Developing novel RNAi-based biopesticides targeting the central nervous system of *Spodoptera littoralis*

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Abstract

The Egyptian cotton leafworm, *Spodoptera littoralis*, is a highly polyphagous lepidopteran capable of damaging > 80 crop species. Most commercial insecticides targeting *S. littoralis* threaten non-target organisms and the environment. RNA interference (RNAi), a natural immune defence mechanism that regulates endogenous gene expression, can be triggered by double-stranded RNA (dsRNA), causing insect mortality. Novel sprayable biopesticides incorporating dsRNA or genetically engineered plants expressing dsRNA offer highly specific pest control methods. This project explored the feasibility of controlling *S. littoralis* via an RNAi approach, with the end goal of developing a dsRNA-based biopesticide targeting this insect. Three genes essential to central nervous system functionality, acetylcholinesterase 1 (*ace-1*), nicotinic acetylcholine receptor (*nAChR*) and voltage-gated sodium channel (*para*) were targeted; NADPH cytochrome P450 reductase (*NADPHcytP450r*) was targeted to elucidate its role in insecticide detoxification. Oral dsRNA delivery to 4th instar larvae via artificial diet and to 5th instar via gavage feeding did not significantly reduce ($P > 0.05$) gene expression, survival, pupation or adult emergence likely because dsRNA is rapidly degraded by nucleases in *S. littoralis* midgut juice, as revealed by *ex vivo* dsRNA incubation. Continuous feeding of dsRNA to neonates significantly reduced larval weight ($P < 0.05$), suggesting reduced nuclease activity in earlier instars, supported by the lack of significant effect on gene expression and survival upon direct dsRNA haemolymph injection into 4th instar larvae compared to significant developmental delays upon 3rd instar injection. Soaking eggs in *ace-1*, *nAChR* and *NADPHcytP450r* dsRNA significantly reduced ($P < 0.05$) hatching, indicating their non-cholinergic roles. Formulation with chitosan, a nanoparticle capable of protecting dsRNA and increasing cellular uptake, did not enhance dsRNA stability in pure midgut juice nor significantly reduce ($P > 0.05$) survival of 4th instar or neonate larvae. Pre-exposure to *NADPHcytP450r* dsRNA reduced the LC_{50} of deltamethrin against *S. littoralis* suggesting its role in detoxification. Bioinformatic analyses suggested the safety of these molecules against non-target organisms, confirmed by bioassay against *Bombus terrestris*. This work highlights the efficacy of targeting these genes whilst also indicating the need for optimised delivery techniques.

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List of abbreviations

˚C degrees Celsius

ACh acetylcholine

AChE acetylcholinesterase

ANOVA analysis of variance

AUD Australian dollars

BCAs biocontrol agents

BLRV Bean leaf roll virus

Bt *Bacillus thuringiensis*

Ca^{2+} calcium ions

CABI Centre for Agriculture and Bioscience international

CCC Climate Change Committee

cDNA Complementary DNA

CNS Central nervous system

CO_2 Carbon dioxide

CQD Carbon quantum dot

Cry crystal protein

CS Chitosan

CS-TPP Chitosan-tripolyphosphate nanoparticles

DEFRA Department for environment, food and rural affairs

DEPC Diethyl pyrocarbonate

DNA Deoxyribonucleic acid

ds*ace-1* dsRNA specific to the *S. littoralis* acetylcholinesterase 1 gene

ds*ace-2* dsRNA specific to the *S. littoralis* acetylcholinesterase 2 gene

dsGFP dsRNA specific to the *Aequorea victoria* green fluorescent protein gene

ds*Kan* dsRNA specific to a bacterial *kanamycin* resistance gene

ds*NADPHcytP450r* dsRNA specific to the *S. littoralis NADPHcytP450r* gene

ds*nAChR* dsRNA specific to the *S. littoralis* nicotinic acetylcholine receptor gene

ds*para* dsRNA specific to the *S. littoralis para* gene

dsRNA Double stranded RNA

dsRNA-CS-TPP dsRNA complexed with chitosan-tripolyphosphate nanoparticles

DvSnf7 RNAi-based biopesticide targeting *Diabrotica virgifera virgifera*

EDTA Ethylenediaminetetraacetic acid

EOSDA EOS Data Analytics

EPA United States Environmental Protection Agency

EPF Entomopathogenic fungi

EPPO European and Mediterranean Plant Protection Organisation

ESTs Esterases

FAO Food and Agriculture Organization of the United Nations

FAPRI Food and agricultural policy research institute

FIGS Fungal induced gene silencing

$GABA_A$ receptor

GluCl Glutamate-gated chloride channels

GSTs glutathione S-transferases

h hour(s)

HIGs host-induced gene silencing

HSE Health and Safety Executive

IRAC Insecticide resistance action committee

kdr knockdown resistance

kr-h1 Krüppel homologue

L:D light:dark

LC_{50} Median lethal concentration

LMIC Low-middle income countries

mg milligram(s)

min minute(s)

ml millilitre

mRNA Messenger RNA

Na^+/K^+ pump sodium/potassium pump

NCBI (National Centre for Biotechnology Information)

ng nanogram

NPF1 Nanocarrier-mediated transdermal gene

NTOs non-target organisms

OECD Organisation for economic co-operation and development

OsDREB1C gene involved in reactive oxygen species (ROS) scavenging in *Oryza sativa*

P450s Cytochrome P450 monooxygenases

PBS Phosphate-buffered saline

PCR Polymerase chain reaction

pcRNA paperclip dsRNAs

PEMV Pea enation mosaic virus

qPCR Quantitative polymerase chain reaction

RHS Royal Horticultural Society

RISC RNA-induced silencing complex

RNA Ribonucleic acid

RNAi RNA interference

RPL13A Ribosomal Protein L13a

rRNA Ribosomal ribonucleic acid

RT room temperature

RT-PCR Reverse transcription polymerase chain reaction

RT-qPCR Reverse transcription quantitative polymerase chain reaction

RYR Ryanodine receptor

SD standard deviation

SEM Standard Error of Mean

SID systemic RNA interference defective genes

SIGS spray-induced gene silencing

siRNA Small interfering RNA

TPP Sodium Tripolyphosphate

TRV Tobacco rattle virus

VGSC voltage gated sodium channel

VIGS virus induced gene silencing

Vip Vegetative insecticidal protein

WRI World resources institute

µg microgram

µl microliter

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Chapter 1. General introduction

1.1 Global food security

Currently, around 770 million people worldwide are classified as food-insecure (Verma and Saxena, 2021) and with a projected population of 9.7 billion by 2050 (UN, United Nations, 2023), the demand for food will inevitably rise. It is estimated that global food production will need to increase by almost 60%, requiring an additional 593 million hectares of land (WRI, World Resources Institute, 2018), to accommodate the growing population. Presently, almost half of all habitable land is used for agriculture (Figure 1.1) but the land available for crop production is finite and decreasing (FAO, Food and Agriculture Organization of the United Nations, 2020) due to commercial, residential and recreational use, and is estimated to decrease a further 20% by 2035 (CCC, Climate Change Committee, 2020). In addition, the percentage of land dedicated to growing bioenergy crops is increasing (DEFRA, Department for environment, food and rural affairs, 2021), further reducing the land available for food production. Consequently, crop yield must be optimised without additional land use.

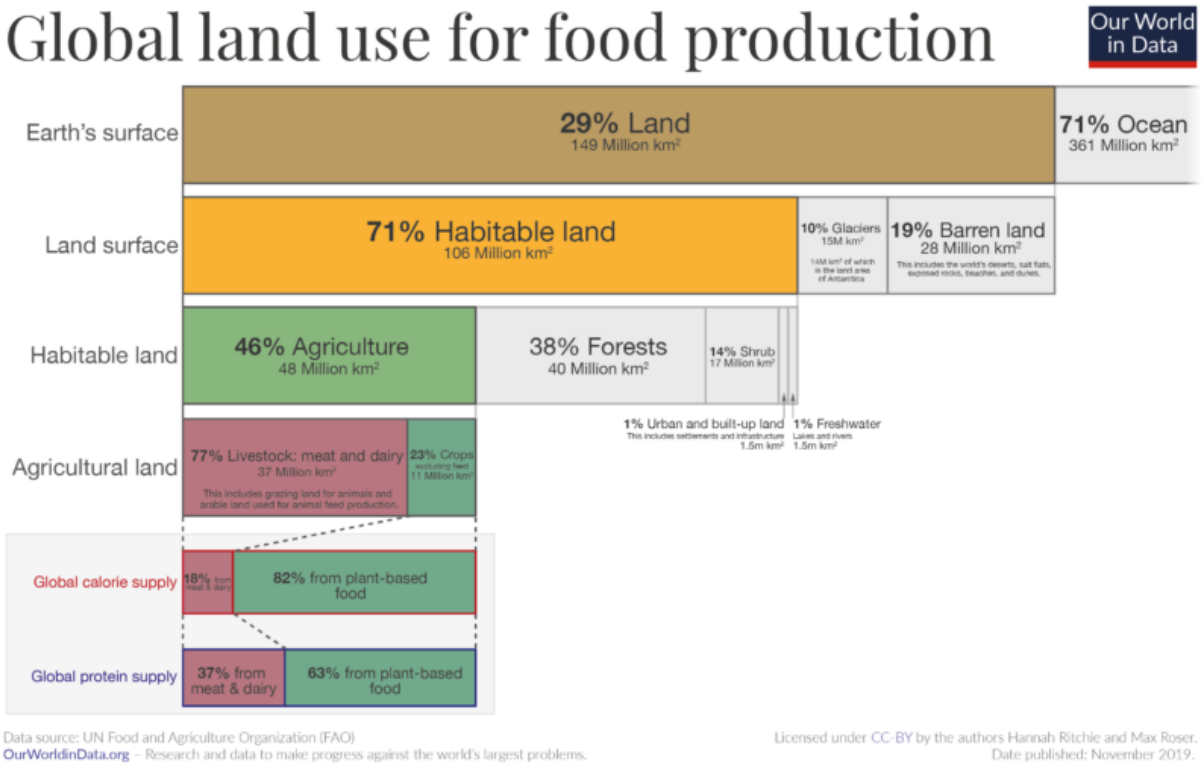

Figure 1.1 Current global land use (as of 2019). Source: Ritchie and Roser (2019).

