng SKTI standards. (C) SKTI/Hv1a X2 PRD. Lanes 1 to 7 show SMD clones 1-7; lanes 8 & 9 show 25 and 50 ng SKTI standards 10 µl culture supernatants loaded. Mw standards (kDa) based on Ponceau S staining are indicated.

**(A) SKTI/Hv1a**




**(B) SKTI(EL)/Hv1a**




**(C) SKTI/Hv1a X2 PRD**

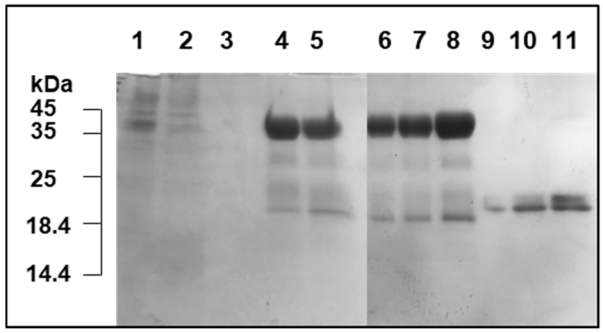



**Figure 6.6.** SDS-PAGE analysis of purified: (A) SKTI/Hv1a, (B) SKTI/Hv1a with Gly-Gly-Gly-Gly-Ser (EL) linker extension and (C) SKTI/Hv1a with X2 Proline rich motif (X2 PRD) linker extension. Lanes 1, 2 & 3, shows a respective 20 µl load of culture supernatant, culture supernatant after being passed through the column and 10 mM imidazole wash; lane 4 indicates the peak fraction and lane 5 shows the collective purified protein; lane 6, 7 & 8 shows 5, 10 & 25 µg of purified fusion protein, respectively and lanes 9 to 11 show respectively 0.5, 2 & 5 µg SKTI standards.

### 6.4 Detection of ingested recombinant SKTI in *Aethina tumida* larvae

To investigate if recombinant SKTI was resistant to proteolytic degradation in the diet and within the gut of *A. tumida* larvae and subsequently also able cross the gut epithelium, haemolymph and gut samples were extracted from insects fed on artificial diet containing recombinant SKTI. Larvae (n=10 per treatment) were allowed to feed *ad libitum* on artificial diet containing SKTI at 5000 ppm for 24 hr. All samples were analysed via western blotting, using anti-SKTI antibodies.

Analysis of 100 ng of diet containing recombinant SKTI showed the presence of an immunoreactive protein of a similar mass to standard SKTI, suggesting SKTI was not subjected to proteolysis after 24 hr (Figure 6.7). Homogenised gut (including gut content) samples showed the presence of recombinant SKTI after 24 hr, with no corresponding immunoreactive band being detected in the control samples. Transport of recombinant SKTI into the haemolymph was evident from the presence of an immunoreactive band corresponding to the size of recombinant SKTI, which was absent in the negative control haemolymph. These results suggest that SKTI could be used as an alternative carrier protein to transport attached toxins to the circulatory system of *A. tumida*.

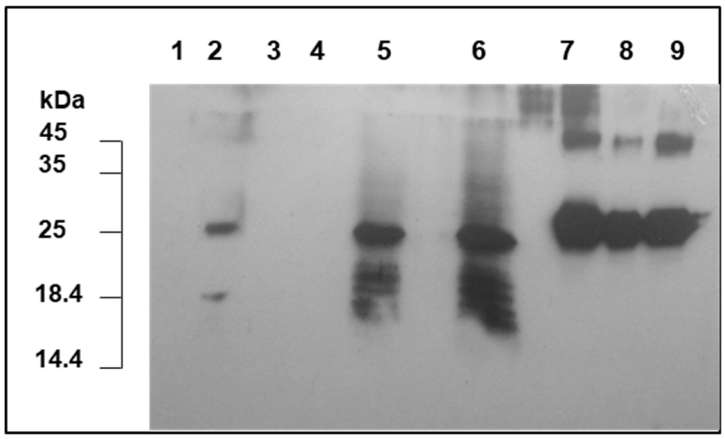



**Figure 6.7**. Western analysis (anti-SKTI antibodies) of 7 day old *Aethina tumida* larval haemolymph and gut samples after feeding on artificial diet containing 5000 ppm recombinant SKTI for 24 hr: lanes 1 & 2 show haemolymph (10 µl) of control and SKTI fed larvae respectively; lane 3 & 4, are replicate control, and 5 & 6 are replicate SKTI gut samples (n=5 guts per sample), approx. 40 µg total protein loaded; lane 7 represents a loading of 100 ng of artificial diet containing SKTI and lane 8 & 9 represents 50 and 100 ng recombinant SKTI standard. Mw standards (kDa) based on Ponceau S staining are indicated.

## 6.5 Biological activity of SKTI/Hv1a, SKTI(EL)/Hv1a and SKTI/Hv1a X2 PRD fusion proteins against *Aethina tumida* wandering larvae after injection

The biological activity of SKTI/Hv1a, SKTI(EL)/Hv1a and SKTI/Hv1a X2 PRD was assessed by injection of wandering *A. tumida* larvae (n=10 per treatment; average weight 17.46 mg) at a dose of 20 µg; this was 10 times the dose required to achieve 100 % mortality after injection with GNA/Hv1a, Hv1a/GNA fusion proteins and K>Q variants (Chapter 4; Figure 4.6). Recombinant SKTI was injected at a dose of 40 µg and SP buffer solution serving as a negative control. Survival was monitored daily for 7 days.

Larvae injected with 40 µg of recombinant SKTI showed a 20 % decline in survival after 7 days, whereas no mortality was recorded for larvae injected with SKTI/Hv1a and SKTI(EL)/Hv1a. In contrast, injection of SKTI/Hv1a X2 PRD at a dose of 20 µg showed a decline in survival 2 days post injection, with 70 % mortality being recorded ($P<0.01$ Mantel-Cox log-rank test). As such wandering larvae were injected with doses ranging from 2.5 to 10 µg of fusion protein, however no mortality was recorded over the 7 day bioassays period (Figure 6.8). Interestingly, all larvae injected with recombinant SKTI, SKTI/Hv1a, SKTI(EL)/Hv1a SKTI/Hv1a X2 PRD fusion proteins showed cuticle mottling 6 days post injection suggesting that the SKTI component was functional and had activated the melanization cascade (Figure 6.9).

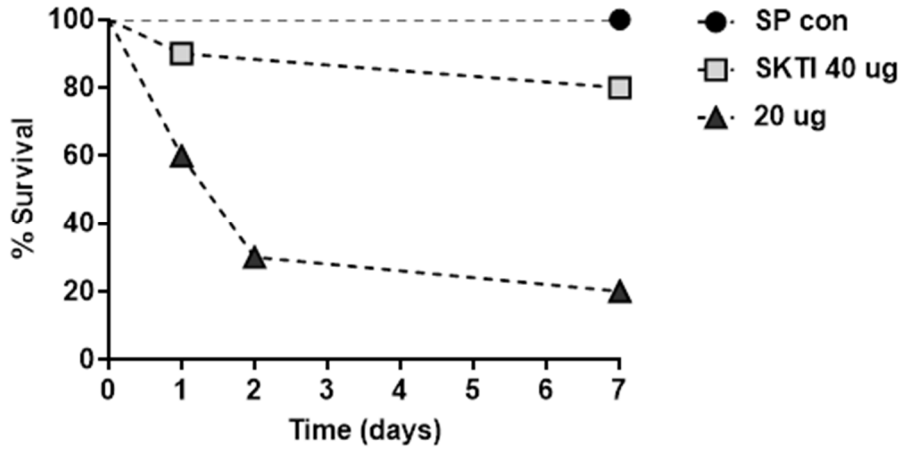



**Figure 6.8**. Survival of *Aethina tumida* wandering larvae injected with 40 µg of recombinant SKTI and 20 µg of SKTI/Hv1a X 2 PRD. Proteins in all cases were re-suspended in sodium phosphate (SP) buffer. Injection volume was 1 µl. SP solution (SP Con) served as negative control. N=10 per treatment.

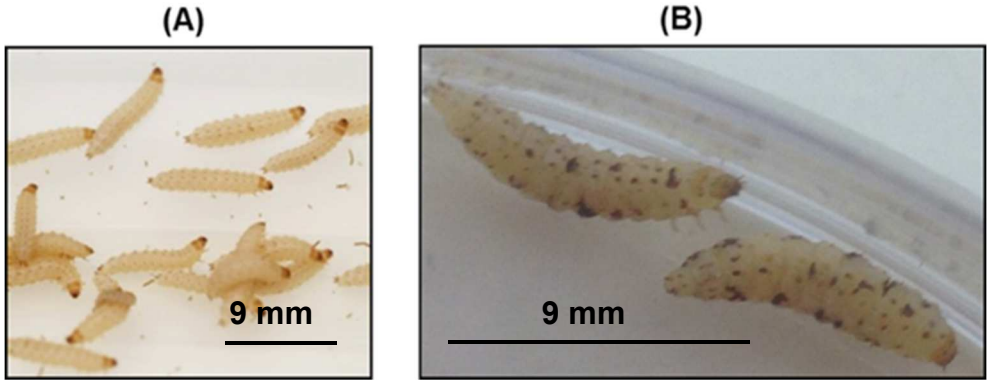



**Figure 6.9.** *Aethina tumida* six days post injection: (A) Controls injected with sodium phosphate buffer and (B) 20 µg of SKTI/Hvla X2 PRD. Scale bars are indicated for the larvae.

### 6.6 Oral delivery of recombinant SKTI and SKTI/Hv1a X2 PRD to *Aethina tumida* feeding larvae via drinking bioassays

Five day old larvae (n= 20 per treatment) were supplied with SP/sucrose solution (13 % v/w) containing recombinant SKTI or SKTI/Hv1a X2 PRD at a final concentration of 10 mg/ml. Negative control larvae were fed on SP/sucrose solution without recombinant proteins. The diet was changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial diet until they entered the wandering stage (i.e. approx. 4 days post liquid feed). As shown in Figure 6.10 no mortality was recorded in the control and SKTI treatment 7 days post feeding, whereas larvae treated with SKTI/Hv1a X2 PRD, showed a 20 % decline in survival after 2 days, with no further reduction in survival observed 7 days post feeding ($P<0.05$; Mantel-Cox log-rank test). The results indicate a significant reduction in the biological activity of Hv1a when linked to the carrier protein SKTI, as 32 times less GNA/Hv1a achieved similar levels of mortality (Chapter 4, section 4.7.3).