1.2 Optimising crop yield

Crop yield is defined as the amount of agricultural product harvested per unit of land area (Fischer, 2015) and can be optimised through the use of high quality seeds (Wimalasekera, 2015), post-sowing treatments (e.g. the addition of growth regulators and micronutrients) (Singh *et al*, 2018), sufficient irrigation (Wang *et al*, 2021), maintaining optimal soil quality (Jalli *et al*, 2021) and fertiliser (Yousaf *et al*, 2017) and pesticide (Washuck *et al*, 2022) application. Yield is highly dependent on the advice given to farmers by breeders and agronomists (Paltasingh and Goyari, 2018) who use various software based technologies to assist with yield optimisation techniques. For example, specialised online mapping tools can identify areas with maximum growth potential ('field zoning') (Torbert *et al*, 2014), monitor crop growth to identify health problems ('remote sensing') (Wang *et al*, 2020) and accurately predict weather patterns ('EOS Data Analytics crop monitoring') (EOS, 2023). However, biotechnological advancements are often hailed as one of the main factors in crop yield improvement (Oluwole *et al*, 2021) with 'Biotech crops' such as transgenic rice, overexpressing the *OsDREB1C* gene (involved in reactive oxygen species scavenging), exhibiting ~70% increased yield due to improved nitrogen use efficiency in experimental field trials (Wei *et al*, 2022). Ultimately, a combination of yield improvement methods meant less than half of the previously estimated 2.78 billion ha of agricultural land was required to fulfil global crop production demands by 2019 (Figure 1.2). However, crop yield is likely to remain relatively unchanged in low-middle income countries (LMIC), where population expansion will increase the most (Ritchie and Roser, 2019) and where climate change is likely to have the biggest effect (McLachlan, 2020), as optimisation techniques are expensive and inaccessible to farmers in these countries.

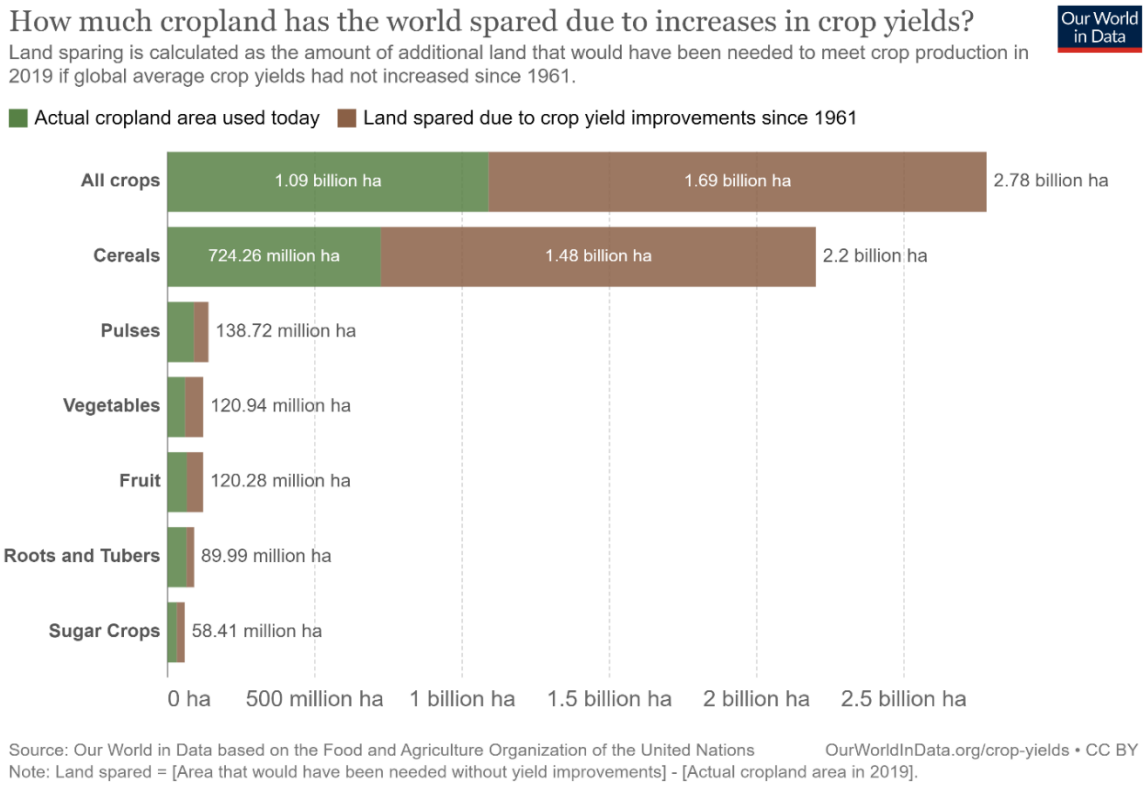

Figure 1.2 Current cropland use. Source: Ritchie and Roser (2019).

1.3 Factors limiting crop yield

Crop yield is reduced by many factors (Pandey *et al*, 2017). Abiotic stresses such as low temperatures, high temperatures, drought and salinity account for almost half of all yield loss worldwide (Ningombam et al, 2021). Furthermore, high salinity levels affect ~ 1 billion hectares, or around 20%, of agricultural land worldwide (Chen, 2023), causing an estimated economic loss of 27 billion US$ (Qadir *et al*, 2014), with even moderate salinity levels capable of causing ~ 55% maize yield loss (Zörb *et al*, 2018). Biotic factors such as fungi, bacteria and viruses reduce global crop yield by ~ 16% (Ficke *et al*, 2018). Furthermore, weeds compete with crops for sunlight, water and nutrients and account for ~ 2.7 million tonnes of annual grain loss in Australia alone, costing farmers ~ AUD 3.3 billion (Llewellyn *et al*, 2016). Insects are one of the most significant biotic factors that reduce the productivity of some of the world's most economically and nutritionally important food crops (Lehmann *et al*, 2020). Insects damage crops directly by feeding on tissues and organs. For example, the cotton bollworm, *Helicoverpa armigera*, causes severe damage to 172 plant species such as cotton, maize and sorghum (Reigada *et al*, 2016) by directly feeding on flowers, bolls and fruits (depending on the species) which also renders them

susceptible to secondary infections from bacteria, viruses and fungi (Li *et al*, 2021). Insects also damage crops indirectly. For example, the pea aphid, A*cyrthosiphon pisum,* not only damages a broad range of crop plants such as faba bean (*Vicia faba*) and alfalfa (*Medicago sativa*) by directly feeding on plant sap, which leads to leaf curling and stunted growth (RHS, 2023), it also acts as a vector for more than 30 plant viruses such as pea enation mosaic virus (PEMV) and bean leaf roll virus (BLRV) (Paudel *et al*, 2018).

Regardless of whether the yield limiting factor is of abiotic or biotic origin, the effects of climate change are likely to exacerbate these problems and drastically impact global crop yield (Ray *et al*, 2019) due to higher temperatures and CO_2 levels, alongside the increasing severity and frequency of floods and droughts (Environmental Protection Agency, EPA, 2023) which all worsen the impact of weeds, pests and pathogens that often thrive in such climates (EPA, 2023). Additionally, rising CO_2 levels could negatively impact crop nutritional value (Beach *et al*, 2019).

1.4 Insect central nervous system

The insect central nervous system (CNS) is comprised mainly of a brain and ventral nerve cord with segmental ganglia (Scharf, 2008) which contain motor neuron cells (presynaptic neuron) that transmit impulses to muscle cells (postsynaptic neuron). At rest, motor neurons are polarised and the resting potential is maintained by the sodium/potassium (Na^+/K^+) pump. A sensory stimulus causes sodium (Na^+) ions to flow out of the cell, thus depolarising it and, upon reaching a threshold (-55 mV), voltage-gated Na^+ channels (VGSC) allow Na^+ ions inside to depolarise the motor neuron further and fire an action potential (AP). The AP is propagated along the axon to its terminals, which causes voltage-gated calcium (Ca^{2+}) channels to open on the presynaptic motor neuron and Ca^{2+} ions to flow inside (Figure 1.3). Consequently, vesicles release the excitatory neurotransmitter, acetylcholine (ACh), into the synaptic cleft (the junction between pre and postsynaptic neurons), via exocytosis (Antranik, 2023), which subsequently binds to nicotinic (nAChR) receptors on the postsynaptic muscle cell. The firing of a further AP allows muscle contraction, and neuronal transmission terminates when ACh is hydrolysed, into acetate and choline, by the enzyme acetylcholinesterase (AChE) (McHardy *et al*, 2017).

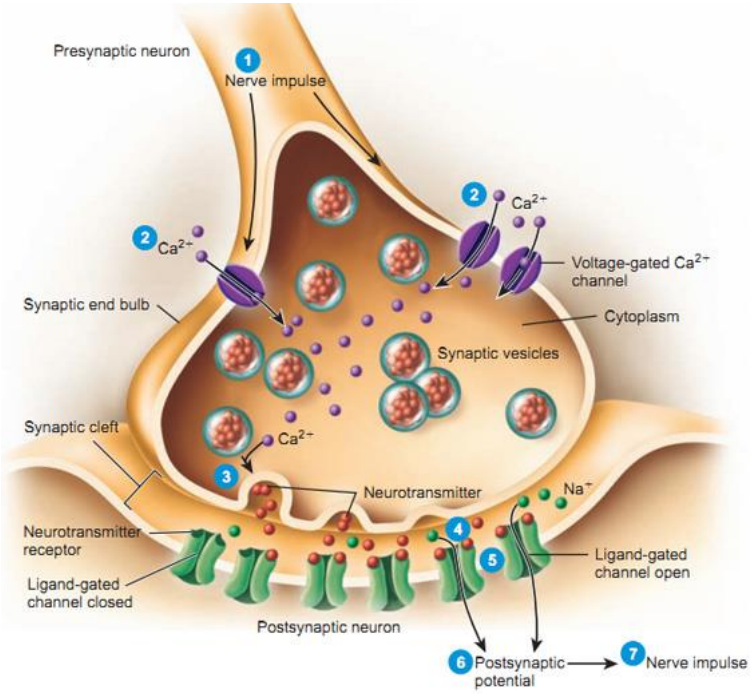

Figure 1.3 Synaptic transmission. Source: Antranik (2023).