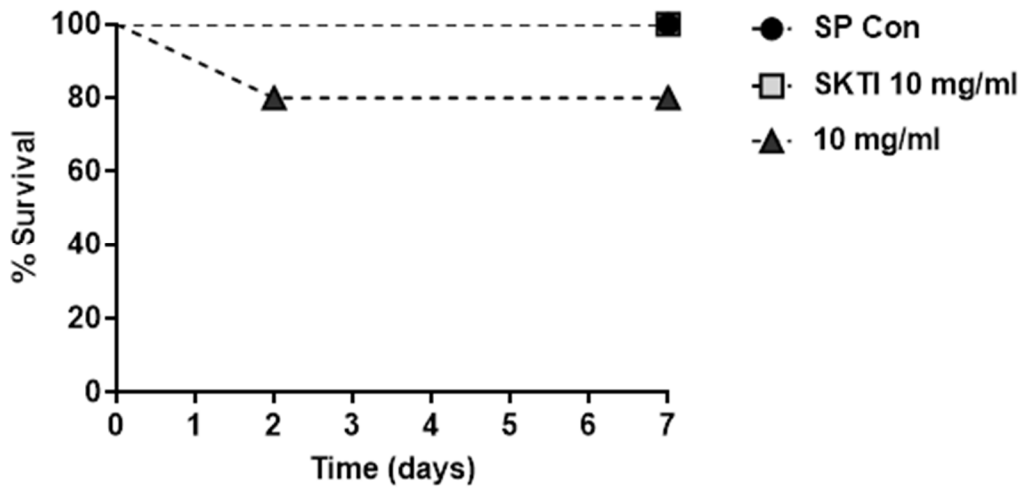



**Figure 6.10.** Survival of *Aethina tumida* 5 day old larvae fed on 10 mg/ml of recombinant SKTI and SKTI/Hv1a X2 PRD. Sodium phosphate (SP Con) buffer served as negative control. N= 20 per treatment. The diet was changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial diet until they entered the wandering stage.

## 6.7 Stability of SKTI/Hv1a X2 PRD within the gut of 5 day old *Aethina tumida* larvae

The presence of SKTI/Hv1a X2 PRD in haemolymph (n=20) and gut samples extracted from larvae (n=5 per replicate) were analysed by western blotting using anti-SKTI antibodies. Figure 6.11 shows western analysis results of haemolymph and gut samples 24 hr post ingestion of SKTI/Hv1a X2 PRD. Analysis of all gut samples after 24 hr confirmed that the fusion protein was cleaved after 24 hr, which was evident by the presence of a single immunoreactive band corresponding to the size of recombinant SKTI, which was absent in the control samples. Western analysis showed evidence for transport of SKTI across the gut epithelium, since SKTI was present in the haemolymph samples 24 hr after feeding.

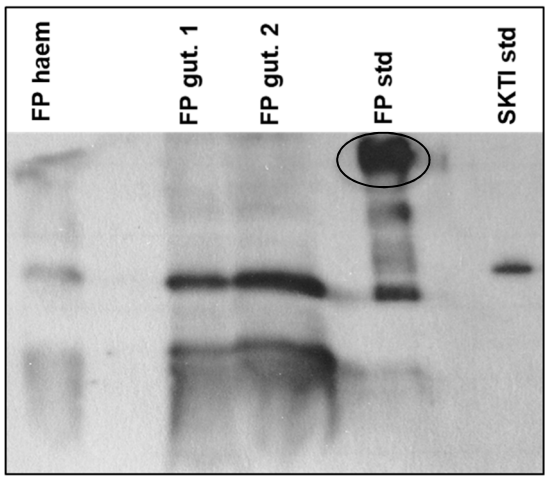



**Figure 6.11.** Western analysis (anti-SKTI antibodies) of 5 day old *Aethina tumida* larval haemolymph and gut samples after feeding on sodium phosphate buffer (SP)/sucrose solution (13 % v/w) containing SKTI/Hv1a X2 PRD at a concentration of 2.5 mg/ml for 24 hr. Fusion protein (FP) haemolymph and gut samples (two replicates depicted as R1 and R2) 10 µl and 10 µg total protein was loaded, respectively. Standards are 25 ng of SKTI/Hv1a X2 PRD (FP std), with the circle indicates intact fusion protein, and commercial SKTI standards (SKTI std).

## 6.8 Discussion

SKTI was selected as a potential alternative carrier to GNA in a fusion-protein based approach. SKTI was successfully produced in *P. pastoris*, with yields of 80 mg/l of culture supernatant being obtained. The potential of SKTI to act as an alternative carrier was demonstrated by the presence of this protein in the haemolymph after ingestion by *A. tumida* larvae, suggesting transport of SKTI across gut epithelium. The ability to transport across gut epithelium requires the ingested protein to be resistance to proteolytic degradation, which like GNA was the case for SKTI. Furthermore, the ability of SKTI to transport into the haemolymph requires a biologically active protein as denatured proteins do not have this ability (Fitches et al., 2001). However, the amount of SKTI detected in *A. tumida* guts and haemolymph samples was approx. 4 and 10 times less, respectively, for SKTI as compared to GNA (Chapter 4, section 4.6). Transport studies using GNA showed an excess of 100 ng of recombinant GNA in both haemolymph and gut samples (including gut content). The reduced levels of SKTI observed in gut samples, could be a consequence of larvae not consuming as much of the diet as compared to GNA fed larvae. It has been suggested that transport across the gut epithelium into the haemolymph can be hindered by the large molecular size of a protein, affecting transport based on passive diffusion or mediated by active cellular processes (Fitches et al., 2008). SKTI is a 21.5 kDa monomeric protein whereas GNA is a 50 kDa tetrameric protein and therefore transport into the haemolymph would not have been affected by the size of SKTI. Overall, this data suggested that like GNA, SKTI has the potential to act as a carrier protein for the delivery of Hv1a to the circulatory system of *A. tumida*.