1.4.1 Insect CNS as a target for insecticides

Most commercial chemical insecticides cause insect paralysis and death and rely on only a few molecular target sites mainly within the CNS (Ffrench-Constant *et al*, 2016). Organophosphates and carbamates irreversibly inactivate AChE (Haddi *et al*, 2017), rendering it unable to hydrolyse ACh which accumulates in the synaptic cleft and leads to repeated muscle stimulation (Haddi *et al*, 2017). Pyrethroids prevent VGSC closure (Silver *et al*, 2018), allowing constant Na^+ ion flow into the axon, causing uncontrolled APs and membrane hyperdepolarization (Haddi *et al*, 2012). Spinosyns and neonicotinoids conformationally change *nAChR*s on the post-synaptic muscle cell which inhibits ACh binding and interferes with synaptic transmission (Aditya and Rattan, 2012). Diamides, a relatively new class of insecticides, cause irreversible muscle contraction by activating the ryanodine receptor (RyR) (in the insect endoplasmic reticulum) which causes rapid Ca^{2+} release, (Troczka *et al*, 2017). Phenylpyrazoles block $GABA_A$- (Gamma-aminobutyric acid) and glutamate- gated chloride channels (GluCls) (Islam and Lynch, 2012) which disrupts chloride ion uptake and AP inhibition and leads to hyper-excitation.

1.5 Insecticide resistance

Many insect pests have developed resistance to chemical insecticides due to their extensive use (Dong, 2007), with some displaying decreased susceptibility to multiple compounds, such as the Green peach aphid, *Myzus persicae*, which is resistant to >70 insecticides (Silva *et al*, 2012); although, this generally comes with fitness costs (Shi *et al*, 2004). Insecticide resistance is mainly genetic or metabolic, although some instances of behavioural (Chareonviriyaphap *et al*, 2013) and penetration (Dang *et al*, 2017) resistance have been documented. However, the evidence regarding these mechanisms has been disputed (Zalucki and Furlong, 2017).

1.5.1 Genetic resistance

Insects acquire knockdown resistance (kdr) through mutations in the target sites of insecticides. Organophosphate kdr is caused by single point mutations in the *AChE1* target site of the Tomato leaf miner, *Tuta absoluta* (A201S) (Haddi *et al*, 2017) and the Chinese malaria mosquito, *Anopheles sinesnsis* (G119S) (Yang *et al*, 2019) (although several insects have a second acetylcholinesterase, *AChE2*, gene; Tmimi *et al*, 2018). Whereas, four mutations, I161V, G265A, F330Y and G368A, confer resistance in the Common fruit fly,

Drosophila melanogaster, with combinations of the four providing stronger resistance (super kdr) (Menozzi *et al*, 2004). Pyrethroid kdr is conferred through point mutations in the sodium channel gene, *para*, which constitutes four domains (I-IV), each with six segments (S1-S6). Low level resistance is commonly associated with leucine (L) substitution with either serine (S), phenylalanine (F) or histidine (H) in segment 6 of domain 2 (IIS6). For example, L1014F in *para* IIS6 reduces *T. absoluta* pyrethroid susceptibility by 10–20 (Haddi *et al*, 2012) but highly resistant populations have further IIS4–IIS6 mutations (M918T and T929I) which confer higher levels of resistance (Haddi *et al*, 2012). Kdr Resistance to both neonicotinoids and spinosyns is due to mutations in the *nAChR* gene, with the R81T mutation in *M. persicae* (Charaabi *et al*, 2018) and *Aphis gossypii* (Hirata *et al*, 2017) conferring neonicotinoid resistance *and* G275E in *T. absoluta* conferring spinosad resistance (Silva *et al*, 2016). Diamide kdr is conferred through point mutations in the ryanodine receptor (Troczka *et al*, 2017). The most common mutation in *Plutella xyostella*, G4946E, provides low resistance but three further mutations Q4594L, E1338D, I4790M confer >2000-fold resistance (Guo *et al*, 2014). Diamides are generally highly potent to lepidopteran insects but less so to other insect species possibly due to most lepidopterans having an isoleucine at amino acid position 4790 of the gene encoding the ryanodine receptor compared to methionine at this site in most other insect species (Guo *et al*, 2014). This was suggested further when Richardson *et al* (2022) *found that* an I4790M variant acquired higher resistance to multiple diamide insecticides when the entire *P. xylostella RyR* coding sequence was integrated into *D. melanogaster*.

1.5.2 Metabolic resistance

Insects also display metabolic resistance, whereby insecticides are sequestered or degraded by detoxification enzymes such as cytochrome P450 monooxygenases (*P450s*), esterases (ESTs) or glutathione S-transferases (GSTs) (Schluep and Buckner, 2021) either by enzyme upregulation or structural changes (Hirata *et al*, 2017). P450s are the primary detoxifying enzymes (Ye *et al*, 2022) and, as most insects have over 100 (Feyereisen, 1999), no singular P450 is responsible for insecticide detoxification both within and between species. For example, *CYP324A12*, *CYP321F3*, and *CYP9A68* are involved in organophosphate resistance in the Asiatic Rice Borer, *Chilo suppressalis* (Zhao *et al*, 2020). In *Anopheles gambiae CYP6Z1*, *CYP6Z2*, *CYP6M2*, *CYP6P3* and *CYP325A3* confer increased resistance to pyrethroids (David *et al*, 2013) but *CYP321E1* and *CYP6BG1* are responsible in *P. xylostella* (Hu *et al*, 2014).

1.6 *Spodoptera littoralis* crop damage

The cotton leafworm, *Spodoptera littoralis*, is a highly polyphagous lepidopteran insect pest (Chen *et al*, 2016) that extensively damages > 80 economically important crop species (Salama *et al*, 1970) including cotton (*Gossypium* spp), wheat (*Triticum aestivum*), maize (*Zea mays*) (CABI, 2023a) and tomato, *Solanum lycopersicum* (Sobhy *et al*, 2015), one of the most nutritionally important food crops in LMIC (Retta and Berhe, 2015) with a positive impact on human health due to its high flavonoid, carotenoid and vitamin C and E content (Ilahy *et al*, 2016). *S. littoralis* development is divided into 4 main life stages: egg, larva (6 instars), pupa and adult, with the larvae being the most destructive (Sobhy *et al*, 2015). Early instars preferably feed on the underside of leaves but will later feed voraciously on most parts of the plant and cause complete defoliation, interfere with plant development and render fruit unsuitable for human consumption due to excrement deposition (Bayer, 2019).

Although native to Africa, *S. littoralis* has spread to most parts of the middle East (Sobhy *et al*, 2015) and certain European countries such as Cyprus and Malta (CAB international, CABI, 2023a). Due to its highly destructive nature, it has been labelled an A2 quarantine pest, or an invasive pest with limited distribution within the European and Mediterranean Plant Protection Organisation (EPPO) region that presents a risk of further spread, in many more countries (EPPO, 2018). Nevertheless, it is not currently considered a threat in areas with fluctuating temperatures as its survival is decreased above 40 ˚C and below 13 ˚C and its optimal reproductive temperature is 25 ˚C (Lopez-Vaamonde, 2008). However, climate change could extend the distribution of the pest species.

1.7 Current strategies for control of *S. littoralis*

1.7.1 Chemical control

As with many other insect pests, chemical insecticides are one of the major forms of *S. littoralis* control, but their widespread use has increased the potential for attack due to the evolution of resistance to many important insecticides, including organophosphates and pyrethroids (Hilliou *et al*, 2021). Also, they are expensive for farmers in economically less developed countries (Sanda *et al*, 2018) and their lack of specificity, and thus ability to threaten non-target beneficial insects (Williams *et al*, 2015), human health and the overall

environment (Atkar *et al*, 2009), has led to more stringent regulations surrounding their use or their complete ban (Health and Safety Executive, HSE, 2023). Consequently, the development of more environmentally friendly and sustainable 'biopesticides', containing bioactive molecules from natural sources, is crucial (Fenibo *et al*, 2021).

1.7.2 Biological control

Biological Control Agents (BCAs) such as *Bacillus thuringiensis* (Bt maize) can be used to control a range of insect pests as certain toxins they produce exhibit insecticidal effects (Latham *et al*, 2017). Crystal (Cry) proteins bind to proteins and glycolipids on the membrane of host gastrointestinal cells (Endo, 2022) which facilitate their insertion into the membrane, leading to pore formation and midgut epithelium destruction (Bravo *et al*, 2023). Vegetative insecticidal proteins (Vip) have a similar mode of action (Gupta *et al*, 2021) and certain Vips, Vip3Aa, Vip3Ab, Vip3Ae and Vip3Af, are effective in of*et alS. littoralis* control (de Escudero *et al*, 2014). However, *S. littoralis* is resistant to many *B. thuringiensis* strains (Salama *et al*, 1989) and target site mutations in midgut receptors can lead to the rapid evolution of resistance (Herrero *et al*, 2016) because Cry1C and Cry1E share a common binding site in *S. littoralis* (Herrero *et al*, 2016), meaning cross-resistance to both toxins can evolve with a single mutation (Pickett *et al*, 2017).

Additionally, entomopathogenic fungi can be used as BCAs as they generate spores with insecticidal activity. Glucans and proteins on the spore surface facilitate attachment to the insect cuticle and aid in the formation of a germ tube which penetrates the cuticle through mechanical and enzymatic action. The fungi then progress through the insect body cavity towards the haemocoel where a transition from germinative to vegetative growth occurs, enabling the fungus to utilise insect nutrients for its own growth and reproduction, thus killing the insect (Barra-Bucarei et al, 2019). *Metarhizium anisopliae* can reduce populations of *S. littoralis* 3rd instar larvae by as much as 80% (Shairra and Noah, 2014). However, because their effects on later, more damaging, larval instars were not investigated and because some *S. littoralis* instars have defence mechanisms against fungal attack (Shairra and Noah, 2014), this approach may not be effective in the field. Furthermore, incredibly high concentrations of fungal spores are needed to attain adequate control (McNeil, 2011) which lowers their cost effectiveness, while increasing their ability to become pests themselves (Bale *et al*, 2008).

Further biological control agents include endoparasitic wasps such as *Trichogramma* spp whose oviposition within host eggs leads to destruction upon hatching. The efficacy of various *Trichogramma* spp against *Spodoptera* spp has been investigated with varying degrees of success. Jaraleño-Teniente *et al* (2020) found that *Trichogramma atopovirilla* had an average parasitism rate of 70.14 % on *Spodoptera frugiperda* (Fall armyworm) eggs compared to just 29.23 % for *Trichogramma pretiosum*, in laboratory conditions, but both species exhibited < 10 % parasitism in the field. Puneeth and Vijayan (2013) found that *Trichogramma chilonis* parasitism rate on *S. frugiperda* eggs was 80.31 % but viability was very low at only 4.91 %. The varying success of *Trichogramma spp* is possibly due to the layered structure of egg masses deposited by *Spodoptera* spp as both Beserra and Parra (2005) and Mohamed (2021) reported that parasitism of *S. frugiperda* eggs by *T. atopovirilia* and *S. littoralis* eggs by *Trichogrammatoidea bactrae* decreased with increasing egg mass layers.

1.7.3 Synthetic sex pheromones

Due to its highly destructive nature, many methods have been deployed to control *S. littoralis* populations. The use of synthetic versions of the sex pheromones naturally produced by *S. littoralis* females can be used to lure males into traps where they become stuck and subsequently die (Sanda *et al*, 2018). However, because *S. littoralis* pheromone composition is complex and because volatile compounds released from both host and non-host plants have a large impact on pheromone detection in males (Borrero-Echeverry *et al*, 2018) this method may not be successful in the field. Furthermore, males are also attracted to the compositionally similar pheromones produced by *Spodoptera litura* (Tobacco cutworm) females (Borrero-Echeverry *et al,* 2018) and because it is not currently understood whether host plant volatiles have a synergistic effect with female pheromones or whether they mediate male attraction towards mating sites before the release of female sex pheromones, males may still be attracted to both their host plants and females of their own species as they share many common host plants (Borrero-Echeverry *et al*, 2018). Also, *S. littoralis* females are able to change the pheromone blend they release (Borrero-Echeverry *et al*, 2018) and males can quickly develop a preference for those (Droney *et al*, 2012). Furthermore, sex pheromone strategies are often expensive (Caparros Medigo *et al*,

2013) and therefore production costs would need to be reduced before they are affordable for farmers in economically less developed countries.

1.8 RNA interference

RNA interference (RNAi) is a natural immune defence mechanism used by most eukaryotic organisms to regulate endogenous gene expression (Maillard *et al*, 2019) but also to defend against viruses and transposable elements (Obbard *et al*, 2009). It can be artificially triggered by the introduction of a specifically designed long dsRNA molecule into an organism of choice, in order to reduce expression of a target gene. Once the dsRNA reaches its site of action in the cell cytoplasm, it is recognised as an invading molecule which the cell seeks to destroy (Obbart *et al*, 2009). To facilitate this, the DICER enzyme cleaves long dsRNA into shorter double-stranded small interfering RNAs (siRNAs), typically 21-25 bp in length, (Figure 1.4) resulting in a collection of siRNAs that cover the entire length of the original dsRNA molecule (Roberts *et al*, 2015). Subsequently, siRNA is unwound to produce a sense (passenger) strand, that is quickly cleaved, and an antisense (guide) strand that is incorporated into the RNA induced silencing complex (RISC) (Elshabir *et al*, 2001) which becomes a template to which complimentary mRNA, transcribed from the target gene, can bind. Once bound, target-specific mRNA is degraded by the catalytic component of the RISC, the argonaute 2 enzyme (Okamura *et al*, 2004). Consequently, the target gene can be transcribed but not translated into a functional protein, leading to an overall reduction in gene expression or complete gene silencing (Arpaia *et al*, 2020). Hence, RNAi has the potential to provide a new generation of biopesticides that target only a specific organism (or small group of organisms) due to the incorporation of specifically designed dsRNA (Tayler *et al*, 2019a).

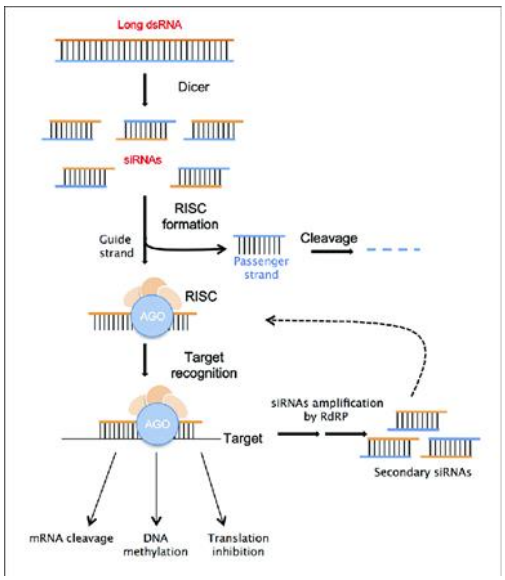

Figure 1.4 RNAi pathway. Source: Limera *et al*, 2017.

1.9 RNAi in insects

As mentioned in section 1.4.1, most commercial chemical insecticides rely on only a few molecular target sites, mainly within the CNS. Consequently, the CNS is also a major target for RNAi studies in insects. For example, Tariq et al (2019) reported a 55% reduction in survival, compared to controls, after 7 days of continuously feeding dsRNA specific to a *VGSC* gene, the target of pyrethroids, to *M. persicae* along with an 8-day reduction in adult longevity. Additionally, Kumar et al (2009) reported 60 % *H. armigera* mortality upon feeding on dsRNA specific to the *AChE* gene alongside developmental delays and 81% growth inhibition and Kola et al (2019) reported a significant reduction in weight and length of the yellow stem borer, *Scirpophaga incertulas*, upon feeding on rice cut stems injected with dsRNA specific to the *AChE* gene.

As mentioned in section 1.5.2, detoxification by *P450s* renders insects less susceptible to insecticides. Hence, *P450s* are commonly targeted by RNAi in an attempt to reduce the capability of an insect to detoxify a specific insecticide, thus reducing the amount of pesticide needed to kill the insect. Due to the presence of more than 100 *P450*s in most insects (Feyereisen, 1999), no singular P450 is responsible for the detoxification of each insecticide neither within nor between species. For example, Dulbecco et al (2021) reported that injection of 1 µg dsRNA specific to *CYP4PR1* into the kissing bug *Triatoma infestans* significantly reduced transcript levels by 93 % and that, upon exposure to the LD_{50}

for deltamethrin, mortality was significantly (20 %) higher compared to controls. Furthermore, Bai-Zhong et al (2020) reported that feeding dsRNA specific to *CYP321A8*, *CYP321A9* and *CYP321B1* via artificial diet to *Spodoptera frugiperda* significantly increased mortality by 43.53 %, 45.79 % and 40.62 %, respectively, upon exposure to chlorantraniliprole.

The NADPH cytochrome P450 reductase gene (*NADPHcytP450r*) is required for electron transfer from NADPH to cytochrome P450 and therefore for the functionality of all *P450*s (Zhu *et al*, 2012). Consequently, this gene has been targeted via RNAi in many insect species in an attempt to target all *P450s* simultaneously, thus potentially increasing sensitivity to a range of commercial insecticides. For example, Moural et al (2020) reported that targeting *NADPHcytP450r in L. decemlineata* via feeding on dsRNA-expressing bacteria led to only 10 % of insects surviving upon subsequent exposure to imidacloprid. Additionally, Tang et al (2023) reported that targeting *NADPHcytP450r* in the cotton aphid (*A. gossypii*) via feeding on dsRNA-incorporated artificial diet led to a 50 % reduction in transcript level and ~20 % increase in mortality upon exposure to Sulfoxaflor; a systemic insecticide that acts as an *nAChR* agonist. Furthermore, Liu et al (2014) reported that targeting *NADPHcytP450r* in *Nilaparvata lugens* via dsRNA injection significantly increased mortality by ~ 30 % and 40 % upon exposure to beta-cypermethrin and imidacloprid, respectively and Ji et al (2019) reported that injection of 3 µg dsRNA specific to *NADPHcytP450r* in *S. litura* increased larval mortality upon exposure to phoxim (an organophosphate) by 34.6% (LC_{15} dose) and 53.5% (LC_{50} dose).

To trigger insect RNAi, dsRNA can be administered via three main methods (i) oral feeding (either directly, applied topically onto or percolated within an artificial diet, gavaging or via genetically modified plants expressing dsRNA); (ii) direct microinjection into the haemocoel or (iii) soaking the organism in a solution containing dsRNA. Triggering an RNAi response via oral feeding is preferential as it indicates how an insect may respond in an open-field situation; where dsRNA is incorporated into sprayable biopesticides via spray-induced gene silencing (SIGs) or expressed by a genetically engineered plant via host-induced gene silencing (HIGs) (Koch *et al*, 2019). Direct haemocoel injection is not feasible outside of the laboratory and is technically difficult due to the high precision and skill necessary but it

does allow RNAi experiments to be conducted in organisms that are refractory to orally induced RNAi (Wang *et al*, 2018).

Generally, RNAi amenability can be grouped by insect order, with the Coleoptera being the most and the Lepidoptera the least amenable. For example, Baum et al (2007) reported that the coleopteran insect, *Diabrotica virgifera virgifera* (Western corn rootworm), exhibited significant mortality, as a result of orally induced RNAi, via dsRNA specific to more than one hundred genes. The presence of a robust RNAi response in this insect has allowed significant progress to be made regarding its control and the commercialisation of transgenic maize expressing dsRNA specific to the *D. virigifera snf7* gene (encoding a protein involved in intracellular trafficking) in the US (Roberts *et al*, 2020). Conversely, lepidopteran insects are generally recalcitrant to, especially orally induced, RNAi with the response usually being localised and transient (Terenius *et al*, 2011).

1.9.1 Factors affecting Insect susceptibility to RNAi

Insect susceptibility to RNAi depends upon many factors. Firstly, to trigger successful gene knockdown, dsRNA must reach its site of action in the cell cytoplasm. Thus, RNAi response in insects is firstly determined by the ability of the silencing effect to spread throughout the body cavity (Katoch *et al*, 2013). There are two main types of RNAi, cell autonomous and non-cell autonomous (Figure 1.5). Cell autonomous RNAi refers to gene silencing only in the cell that is directly exposed to experimentally introduced dsRNA (Whangbo and Hunter, 2008), whereas non-cell autonomous RNAi relates to the spread of the silencing signal to distant cells. Non-cell autonomous RNAi encompasses both systemic and environmental RNAi. Systemic RNAi refers to the silencing signal crossing into neighbouring cells and was first identified when target-specific dsRNA was injected into the body cavity of the nematode *Caenorhabditis elegans* which led to gene silencing in tissues throughout the insect as well as its offspring (Fire *et al*, 1998). *C. elegans,* along with a select group of other organisms (Whangbo and Hunter 2008), also exhibits 'environmental RNAi', whereby gene silencing occurs when the organism is exposed to environmentally encountered RNAi (i.e. through soaking or feeding). Environmental RNAi involves dsRNA importers which take dsRNA molecules from the intestinal lumen into intestinal cells, leading to silencing in intestinal cells which is then spread via systemic RNAi to distant cells (Maruekawong *et al*, 2022).