The insecticidal activity of an injected and orally ingested fusion protein requires both lectin functionality, for transport and toxin functionality for insecticidal activity. Injection of *A. tumida* larvae with SKTI/Hv1a at a dose of 20 µg failed to induce any mortality, however cuticle mottling was evident 6 days post injection. This provided further evidence for functionality of the SKTI component, as the observed cuticle mottling suggested that SKTI had proteolytically cleaved the phenoloxidase enzyme which is key to the melanisation cascade, thereby activating this cascade, causing unwanted melanisation of the cuticle (Ashida and Dohke, 1980). It was evident from the lack of mortality after injection of 20 µg of SKTI/Hv1a that the Hv1a peptide was not functional, as by comparison injection of GNA based fusion proteins at a

concentration of 2 µg (10-fold reduction in dose) resulted in 100 % mortality after 7 day. Fitches et al., (2008) showed that GNA/ButaIT was toxic to *M. brassicae* after injection and when fed, had negative effects on larval survival and growth. However, when ASAII, a similar lectin derived from the garlic bulb, was linked to ButaIT peptide no effects were observed after injection and oral delivery of this fusion protein towards *M. brassicae*. The author hypothesised that the lack of insecticidal activity may be a consequence of the ButaIT part of the fusion protein not folding correctly or may be constrained by the lectin domain so that it is unable to adopt the correct conformation to interact with its normal target (Fitches et al., 2008).

Consequently, the use of naturally occurring linker regions was investigated as linker regions serve to connect protein moieties maintaining functions such as cooperative inter-domain interactions or preserving biological activity (Gokhale and Khosla, 2000; Ikebe et al., 1998). The incorporation of linker regions in some cases, has led to the successful production of a fusion protein by providing an appropriate distance between the two domains thereby reducing their interaction and restoring/improving protein folding, ultimately maintaining biological activity (Bai et al., 2005; Zhang et al., 2009). Flexible linkers are usually used when joined domains require a degree of movement or interaction and have been shown to increase stability and folding (Argos, 1990; Chen et al., 2013). The most common flexible linker used in structural studies is Gly-Gly-Gly-Gly-Ser, with varying repeats of the motif (Klein et al., 2014). Sabourin et al. (2007) placed flexible Gly linkers between the epitope and tagged protein, which resulted in increased sensitivity and accessibility of the epitope without compromising protein folding and function. As such a second fusion protein construct was designed using a single Gly-Gly-Gly-Gly-Ser (EL) motif. Analysis of the purified fusion protein showed the presence of a protein corresponding to the predicted molecular weight of approx. 27 kDa. However, like SKTI/Hv1a, injection of SKTI(EL)/Hv1a at a dose of 20 µg failed to induce any larval mortality, suggesting the incorporation of the linker region did not prevent the interaction of the SKTI domain with Hv1a peptide, enabling correct folding of this peptide.

Proline amino acids are common to many naturally derived interdomain linkers, and proline rich sequences tend to form rigid linkers, preventing unfavourable interaction between the domains and allowing the protein domains to function independently (Radford et al., 1987; Williamson, 1994). Rigid linkers have successfully

been applied to fusion proteins, where flexible linkers have failed, as they have been able to maintain the distance and reduce/prevent the interference between the proteins domains (Chen et al., 2013). Bonning et al., (2014) successfully used an aphid plant luteovirus coat protein to deliver Hv1a to four aphid species, by inserting a Proline rich motif spacer between the C-terminus coat protein read-through domain and the N-terminus of Hv1a, allowing them to fold correctly, retain solubility and function. Furthermore, it has been demonstrated that adjusting the copy number of a linker region allows for the optimum separation of the functional domains and/or for the necessary inter-domain interaction (Chen et al., 2013). Finally, a third fusion protein incorporating two repeats of the Proline motif (X2 PRD), mentioned above, was inserted between SKTI carrier protein and Hv1a peptide. Analysis of purified SKTI/Hv1a X2 PRD showed the presence of proteins of approx. 40 kDa, suggesting the fusion protein was heavily glycosylated or the α-factor prepropeptide was not completely cleaved from the expressed protein prior to leaving the yeast cell (Brakes, 1990). Injection of SKTI/Hv1a X2 PRD at a dose of 20 µg showed a significant 70 % decline in 2 days post injection. However, further injections with lower doses (2.5 to 10 µg protein) did not result in reduced survival. In contrast, 100 % mortality was recorded against *A. tumida* larvae after injection of 2 µg GNA based fusion proteins (Chapter 4, section 4.4).

Oral delivery of SKTI/Hv1a X2 PRD at a dose of 10 mg/ml, with diet being changed every 24 hr for 72 hr and thereafter larvae were supplied with artificial diet until they entered the wandering stage, resulted in a 20 % decline in larval survival over 7 days. The dose of SKTI/Hv1a X2 PRD administered in the drinking assays was 32 times the dose required to achieve similar levels of mortality in larvae fed on solutions containing GNA/Hv1a in comparable assays. Western blotting experiments to investigate if intact fusion protein was present in haemolymph of larvae fed for 24 hr on fusion protein solutions were carried out. The results showed only the presence of SKTI in both haemolymph and gut samples for SKTI/Hv1a X2 PRD. Ozawa and Laskowski (1966) have reported that the interaction between trypsin and SKTI results in the cleavage of the peptide bond between Arg64 and Ile65 in the reactive site of SKTI, resulting in two peptides of approx. 6.8 kDa and 15.1 kDa, respectively. However, the results of the western blotting of ingested SKTI/Hv1a X2 PRD, indicated the presence of 20 kDa immunoreactive band corresponding to the size of intact SKTI. Trypsin proteases have

been identified in the gut of *A. tumida* larvae (Chapter 5, section 5.3) and it is possible these proteases are assisting in the cleavage of the Hv1a peptide from the carrier protein SKTI. No further stability assays were carried out, due to the poor oral toxicity of SKTI/Hv1a X2 PRD towards *A. tumida* larvae.