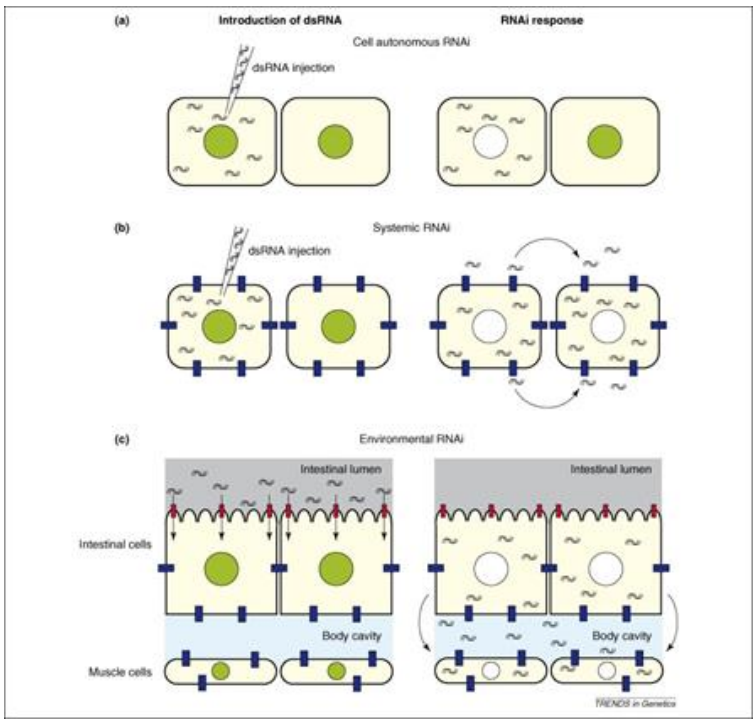

Figure 1.5 Cell autonomous and non-cell autonomous RNAi. Source: Whangbo and Hunter (2008).

Silencing spread throughout the insect body cavity depends largely upon the presence of systemic RNA interference deficient (*SID*) genes which were first identified in C. *elegans* (Conte Jr *et al*, 2015). *SID-1* genes are responsible for uptake and release of dsRNA among cells whereas *SID-2* genes initiate the import of dsRNA from the intestinal lumen and into nearby cells via endocytosis (Joga *et al*, 2016). *SID* genes have been identified in various coleopteran insects such as *T. castaneum* and *Leptinotarsa decemlineata* (Colorado potato beetle) which are both highly amenable to orally-induced RNAi (Knorr *et al*, 2018 and Cappelle *et al*, 2016 respectively), however *SID-2* genes have not been identified in any insect species thus far (Joga *et al*, 2016), perhaps suggesting *SID-2* genes are not essential to the insect RNAi response and that cellular dsRNA uptake relies solely on the *SID-1* genes. However, the *T. castaneum SID-1* gene is not involved in dsRNA uptake (Tomoyasu *et al*, 2008) and RNAi is still achievable in the dipteran insect *D. melanogaster*, even though this insect lacks *SID-1* genes (Saleh *et al*, 2006). Moreover, the lepidopteran insect *Bombyx mori* (Domestic silkworm) has three *SID* genes, yet inducing RNAi via oral-feeding is difficult in this insect (Yamaguchi *et al*, 2011) but this is perhaps because the *B. mori SID-1* gene is not orthologous to the *C. elegans SID-1* gene and is instead more similar to the nematode gene, *tag130*, which is not necessary for systemic RNAi (Kobayashi *et al*, 2012). However, the introduction of the *C. elegans SID-1* gene into *B. mori* enhanced *dsRNA* uptake (Kobayashi

et al, 2012). Therefore, the inter-order variation in RNAi susceptibility may be explained by differences in uptake efficiency pertained to the lack of *SID* genes but the presence of *SID* genes does not automatically suggest essentiality to the systemic RNAi response in all insect species.

For successful knockdown, dsRNA must reach its site of action intact but this is often hindered by nuclease degradation, especially in lepidopteran insects who often exhibit high nucleolytic activity in their saliva, haemolymph and midgut. Although significant knockdown has been reported in some lepidopteran insects (Poreddy *et al*, 2017 and Zhang *et al*, 2015), the effect is often transient, with transcript levels able to rebound within 48 h (Zhang *et al*, 2015), possibly due to nucleolytic activity (Singh *et al*, 2017). Guan *et al* (2018a) found that a specific nuclease, *REase*, in the saliva of the lepidopteran insect *Ostrinia furnacalis* (the Asian corn borer) is upregulated in the presence of dsRNA. This phenomenon is also true of various hemipteran insects such as *A. pisum* which is also notoriously recalcitrant to RNAi (Christiaens *et al*, 2014). Wang *et al* (2016) found that haemolymph degradation of template dsRNA was more than 4-fold higher in the lepidopteran insect *S. litura* than the coleopteran American cockroach (*Periplaneta americana*) and 10-fold higher than in the blattodean insect *Zophobas atratus* (Giant mealworm beetle) which also coincided with a 4-fold higher gene knockdown in *P. americana* and *Z. atratus* compared to that of *S. litura*. Wang *et al* (2016) also reported high gene knockdown (76 %) with dsRNA haemolymph injection compared to only 5% knockdown with dsRNA oral feeding in the orthopteran *Locusta migratoria* (Migratory locust) compared to only 20 % and 1 % gene knockdown when dsRNA was injected and fed, respectively, to *S. litura*. This coincides to the lower nuclease activity in *L. migratoria* haemolymph compared to its midgut (Peng *et al*, 2018) and the high nuclease activity in both the gut and haemolymph of *S. litura* (Peng *et al*, 2018). Furthermore, significant gene knockdown was observed in *P. americana* through both dsRNA injection and feeding, 82 % and 47 % respectively, likely pertaining to low nuclease activity in the gut and haemolymph (Peng *et al*, 2018). Alongside nucleolytic activity, the lepidopteran gut also presents a very hostile environment for dsRNA due to an extremely high alkaline pH (>9.0) (Christiaens *et al*, 2018a) which causes chemical hydrolysis of dsRNA.

RNAi response can also vary within an insect order and even within a species. For example, targeting Vacuolar-type ATPase (*V-ATPase*) subunit A in four closely related lady beetle species led to only 5 % mortality in the Harlequin ladybird (*Harmonia axyridis*) but 80 % in the Convergent lady beetle (*Hippodamia convergens*) (Pan *et al*, 2020). Furthermore, Whyard *et al* (2009) reported an LC_{50} of just 0.0025 µg dsRNA/mg diet after 7-day dsRNA exposure to dsRNA targeting different subunits of the *V-ATPase* gene in *T. castaneum* but Cao *et al* (2018) reported a more delayed response even with considerably higher dsRNA concentrations.

1.10 Design considerations for RNAi experiments

The variation in RNAi response within a particular order, and even specific species, suggests there are many barriers to successful RNAi, other than those specific to Lepidoptera, and highlights the need for carefully designed RNAi experiments.

1.10.1 Target gene selection

Target selection is important as RNAi sensitivity varies between genes (Silver *et al*, 2021). For example, although Baum *et al* (2007) reported that *D. virgifera virgifera* was highly susceptible to dsRNA targeted to an array of genes, dsRNA specific to a further 165 genes did not induce a robust RNAi response in this insect species. Hence, choosing an 'ideal' gene can increase RNAi efficiency without the use of more complex techniques. However, gene selection relies on genome-wide screening or previous literature regarding RNAi experiments in closely related species and, as RNAi is a relatively new technology, further research is necessary to help identify candidate gene pools (Silver *et al*, 2021). It is also important to avoid genetic redundancy to ensure that a secondary gene with a similar function cannot compensate for target gene silencing (Li *et al*, 2013).

1.10.2 Sequence specificity and gene region

The level of similarity necessary between dsRNA and target gene sequence varies between species. For example, *L. decemlineata* requires match rates to be at least 97% for maximum efficiency (He *et al*, 2020a) and different dsRNA transcripts targeting a VGSC gene in the Yellow fever mosquito, *Aedes aegypti*, caused varying levels of mortality (Melhorn *et al*, 2020). Contrastingly, Baum *et al* (2007) reported no variation in RNAi efficiency when different regions of the *D. virgifera virgifera* V-ATPase gene were targeted. Differences in

the preferred level of sequence similarity may be attributed to varying DICER cleavage patterns. For example, *O*. *furnacalis* and *H. armigera* dsRNA is often specifically cut at GGU nucleotide sequences but *T. castaneum* dsRNA is cleaved at AAG, GUG, and GUU sites. Furthermore, evidence that removal of these consensus sites reduced cleavage events and thus the amount of siRNAs produced (Guan *et al*, 2018b) suggests that RNAi efficiency may be reduced if the dsRNA sequence is not carefully considered.

Nevertheless, the dsRNA sequence should be highly specific to that of the gene being targeted as the plethora of siRNAs produced by DICER increase the chances of both off-target effects (silencing an unintended gene within the specific organism) (Jackson and Linsey, 2010) and accidental silencing of genes within non-target beneficial insects (Vogel *et al*, 2019). Thus, when designing dsRNA, it is important to consider not only the similarity between the dsRNA molecule and gene sequences within non-target organisms (NTOs) but also each individual siRNA molecule.

1.10.3 dsRNA concentration and length

The necessary dsRNA concentration for RNAi varies between each gene target and insect species (Joga *et al*, 2016) and increasing the concentration of dsRNA may not optimise gene silencing (Shakesby *et al*, 2009). Additionally, if multiple dsRNAs are administered, the concentration of both need to be considered to avoid dampened cellular uptake as a result of competition (Barik, 2006) and oversaturation of the RNAi machinery which can lead to mortality in an unintended manner (Tomoyasu *et al*, 2008). Furthermore, optimal dsRNA concentration can depend on development stage (Vogel *et al*, 2019) as most genes are not stably expressed throughout the insect life cycle (Griebler *et al*, 2008) therefore it is important to determine baseline mRNA transcript levels at each life stage prior to RNAi. Additionally, the length of dsRNA necessary to trigger RNAi varies from one insect to another (Bolognesi *et al*, 2012). Long-dsRNA ranging from 140-500 bp has generally been considered most successful in initiating RNAi (Joga *et al*, 2016) although much shorter (Miller *et al*, 2012) and longer (Huvenne and Smagghe, 2010) lengths have resulted in success.

1.10.4 dsRNA administration method

As mentioned, dsRNA is mainly administered to insects via oral feeding or direct haemolymph injection. In feeding assays, the artificial diet must be able to support the life cycle and development of the insect and not contain lethal substances (Mehlhorn *et al*, 2021) or interfere with the stability of dsRNA (Bachmann *et al*, 2020) to ensure mortality is the result of gene knockdown. Oral feeding is cost-effective and easy to perform (Tian *et al*, 2009) while being more practical for smaller insect species and early instars (Walshe *et al*, 2009). However, its main drawback is the variable ingestion of dsRNA (Yu *et al*, 2012). This can be overcome by haemolymph injection which delivers known dsRNA concentrations (Yu *et al*, 2012) and is also favourable for insects with specific barriers to RNAi, i.e. the presence of salivary and midgut nucleases. However, it is relatively more difficult, time-consuming and requires optimisation because needle size, injection site (Yu *et al*, 2012) and optimal injected volume (Jaubert-Possamai *et al*, 2007) vary between organisms. At best it provides 'proof of concept'.

1.11 Improving RNAi efficiency in lepidopteran insects

Although RNAi demonstrates great potential in insect pest control, barriers to its efficiency mean improvements are necessary, especially in the Lepidoptera. In particular, improving the susceptibility to orally induced RNAi is important to ensure that SIGS and HIGS-based approaches will be effective in the field.

1.11.1 Manipulation of dsRNA molecules

RNAi efficiency can be increased via various dsRNA manipulation methods. For example, in *A. aegypti*, paperclip dsRNAs (pcRNA), where the dsRNA sequence folds back on itself, exhibited higher stability in the presence of endonucleases and could reduce gene expression even when the primary dsRNA uptake mechanism was silenced (Abbasi *et al*, 2020) suggesting pcRNA are taken into cells through a different mechanism (Khajuria *et al*, 2018) and could overcome uptake issues. Chemical modifications such as the addition of methyl groups to dsRNA may also enhance RNAi efficiency but cost and various safety ethics must be considered (Nitnavare *et al*, 2021).

1.11.2 Nuclease inhibition

Chelating agents such as EDTA (ethylenediaminetetraacetic acid) can inhibit proteinases such as the nucleases present in the saliva, midgut and haemolymph of many insects (Castellanos *et al*, 2018) however EDTA could only cause increased mortality (compared to control) for one of two target genes in *Euschistus heros*, so further research into its effectiveness is necessary (Castellanos *et al*, 2018).

1.11.3 Polymer-based nanoparticles

Polymer-based nanoparticles, such as those containing chitosan, can encapsulate, protect and increase the stability of dsRNA to enable orally-fed dsRNA to be delivered to its target site without nuclease degradation (Christiaens *et al*, 2018a). Chitosan-dsRNA polymer complexes protect dsRNA via the interaction of electrostatic forces between the positively charged amino groups in chitosan and negatively charged phosphate groups on the dsRNA. They are technically simple, inexpensive to produce (Zhang *et al*, 2015), biodegradable and unlikely to harm non-target beneficial insects as they are a natural component of arthropod exoskeletons (Silver *et al*, 2021). However, they rely on incorporation into foliar sprays (or topical application) and therefore may not be effective in insects that burrow through stems/leaves that may not come into contact (Silver *et al*, 2021). Nevertheless, chitosan-based nanoparticles have enhanced gene silencing, increased mortality and reduced adult emergence in *A. aegypti* (Kumar *et al*, 2016), while enhancing dsRNA stability in the midgut of *S. frugiperda* (Gurusamy *et al*, 2020) and the European corn borer, *Ostrinia nubilalis* (Cooper *et al*, 2020a). Furthermore, *S. frugiperda* mortality was enhanced and transcript levels were suppressed (Gurusamy *et al*, 2020) but these remained unchanged in *O. nubilalis* (Cooper *et al*, 2020a) suggesting their ability to enhance RNAi efficiency is insect-specific.

The efficacy of other polymers in enhancing RNAi efficiency has also been tested, such as core-shell nanoparticles and star polycations which, like chitosan, are attracted to negatively charged dsRNA molecules. Core-shell nanoparticles enabled dsRNA targeted to the chitinase *CHT10* gene to cross *O. furnacalis* cell membranes intact, leading to stunted growth and 100% larval mortality (within 5 days) in feeding trials (He *et al*, 2013) but further research into their impact on other pest species is necessary. Star polycations incorporating dsRNA targeting the *V-ATPase* gene caused 80% transcript level reduction as

well as > 70% mortality in the aphid species *Aphis glycines* (Soybean aphid) (Yan *et al*, 2020). Interestingly, these polymers seem to penetrate the aphid cuticle, which potentially could overcome current RNAi barriers. However, further research is necessary to determine their use in other insect species as well as their stability and efficacy in the field (Silver *et al*, 2021).

Another alternative is the use of guanylated polymers which can protect dsRNA against nucleolytic activity even in high pH environments, such as that displayed by the lepidopteran midgut, for up to 30 h (Christiaens *et al*, 2018a). These polymers were taken up by midgut cells and enhanced dsRNA stability in the presence of *Spodoptera exigua* (Beet armyworm) nucleases. Furthermore, transcript levels were reduced (albeit moderately), larval development was stunted with slower weight gain, mortality increased by ~ 30% and none of the surviving individuals reached adulthood. However, the effect of these polymers against other pest insects is unknown (Silver *et al*, 2021), highlighting the need for further research.

1.11.4 Peptide-based nanoparticles

Peptides can also be complexed with dsRNA to improve RNAi efficiency in insects. When dsRNA specific to the *BiP* gene was integrated within aliphatic peptide capsules, mortality and transcript suppression improved compared to naked dsRNA administration in *A. pisum* (Avila *et al*, 2018), an aphid species generally recalcitrant to orally induced RNAi (Cao *et al*, 2018). Furthermore, cell-penetrating peptides such as the peptide transduction domain (PTD) can be fused with the dsRNA binding domain (DRBD), to create the chimeric protein PTD-DRBD which, when combined with dsRNA, creates ribonucleotide particles (RNPs) (Gillet *et al*, 2017). RNPS can protect dsRNA from gut nucleases and enhance cell internalisation to allow endosomal escape and deliver dsRNA to its site of action (Wadia *et al*, 2004; Eguchi *et al*, 2009). Gillet *et al* (2017) reported that the complexation of PTD-DRBD with dsRNA specific to the *chitin-synthase 2* gene improved dsRNA stability and significantly reduced gene expression in larvae of the boll weevil *Anthonomus grandis*.

1.11.5 Liposomes

Liposomes can encapsulate and protect dsRNA as their lipid bilayer can be loaded with nucleic acids (Hirko *et al*, 2003). Although the method by which liposomes enhance the

RNAi effect is not well documented (Lin *et al*, 2017), they are able to delay degradation and thus increase the efficiency of dsRNA in insects (Castellanos *et al*, 2018). Lin *et al* (2017) found a significant reduction in the expression of α-tubulin in the German cockroach, *Blattella germanica*, when dsRNA targeted to this gene was contained within a lipoplex, however protection from midgut nucleases was time dependent and declined significantly after 24 h, although significant mortality was still achieved after 16 days of continuous feeding. Furthermore, Taning *et al* (2016) found liposome-mediated dsRNA delivery led to significant silencing of three genes and also significant mortality in the Spotted wing drosophila, *Drosophila Suzukii*, an insect that lacks SID genes and therefore most likely relies on endocytosis (Wynant *et al*, 2014a) which may be too slow to allow for a robust RNAi response (Taning *et al*, 2016). However, liposome-based methods are expensive and higher-throughput techniques are needed to make largescale open-field dsRNA delivery affordable (Tayler *et al*, 2019b), especially for farmers in economically less developed countries. Also, as dsRNA complexed with liposomes can degrade within just 2 h in the presence of salivary nucleases (Castellanos *et al*, 2018) they may not be effective in all species.

1.11.6 Immune system priming

Immune system priming can also increase RNAi efficiency. For example, Fan *et al* (2022a) reported that pre-injection of dsRNA specific to the Aequorea Victoria green fluorescent protein (e*GFP*) gene into *O. furnicalis* led to upregulation of two genes responsible in the RNAi mechanism (*OfDicer2* and *OfAgo2*). Consequently, the expression of two experimental genes, *OfEF1α and OfCTP8,* was reduced by 46.9% and 44.1% in the haemolymph and midgut, respectively for *OfEF1α*, and 91.9% and 80.0% in the haemolymph and integument,respectively for *OfCTP8*. However, mortality or changes to the phenotype were not recorded so further research into the efficacy of this method in the control of insect pests is necessary.

1.11.7 dsRNA-expressing bacteria

Engineering bacteria to express target-specific dsRNA can also enhance RNAi efficiency as, upon ingestion by the insect, the bacteria provide a more sustained dsRNA release which may help overcome rapid nuclease degradation (Christiaens *et al*, 2020b). *Escherichia coli* is often used but, to avoid dsRNA degrading nucleases (e.g. RNA III), the correct strain must

be chosen (e.g. HT115) (Timmons *et al*, 2001). Ganbaatar *et al* (2017) found that orally fed bacteria expressing two dsRNAs specifically targeted to two chitinase genes caused reduced body weight and increased mortality in *Mythimna se*para*te* (Northern armyworm) larvae. Furthermore, bacteria expressing dsRNA targeted to the immune suppressive gene *Sl102* in *S. littoralis* enhanced the mortality caused by a *B. thuringiensis* based biopesticide (Caccia *et al*, 2020). Dhandapani *et al* (2020) also reported 100% mortality in *Anoplophora glabripennis (Aisna long-horned beetle)* larvae and adults through bacterially expressed dsRNAs targeting the *Snf7* gene and Bento *et al* (2020) reported bacteria expressing dsRNA specific to the *arginine kinase* gene caused 70% larval mortality. Overall, bacterial expression of dsRNA has enhanced RNAi efficiency in a variety of insect orders, highlighting that only optimization of the target gene may be necessary (Silver *et al*, 2021).

1.11.8 Engineering symbionts

Symbiont-mediated dsRNA delivery can also increase RNAi efficiency. For example, Whitten *et al* (2016) reported systemic knockdown of two salivary Nitrophorin genes by engineering the bacterial symbionts of the kissing bug (*Rhodnius prolixus*) and the Western flower thrip (*Frankliniella occidentalis*) to produce target-specific dsRNA. The silencing effect was long-lasting and significantly higher than when the same transcript was injected or orally delivered. This method could improve dsRNA delivery in a range of smaller insects, such as aphids or early larval instars, in which haemocoel injection is difficult and causes trauma to the insect (Silver *et al*, 2021).