SKTI/Hv1a X2 PRD showed limited biological activity after injection requiring approx. 40 times the dose to achieve similar levels of mortality compared to GNA based fusion proteins. The suitability of GNA as a carrier protein, in contrast to SKTI, is two-fold. It is thought that GNA assists in toxin folding during production leading to the generation of a biologically active fusion protein (Fitches pers com, 2018). SKTI consisting of 12 criss-crossing antiparallel β-strands proteins linked by long loops, with two disulfide bridges involving Cys $^{39\text{-}86}$ and Cys $^{138\text{-}145}$ (Steiner et al., 1965; Lehle et al., 1994; Tetenbaum and Miller, 2001). Song and Suh et al. (1998) suggested that these disulfide bonds are responsible for reducing the flexibility of the loop region by cross-linking them. It is possible the presence and function of the two disulfide bonds, which are absent in GNA, are responsible for SKTI's inability to assist in toxin folding during production. SKTI acts by inhibiting the gut proteases of insects by irreversibly binding tightly to the active sites preventing utilisation of the ingested protein and consequently resulting in amino acid deficiency which affects growth, development and survival (Ryan 1990; Richardson, 1991; Gatehouse et al., 1993; Solomon et al., 1999; Carlini and Grossi-de-Sá, 2002). It is possible that SKTI's ability to bind irreversibly to active site of the protease in a substrate-like manner in the gut of insects, prevented the transport of Hv1a toxin to the haemolymph of *A. tumida*, therefore preventing SKTI/Hv1a fusion proteins from being biologically active. Fitches et al. (2012) showed that GNA may act as an anchor, by binding to the nerve cord of *M. brassicae* increasing the localisation of the Hv1a peptide resulting in a higher efficacy. It is possible that the absence of biological activity of the SKTI/Hv1a fusion proteins variants may well be a consequence of SKTI's affinity for proteolytically cleaving the phenoloxidase enzyme which is key to the melanisation cascade, therefore diverting the Hv1a peptide from the CNS in *A. tumida* and preventing the localisation of the toxin. The co-feeding of GNA/Hv1a with SKTI, holds enormous potential for hypothetically reducing proteolysis in the gut environment (Chapter 5; Figure 5.6) and thereby increasing levels of Hv1a toxin that can be delivered to the haemolymph of *A. tumida*.

The data presented in this Chapter clearly showed that fusion to SKTI, even with the incorporation of a natural rigid linker, significantly reduced the biological activity of the fused recombinant toxin. Future work to investigate the potential use of alternative carrier proteins requires careful consideration of the factors that determine and maintain toxicity of the attached peptide. Alternative, natural linkers such as hydroxylamine-rich cellulase and xylanase linkers could be considered as they maintain an extended conformation and are protected from proteolysis because of the O-glycosylation, promoting protein folding and ultimately enhancing biological activity (Rizk et al., 2012).

# CHAPTER 7 | GENERAL DISCUSSION

## 7.1 Introduction

There is a pressing need to develop effective control options for *A. tumida* that pose little to no hazards to honey bees. Current control measures used against *A. tumida* are inadequate, suffering variability in levels of pest control. Furthermore, *A. tumida* control largely relies on the use of in-hive organophosphate, CheckMite + Strips™ (10 % w/w Coumaphos) used in conjunction with GardStar7 (40 % permethrin), a soil treatment product (Delaplane, 1998; Hood and Miler, 2003). Organophosphates are highly toxic to bees, wildlife and humans (Carson, 1962) and hence all hive honey combs have to be removed prior to treatment. Additionally, the continued use of pyrethroids such as permethrin can give rise to resistance, and upon contact, is deleterious to honey bees (De Guzman et al., 2011). Effective pest control in the UK is coming under increased pressure due to the loss of effective chemical control options resulting from withdrawals arising from EU Directive 91/414, therefore limiting the range of products that could be employed in the event of *A. tumida* outbreak (EFSA, 2015). Given these issues alternative control strategies are urgently required. In this thesis the potential use of RNAi and fusion protein technology as target specific and effective novel strategies for the control of *A. tumida* was assessed in quarantine laboratory conditions.

## 7.2 RNAi-mediated control of *Aethina tumida*

RNAi is a post translational gene silencing phenomenon mediated by exogenous or endogenous dsRNA. The RNAi pathway is a well-conserved mechanism in insects and holds immense potential as an alternative control method for insect pests (Hannon, 2002). RNAi efficacy is generally high in coleopterans, with systemic RNAi responses being documented in most reported coleopteran studies (Huvenne and Smagghe, 2010; Scott et al., 2013). Consequently, the use of RNAi as an alternative control method for *A. tumida* was investigated in Chapter 3. *Laccase* 2 and *V-ATPase subunit A* were selected as target genes on the basis of previous successful RNAi studies against coleopteran insect pests (Arakane et al., 2005; Baum et al., 2007; Forgac, 2007; Nui et al., 2008; Laudani et al., 2017).

In this Chapter, a robust systemic RNAi response was observed in *A. tumida* feeding and wandering larvae after injection of *V-ATPase subunit A* and *Laccase 2* dsRNAs. A phenotype resulting from dsRNA introduced by injection was observed and qPCR confirmed significant decreases and enhanced suppression of transcript levels over time, indicative of systemic RNAi. The results of the injection bioassays are in agreement with previous RNAi studies carried out against coleopteran insect pests (Tomoyasu and Denell, 2004; Arakane et al., 2005; Nui et al., 2008; Prentice et al., 2015). Oral delivery of *Laccase 2* in solution failed to evoke a phenotypic effect on either survival or fitness parameters. Feeding *V-ATPase subunit A* dsRNA at a concentration of 100 ng/µl (100 µg/ml) to larvae did result in 50 % mortality and deformities in surviving adults, however gene suppression could not be verified. In contrast, Bolognesi et al., 2012 reported that oral delivery of DvSnf7 dsRNA provided respective $LD_{50s}$ of 4.3 and 1.2 ng/ml diet for *D. v. virgifera* and *D. undecimpunctata howardii*. Additionally, Zhao et al. (2008) has reported that spraying leaves with target specific Arginine kinase (AK) dsRNA resulted in $LD_{50}$ value of 0.80 ng/ml for the stripped flea beetle, *Phyllotreta striolata*. It is clear that *A. tumida* larvae are highly susceptible to dsRNA delivered via injection, but consistent RNAi effects after oral delivery of dsRNA could not be achieved. To understand the lack of sensitivity to orally delivered dsRNA in *A. tumida* the persistence of dsRNA in the presence and in the gut of *A. tumida* larvae was investigated. Rapid and complete degradation of dsRNA was observed in *A. tumida* gut extracts, suggesting the involvement of ribonuclease activity. This has been demonstrated in *S. gregaria*, as like *A. tumida* they are highly sensitive to dsRNA when delivered via injection, but oral delivery of dsRNA has proved unsuccessful. Studies performed by Luo et al. (2012) and Wynant et al. (2014) showed rapid degradation of dsRNA in the gut extracts of *S. gregaria* and suggested this was a consequence of *Sg-dsRNAses 2*. The Colorado potato beetle, *L. decemlineata*, is highly sensitive to ingestion of *V-ATPase subunit A* dsRNAs ($LD_{50}$ of 5.2 ng/cm$^2$; Baum et al., 2007). Shukla et al., (2016) has recently demonstrated that incubation of dsRNA in *L. decemlineata* lumen content (diluted 50 %) did not cause significant degradation of dsRNA after 90 min. In contrast, when *A. tumida* gut extracts were diluted to 10 %, complete degradation of dsRNA was observed after 60 min. Thus, it is highly likely that dsRNA degradation within the gut of *A. tumida* was responsible for preventing sufficient uptake of dsRNAs by epithelial cells to induce an RNAi response. Another

factor that could contribute to these inconsistent results was the delivery method used. Oral delivery of dsRNA via a soaking assay made it impossible to determine the amount of dsRNA consumed by each *A. tumida* larvae.

Sequence specificity of dsRNA is vital for a target specific approach against insect pests, as identical mRNA regions of 20-25 nucleotides can elicit non-target effects. This was evidence by Braun et al. (2008) who showed that when dsRNAs targeting *D. virgifera* genes were tested on other insect pests, an effective oral RNAi effect was observed. An explanation for these non-target effects is the presence of 3 identical regions of 20-29 nucleotides that can be identified in the published sequence alignment. Alignment of *A. mellifera* and *A. tumida Laccase 2* and *V-ATPase subunit A* mRNAs indicated at most conserved regions of 15 bp and sequence identities were 74 % and 68 %, respectively. The lack of effect on survival or gene expression in honey bees was confirmed via the injected of *A. tumida* dsRNAs.

In this thesis convincing evidence for the potential of RNAi based biopesticides for target specific and environmentally benign control of *A. tumida* in apiculture is presented. Prevention of dsRNA degradation in the environment and gut of an insect is key to successfully inducing an RNAi response. Coating of dsRNA molecules could afford protection of introduced dsRNA from nucleases and gut pH variations (Huvenne and Smagghe, 2010). Liposome or nanoparticle-based delivery systems allow for the stabilisation of dsRNA molecules during delivery, subsequently increasing RNAi efficiency (Mamta and Rajam, 2017). Whyard et al. (2009) orally delivered non-encapsulated $_{Y}$*TUB23C* dsRNA to four *Drosophila* spp. with no evidence of an RNAi effect being observed, however when liposome encapsulated dsRNA was fed, all species suffered high mortalities following ingestion. Similarly, Taning et al. (2016) showed that oral delivery of liposome encapsulated dsRNA to *D. suzukii* led to a significant increase in gene silencing and insect mortality, whereas naked dsRNA failed to induce an RNAi effect. Regarding synthetic nanoparticles, it has been demonstrated that the incorporation of dsRNA into chitosan, a natural biodegradable polymer that can be prepared cheaply by deacetylation of chitin, resulted in effective induction of RNAi in mosquitoes by feeding (Zhang et al., 2010; Zhang X. et al., 2015). Zhu et al. (2011) used RNAase II-deficient *E. coli* to produce dsRNA and after *L. decemlineata* ingested the bacteria, significant mortality and loss of body weight was observed. Apse RNA Containers™ (ARCs) is a biotechnology company that has

recently developed a method to mass-produce encapsulated dsRNA using bacteria, which holds enormous potential for the future use of RNAi (Joga et al., 2016).

The mechanisms that facilitate dsRNA uptake in the insect gut are still largely unknown. The *sid-1* gene product is responsible for mediating systemic RNAi effects *C. elegans* (Winston et al., 2002). A survey of systemic RNAi in *T. castaneum* identified three putative SID-like genes, but the results of the study showed that they were not required for systemic RNAi (Tomoyasu et al., 2008). Where robust systemic RNAi responses are observed in insects such as *T. castaneum* additional or different genes with similar functions, or possibly even different mechanisms, such as endocytosis, could be responsible for the cellular uptake of dsRNA (Tomoyasu et al., 2008; Zhang et al., 2010). It has been suggested that endocytosis mediated uptake of dsRNA may be too slow to facilitate a strong RNAi response without the use of transfection reagents to improve delivery to the gut cell (Taning et al., 2016). It is possible that *A. tumida* may rely on an endocytosis pathway for the uptake of dsRNA and together with dsRNA degradation in the gut, the total amount of dsRNA available for uptake by epithelial cells was significantly reduced preventing an RNAi response (Taning et al., 2016). The translation of RNAi technology into a viable control strategy for *A. tumida* requires further research into the molecular mechanisms that may facilitate dsRNA uptake and the characterisation of gut nucleases, as the choice of formulation will depend on these factors.