1.11.9 Fungal-induced gene silencing

Fungal-induced gene silencing (FIGS) can also enhance RNAi efficiency in insect pests. Firstly, fungi can be engineered to express target-specific insect dsRNA (Van Ekert *et al*, 2014) e.g. dsRNA production by *Saccharomyces cerevisiae* (Mysore *et al*, 2019) and *Pichia pastoris* (Van Ekert *et al*, 2014) significantly increased larval mortality and delayed development in the dipteran *A. aegypti*. However, their effectiveness against other insect orders is yet to be investigated (Silver *et al*, 2021). Fungi can also be engineered to express dsRNA that enhances their own virulence (Chen *et al*, 2015). For example, the fungal species *Isaria fumosorosea* was engineered to express dsRNA targeting the *Bemisia tabaci* (Silverleaf whitefly) toll receptor gene, *TLR7* (important in pathogen recognition) and its ingestion led to a 20 % increase in nymph mortality and a 40 % reduction in gene

expression (Chen *et al*, 2015). Similarly, expression of the *L. migratoria ATP synthase* gene by *Metarhizium acridum* increased mortality and reduced transcript suppression in *L. migratoria* (Hu *et al*, 2019), however as administration was via haemocoel injection in this case, further research into its effectiveness via regular fungal infection routes is necessary (Silver *et al*, 2021).

1.11.10 Virus-induced gene silencing

Virus-induced gene silencing (VIGS) can also enhance RNAi efficiency in insects. For example, engineering an *A. aegypti* densovirus to produce short hairpin RNAs targeting the *V-ATPase subunit A* gene led to systemic viral infection, suppressed V-ATPase transcript levels and reduced survival in *Aedes albopictus* (Gu *et al*, 2011). Another approach is to engineer plant viruses to produce dsRNA specific to an insect pest. Mulot *et al* (2016) reported suppressed transcript levels of two target genes in *M. persicae* when tobacco plants were infected with engineered tobacco rattle virus (TRV) expressing *M. persicae* specific dsRNA. Therefore, VIGS shows potential in RNAi-based pest control methods, especially considering the range of viruses that either directly infect insects or plants that are eaten by insects. However, as this approach involves infecting a plant with a live virus, concerns surrounding safety as well as possible impacts on yield or crop appearance may arise (Silver *et al*, 2021).

1.11.11 Virus like particles

An alternative to VIGS are viral-like particles (VLP), where dsRNA is enclosed in viral capsid proteins (Silver *et al*, 2021), which similarly protect dsRNA and increase cellular uptake (Christiaens *et al*, 2020b). Although the use of VLPs has not been investigated in insects, they show promise in vertebrate systems (Christiaens *et al*, 2020b) and are potentially advantageous as, unlike viruses, they cannot replicate and are therefore unlikely to negatively affect crop productivity or suppress plant RNAi machinery (Kolliopoulou *et al*, 2020) which may overcome the biosafety or public acceptance concerns associated with genetically engineered viruses (Christiaens *et al*, 2020b).

1.12 RNAi biosafety considerations

RNAi has the potential for species-specific targeting of insect pests via dsRNA-based biopesticides and therefore the ability to overcome many of the environmental safety concerns associated with chemical insecticides. As dsRNA molecules are naturally involved in eukaryote immunity, they are widespread throughout animals and plants and therefore already safely consumed by humans (The organistaion for Economic Co-operation and Development, OECD, 2020) and due to the presence of nucleases and barriers to cellular uptake in the gut of both humans and vertebrates, dsRNA ingestion is unlikely to cause harm (Petrick *et al*, 2013). However, all new technologies have potential risks which must be evaluated before implementation in the field (Fletcher *et al*, 2020). Furthermore, as genetically modified organisms (GMOs) are perceived negatively by the public, the cost and time associated with regulatory approval is high, so the use of SIGS is generally preferred over HIGS (Fletcher *et al*, 2020).

1.12.1 Regulatory framework and risk assessments

Before SIGS-based biopesticides can be used in the field, it important to have risk assessments and regulatory frameworks in place (De Schutter *et al*, 2021). The definition of dsRNA based biopesticides varies between countries, with the USA regarding SIGS-based products as biochemical pesticides as, compared to chemical pesticides, they are inherently less toxic due to the inclusion of natural compounds (Dietz-Pfeilstetter *et al*, 2021). However, these novel biopesticides still require EPA registration which bases approval on a risk/benefit basis meaning that there should be no adverse effects to humans or the environment (Dietz-Pfeilstetter *et al*, 2021). In Europe, HIGS-based products are considered as genetically modified organisms and different regulations have to be considered if these plants are intended for food or livestock feed (De Schutter *et al*, 2021) and SIGS-based products are authorised differentially depending on whether or not they contain viable genetically modified (GM) organisms or just purified molecules (Schenkel and Gathmann, 2021).

1.12.2 Exposure routes

The biosafety of a dsRNA molecule against a specific NTO depends largely upon whether the NTO will come into contact with it. The various exposure routes are outlined in Figure 1.6 and include direct exposure either through topical application or via consumption of plant material or contaminated pollen. Natural enemies such as predators, parasites and

parasitoids may also be exposed via feeding on the target pest and soil or aquatic organisms could be exposed via dsRNA leeching, spray drifting and surface run-off (Bachman *et al*, 2020) into the surrounding environment. These effects can also be expanded to non-target vertebrates and humans.

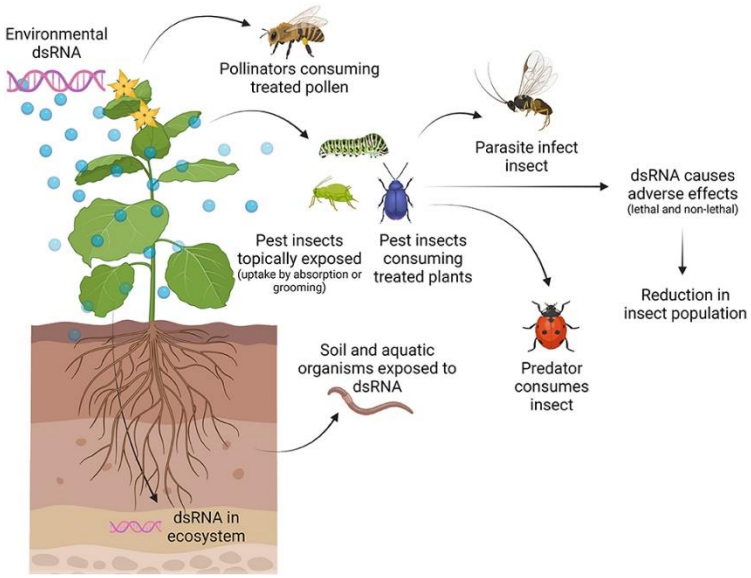

Figure 1.6 Exposure pathways of target and non-target organisms to externally applied dsRNA. Source: De Schutter *et al* (2022).

1.12.3 Environmental persistence

Exposure of an NTO to a dsRNA molecule is also dependent upon how long dsRNA persists in the environment post-application. Dubelman *et al* (2014) reported that time taken for dsRNA specific to the *D. virgifera virgifera snf7* gene to degrade by 90% in three soils with differing physiochemical properties was < 35 h and also concluded that dsRNA is unlikely to accumulate in soils regardless of clay content or pH. Joaquim *et al* (2019) also reported similar degradation in Brazilian tropical soils and Fischer *et al* (2016) reported that two dsRNAs of different sizes and structures (linear or hairpin) also degraded rapidly in soil. Furthermore, although dsRNA can persist in sterile water in laboratory settings, it is quickly degraded in aquatic environments where its half-life is less than three days (Fischer *et al*, 2017), likely due to microbial degradation (Bachman *et al*, 2020). However, product formulation, as well as the formulation itself, may impact the persistence of dsRNA and therefore will need to be considered in risk assessments (De Schutter *et al*, 2021).

1.13 Avoiding unintended gene silencing in NTOs

There are two main ways to explore, and aim to minimise, the chance of unintended gene silencing in NTOs. The first is via an *in silico* bioinformatics approach and the other involves the use of toxicology tests to determine the effects of dsRNA exposure on NTOs. Bioinformatics can aid in the avoidance of dsRNA sequences with significant off-target NTO complementarity (Vaishnaw *et al*, 2010) by assessing sequence similarity using online tools such as BLAST (Basic Local Alignment Search Tool) (Altschul *et al*, 1990), thus minimising off-target binding (Christiaens *et al*, 2018b) and reducing the uncertainty surrounding the risk associated with a specific dsRNA molecule. However, this approach has various problems such as its reliance on genomic sequence data which is not available for all NTOs and, as the degree of sequence similarity necessary to trigger RNAi is not currently known, there is potential for silencing even with mismatches (OECD, 2020). Furthermore, Santos *et al* (2019) reported that the sequence length of siRNA produced by the DICER enzyme is species specific and therefore, because the abundance of each siRNA is an important factor in determining RNAi susceptibility (OECD, 2020) the production of more siRNAs (as a result of shorter sequences) could affect the insect response to dsRNA exposure. Furthermore, the level of base-pairing between the siRNA molecules and guide strand is not the only factor determining silencing effect and high sequence similarity does not necessarily infer a hazard (OECD, 2020). As previously mentioned, not all organisms are susceptible to RNAi due to the presence of uptake barriers and endonucleases and therefore the dsRNA may not reach the site of action and therefore not elicit a response even if a sequence is highly similar. Examples include the lack of detrimental effect on larval development, survival, life span and gene expression observed when the beneficial pollinator *Apis mellifera* (Western honeybee) was exposed to dietary *V-ATPase subunit A* dsRNA specific to both *D. virgifera virgifera* and *A. mellifera* (Vélez *et al*, 2016) and the lack of response to dietary *V-ATPase subunit A* dsRNA specific to *D. virgifera virgifera* or *Danaus plexippus* (the monarch butterfly) (Pan *et al*, 2017). Therefore, bioinformatics analyses are not a sufficient basis for hazard risk assessment on their own (Dix *et al*, 2006) and further testing (e.g. via toxicology tests) is essential to determine potential hazards of dsRNAs on NTOs. However, bioinformatics studies can be useful in the design and spectrum of toxicology tests (OECD, 2020) by identifying organisms with the highest sequence similarity, that should therefore be included in toxicology tests, and those with none/very little sequence similarity that

should be omitted (Christiaens *et al*, 2018b), which consequently saves labour while reducing costs.

1.14 Progress towards commercial RNAi-based products

RNAi shows great potential for the control of insect pests. Consequently, the first HIGs based product to aid in pest control, "SmartStax Pro" by Bayer, was approved by US and Chinese regulators in 2017 and 2021, respectively. This transgenic maize combines the expression of *B. thuringiensis* Cry3Bt1 toxin with glyphosate resistance alongside the expression of dsRNA targeting the *Snf7* gene in *D. virgifera virgifera*. SmartStax Pro will be available to farmers in the US in 2022 and Canada in 2023 and has been authorised in Europe for all uses except cultivation (De Schutter et al, 2022). However, the use of RNAi is not constrained to pest control. For example, several HIGs based products, using RNAi to improve crop quality, have been authorised for commercialisation. For example, Bayer's "Vistive gold", a genetically modified high oleic and glyphosate resistant soybean variety, has been approved for use as food and feed in the EU market and for food, feed and cultivation in the USA, Canada, and Japan (ISAAA, 2021). Also, the U.S. department of agriculture (USDA) recently deregulated Simplot's GM potatoes, in which RNAi technology prevents potato bruising and improves starch quality through targeting the PPO5 (enzyme polyphenol oxidase) gene (Waltz, 2015).