### 7.3 Fusion protein technology as a control method for *Aethina tumida*

Current chemical pesticides tend to act on a single target within the insect nervous system by blocking or inhibiting one of five main targets: voltage gated sodium ($Na_V$) channels, glutamate receptors, γ-aminobutyric acid (GABA) receptors, nicotinic acetylcholine receptors and acetylcholinesterases (Casida, 2009). Consequently, the use of chemical compounds targeting few receptors has led to the development of resistance to several insecticidal families (Feyereisen, 1995; Brogdon and McAllister, 1998). Insecticidal venom peptides derived from insect predators such as scorpions (Wugargiri et al., 2001), parasitic wasps (Gould and Jeanne, 1984), predatory mites (Tomalski et al., 1988) and spiders (Lipkin et al., 2002; Tedford et al., 2004) have received a great deal of interest as they provide an extensive source of highly

insecticidal toxins. Many of these insecticidal toxins are active against neuronal voltage dependent $Na^+$, $K^+$, $Ca^{2+}$ or $Cl^-$ ion channels (Fajloun et al., 2000), providing alternative targets to traditional chemical control options. Spider venoms particularly have received much attention as they comprise an extensive library of neurotoxic peptides, and, to date, in the region of 1550 peptide toxins from 78 spider species have been described (Windley et al., 2012; www.arachnoserver.org). Furthermore, many spider peptide toxins are selectively insecticidal, making them ideal candidates for development as bioinsecticides (Windley et al., 2012). The use of ω-Hexatoxin-Hv1a (Hv1a) targeting voltage gated calcium channels as an alternative target to conventional chemical pesticides, could have the potential to improve the efficacy of existing pest management strategies and possibly exhibit synergism with current control programmes (Wratten, 2009). The oral activity of Hv1a alone is limited, however Fitches et al. (2012) demonstrated that linking this peptide to the carrier protein GNA facilitates transport of the Hv1a peptide across the gut epithelium into the circulatory and may localise the toxin to the CNS, enhancing toxicity. Subsequently, Hv1a/GNA has been used to successfully reduce survival and larval weight of *T. castaneum* and in glasshouse trials has induced 100 % mortality in *L. decemlineata* larvae after potato plants were sprayed with 350 ppm (0.35 mg/ml) fusion protein solution (Back, 2011; Fitches pers com, 2018).

In Chapter 4, the biological activity of GNA/Hv1a, Hv1a/GNA fusion proteins, and K>Q variants were investigated towards *A. tumida* via injection and feeding bioassays. Injection of GNA into wandering larvae elicited an $LD_{50}$ after 7 days of 788.09 µg/g insect. In contrast, recombinant pro-Hv1a resulted in a $LD_{50}$ of 43.53 µg/g insect or 11 nmoles/g insect against *A. tumida* larvae, which is typically higher than values reported for recombinant Hv1a (Atkinson et al., 1998; Bloomquist et al., 2003). These data tend to suggest that recombinant pro-Hv1a does not fold as efficiently in yeast and/or hyperglycosylation disrupts binding to ion channels, reducing the toxicity of pro-Hv1a towards *A. tumida* wandering larvae. The $LD_{50}$ values for GNA/Hv1a and GNA/Hv1a(K>Q) were a comparable 25.20 and 26.92 µg/g insect (equivalent to 6.3 and 6.7 µg/g insect of Hv1a), whilst Hv1a/GNA and Hv1a(K>Q)/GNA $LD_{50}$'s were slightly lower at 18.90 and 14.32 µg/g insect (equivalent to 4.7 and 3.58 µg/g insect of Hv1a), respectively. The $LD_{50}$ for pro-Hv1a was approx. 6 to 12-fold less toxic against wandering larvae, compared to the fusion protein variants. Fitches et al. (2012)

provided direct evidence for GNA localisation to CNS by injecting FITC-labelled GNA and subsequently showed binding of GNA to the nerve cord of *M. brassicae*, suggesting that GNA may assist in localising Hv1a to the CNS of exposed insects. These injection results are in agreement with Fitches et al. (2012), showing that the fusion of Hv1a to the carrier protein GNA can enhance biological activity of the recombinant toxin.

Oral delivery of all four fusion proteins showed that GNA/Hv1a had the highest toxicity towards *A. tumida* larvae with an $LC_{50}$ value after 7 days of 0.52 mg/ml which was 1.7 to 2.3-fold lower than that recorded for the other fusion protein variants. Consequently, stability assays were carried out to determine the length of time the fusion proteins remained intact *in vitro* in the gut and *in vivo* in the presence of feeding *A. tumida* larvae. The results of both the stability assays confirmed that GNA/Hv1a remained intact for the longest period, suggesting increased delivery of the Hv1a toxin to the haemolymph; thereby achieving an $LC_{50}$ approx. two times lower than the other fusion protein variants. Based on the results generated in the feeding bioassays, GNA/Hv1a was selected for use in “applied” bioassays whereby bee brood, or eggs and bee brood, were sprayed with fusion protein. Significant levels of mortality were achieved, however they were not as high as observed in the drinking assays when the larvae were fed on sucrose solutions containing fusion protein. The emerging larvae in the brood assays were exposed to protein rich brood and thus the ingested GNA/Hv1a may be more prone to gut proteolysis thereby reducing levels of toxin delivery to the CNS. In Chapter 5 we identified trypsin as the dominant gut protease in *A. tumida* larvae and demonstrated that the addition of a trypsin inhibitor, SKTI, prevented the degradation of intact GNA/Hv1a and Hv1a/GNA over a 24 hr time period. Further research is required to determine if co-feeding fusion proteins with PIs such as SKTI, could trigger a synergistic effect and significantly increase the effectiveness of GNA/Hv1a. Alternatively, modifications to the linker region between GNA and Hv1a could potentially increase the stability of the fusion protein, thus minimising cleavage following ingestion by *A. tumida*.

The use of SKTI as an alternative carrier protein, could hold enormous potential for hypothetically reducing proteolysis in the gut environment and thereby increase levels of Hv1a toxin that can be delivered to the haemolymph of *A. tumida*. SKTI was evaluated as carrier protein and preliminary results showed transport into the

circulatory system of *A. tumida*, suggesting it could be used as an alternative carrier protein to transport attached toxins to the circulatory system of *A. tumida*. (Chapter 6 section 6.4). However, fusion of Hv1a to SKTI, incorporating a rigid linker, significantly reduced the biological activity of the fused recombinant toxin. The lack of insecticidal activity following the use of an alternative carrier protein to GNA is not limited to SKTI. When GNA based fusion proteins were compared to a similar lectin derived from the garlic bulb, GNA was able to form functional recombinant proteins, however when same toxin was fused to the garlic lectin no biological activity was observed (Fitches et al., 2008). It is clear that GNA seems to be vital in assisting in the folding of these toxin, however the mechanisms responsible for this interaction remains unclear (Fitches pers com, 2018).

Alternative methods to deliver Hv1a have been demonstrated by Bonning et al. (2014) who fused a coat protein of a luteovirus (an aphid-vectored plant virus) to Hv1a and demonstrated successful delivery of the peptide to the haemolymph of four aphid species. The author demonstrated that ingestion of the fusion protein, via membrane sachet or in transgenic Arabidopsis plants, caused significant mortality in *Acyrthosiphon pisum*, *Rhopalosiphum padi*, *Aphis glycines* and *Myzus persicae* in comparison to control groups. Recently, Hv1a has been linked N- and C-terminally to an onion leaf lectin and expressed in *Nicotiana tabacum*, using a *Potato Virus X vector*, causing 65-83 % mortality in the mealy bug, *Phenacocuss soleopsis* (Javaid et al., 2018). Interestingly, fusion of Hv1a linked to alternative carrier proteins expressed in plants has resulted in functional fusion proteins, which we have failed to achieve in a yeast expression system. This could be a consequence of the folding environment in plants being better suited to the production of a functional toxin. A further method of delivering insecticidal arthropod toxins is to engineer entomopathogens, such as entomopathogenic fungi, to express transgenes encoding these toxins. Wang and St Leger (2007) demonstrated that engineering the fungus *M. anisopliae* to express the scorpion venom peptide AahIT, resulted in a 22-fold increase in fungal toxicity against *M*. *sexta* and adult yellow fever mosquitoes, *Aedes aegypti*, without compromising host specificity. Entomopathogenic fungal isolates have been screened against *A. tumida* with varying levels of success (Leemon and McMahon, 2009), however genetically engineering them to express Hv1a could greatly enhance fungal toxicity.

For application in apiculture, the only viable approach would be the development of fusion proteins as a product formulated for spray application, so it can be applied directly to combs within a hive. For this to be a successful approach, the fusion protein would need to be sufficiently resistant to degradation in the environment. Additionally, following ingestion, resistance to proteolysis within the insect gut is essential to allow sufficient toxin to be delivered to the CNS, achieving effective insect control. However, engineering fusion proteins to be too stable could potentially prevent the free toxin from interacting with ion channel targets, once delivered to the haemolymph (Back, 2011). Back (2011) reported that in lepidopteran larvae the breakdown of ButaIT-GNA within the haemolymph is directly comparable with insecticidal activity. Therefore, careful consideration needs to be given to the type of product used for the formulation of fusion proteins so that the stability in the environment and gut of an insect is enhanced, but the biological activity is not compromised. This could be achieved by dissolving the recombinant proteins in oil-water emulsions, which may afford protection against degradation. Furthermore, the cost of fusion protein production will need to be reasonable enough to compete with chemical insecticides. Advances in this regard have been demonstrated by Vestaron Corporation who have developed a commercially available biopesticide called SPEAR$^{TM}$, with the active ingredient being derived from spider venom. SPEAR$^{TM}$ is currently being used for treatment of thrips, whiteflies, two spotted spider mites, broad mites and aphids in vegetables in greenhouses. The mode of action is presumed to be via the spiracles as oral delivery of this product has not been particularly effective (www.vestaron.com).

## 7.4 Conclusions

In summary, the results presented in this thesis have shown that both RNAi, targeting *Laccase 2* and *V-ATPase subunit A* genes, and fusion proteins incorporating venom-derived neurotoxins, offer enormous potential for the development of new target specific and environmentally benign biopesticides, as alternatives to broad range pesticides. Like all insects, *A. tumida* have several lines of defence to introduced molecules, with regards to RNAi ribonucleases play an important role in the degradation of dsRNA. In contrast fusion protein cleavage seemed to be a consequence of the presence of gut proteases. In order to translate these approaches

into a viable control strategy for target specific control of *A. tumida* in apiculture further research is needed to develop suitable formulation options to enhance the oral toxicity of introduced dsRNA or fusion proteins.

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**Appendix 1:** Powell, M.E., Bradish, H.M., Gatehouse, J.A. & Fitches, E.C. (2017). Systemic RNAi in the small hive beetle *Aethina tumida* Murray (Coleoptera: Nitidulidae), a serious pest of the European honey bee *Apis mellifera*. *Pest Management Science* 73(1), 53-63.