Due to negative public opinion regarding the safety of genetically modified plants (Zotti et al, 2018), the time taken for regulatory approval (De Schutter et al, 2022) and the lack of technology available for the genetic transformation of some species (Rank and Koch, 2021) non-transgenic SIGs based products are currently favoured (He et al, 2022). Nevertheless, mass commercialisation of these products will require stringent risk assessments to minimize possible risks posed to NTOs and the environment. Although genetically modified plants utilising RNAi technology are assessed using existing regulatory framework, there are currently no appropriate safety evaluations or authorization procedures for SIGS-based products (De Schutter et al, 2022). Consequently, the European Food Safety Authority (EFSA), the Australian Pesticides and Veterinary Medicines Authority (APVMA) and the US Environmental Protection Agency (EPA) utilize existing regulatory frameworks for plant protection products, agricultural chemical products and biochemical pesticides, respectively (He et al, 2022). Although a set of recommendations for the assessment of risks associated with dsRNA based products was recently developed by the OECD (OECD, 2020), the

development of a consensus regulatory framework will aid worldwide commercialization of SIGs based products.

1.15 Research rationale and aims

Most current commercial insecticides harm NTOs and the environment, highlighting the importance for safer, more sustainable 'biopesticides'. Consequently, this project will exploit the targets of the chemical insecticides most commonly used to control *S. littoralis*, pyrethroids, organophosphates (Hilliou *et al*, 2021) and spinosyns (El-Sayed et al, 2023),via RNAi based strategies (Figure 1.7). It is hypothesised that targeting these genes will elicit mortality similar to that achieved by chemical insecticides but, due to careful dsRNA design, with less impact on NTOs. Furthermore, novel methods of increasing the efficacy of RNAi in a recalcitrant species will be explored.

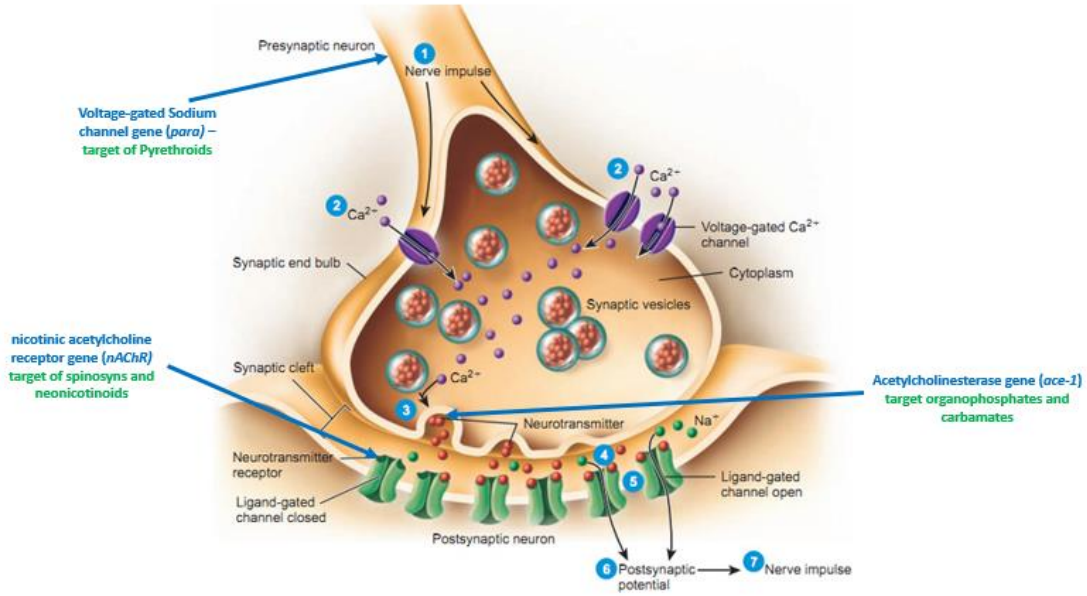

Figure 1.7 **Location of gene targets used in the present study. Blue arrows and text indicate the name of the target gene and its positioning within the CNS and green text indicates the most common insecticide class(es) that act on the specific gene. Source: Antranik (2023).**

1.16 Research objectives

1. To determine the effect of administering dsRNA specific to three genes essential to central nervous system functionality (acetylcholinesterase, *ace-1*; voltage-gated sodium channel gene, *para* and nicotinic acetylcholine receptor, *nAChR*) on the survival of *S. littoralis* and to decipher the role of the *NADPH cytochrome P450 reductase* gene in insecticide detoxification (**Chapter 3**).

2. To increase RNAi efficacy in *S. littoralis* using a nanoparticle-based delivery system and immune system priming (**Chapter 4**).
3. To Determine the biosafety of dsRNA specific to the *S. littoralis ace-1*, *para, nAChR* and *NADPH cytochrome P450 reductase* genes (ds*ace-1*, ds*para*, ds*nAChR* and ds*NADPHcytP450r*) against the non-target beneficial insect *Bombus* terrestris (Buff-tailed bumblebee) (via *in vitro* studies) and a range of non-target organisms (via bioinformatics) (**Chapter 5**).

Chapter 2. Materials and methods

2.1 *S. littoralis* rearing

An *S. littoralis* culture was provided by the University of Napoli Federico II (Italy). Larvae were reared on an agar-based artificial diet (125 g pinto bean, 100 g wheat germ, 50 g soy protein, 50 g casein, 62.5 g torula yeast, 6 g abscorbic acid, 5 g methyl paraben, 3 g sorbic acid and 17.5 g/l agar) at 25 ± 1° C, 70 ± 5 % RH, with 16:8 h light-dark period. Adults were maintained on a liquid-based diet (50 g sucrose, 60 g honey, 1 L distilled water) under the same rearing conditions.

2.2 RNA extraction and cDNA synthesis

RNA was extracted from *S. littoralis* larva (instars 1-6), pupa or adults or *B. terrestris* adults, depending on specific experiment, using the PureLink® RNA Mini Kit with Trizol® reagent (Ambion). Genomic DNA was subsequently digested with PureLink® DNase (Invitrogen), per manufacturer's instructions. The purity and concentration of extracted RNA were confirmed spectrophotometrically with a NanoDrop® (ND-1000) (Thermo Fisher Scientific) and cDNA was synthesised from 1 µg of RNA (SensiFAST™ cDNA Synthesis Kit) per manufacturer's instructions (Bioline).

2.3 Primer design

The National Centre for Biotechnology Information (NCBI) database identified mRNA sequences for the *S. littoralis ace-1* (GenBank accession KC961944.1)*, ace-2* (GenBank accession KC961945.1) *NADPHcytP450r* (GenBank accession JX310073.1) and bacterial kanamycin resistance genes (*Kan*) (GenBank accession JN638547.1). The mRNA sequences for the *para* and *nAChR* as well as the housekeeping genes *RPL13A*, *EF1 α-factor*, and *β-actin* were provided by supplementary material in Roy *et al* (2016). Primers for use in RT-qPCR experiments were designed using Primer3plus (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Table 2.1) and those used to amplify dsRNA template regions were designed using E-RNAi (https://www.dkfz.de/signaling/e-rnai3/). To enable subsequent dsRNA synthesis, T7 tags were appended to these primer sequences. Primers for use in RT-qPCR were designed to bind to the 3' end of the target sequence to avoid binding to the dsRNA construct. The specificity of all primer pairs were assessed using the 'ThermoFisher multi primer analyzer' (ThermoFisher Scientific, 2023) and 'PCR primer stats' (SMS, 2023).

2.3.1 Degenerate primer design

To design degenerate primers for use in subsequent PCR experiments, the NCBI database identified *nAChR* amino acid sequences in phylogenetically closely related lepidopteran insects; *T. absoluta* (GenBank accession ALM23508.1), *Plutella xylostella* (GenBank accession AMH87607.1), *Cydia pomonella* (GenBank accession AJA39821.1), *C. suppressalis* (GenBank accession AKQ12751.1) and *B. mori* (GenBank accession NP_001091842.2). The *nAChR* sequence of the dipteran *Drosophila melanogaster* was included (GenBank accession >NP_995674.1) to ensure a highly conserved region was identified. Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) aligned the sequences to enable identification of highly conserved regions. The regions 5'-FITNGEW-3' and 5'-MFMICNF-3' were used for degenerate primer design due to reduced degeneracy compared to other regions and the forward '5'-TTYATHACNAAYGGNGARTGG-3' and reverse 5'-CATRAACATDATRCARTTRAA-3' primers were designed using IUPAC nucleotide codes (https://www.bioinformatics.org/sms/iupac.html).

Table 2.1 Gene specific primers used in RT-qPCR, degenerate PCR and to synthesise gene specific dsRNA templates.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product size (bp)
ace-1	CCCCAAGGAAGAGAATGTAGG	CGAACACAATAGCCTGTCTGC	104
ds*ace-1*	ATCCCAACACAGACATGCAG	TCGGATTCCTCTCAAAATGC	484
ds*ace-1*_T7	TAATACGACTCACTATAGGGAGA ATCCCAACACAGACATGCAG	TAATACGACTCACTATAGGGAGAT CGGATTCCTCTCAAAATGC	530
ace-2	TGGGGCGTTCGGATTTTTATACT	ACGAATAGCGAGTTGTTGATCCC	100
para	AAAACGATGAGAACGCTGCG	CACGTTGAAGATGGACGGGA	114
ds*para*	CAGTGATAACGAAGCCATGC	TCAGTTGGTATTGCGGTCAG	499
ds*para*_T7	TAATACGACTCACTATAGGGAGA CAGTGATAACGAAGCCATGC	TAATACGACTCACTATAGGGAGAT CAGTTGGTATTGCGGTCAG	545
nAChR	CTTCACTGGTCGGAAGTCGT	GCCCCTTGTGACTTGTGACT	112

ds*nAChR*	TGTCCATCTCACTTGGGTCA	CAGGCTGGAAATTGCTGAAC	486
ds*nAChR*_T7	TAATACGACTCACTATAGGGAGA TGTCCATCTCACTTGGGTCA	TAATACGACTCACTATAGGGAGAC AGGCTGGAAATTGCTGAAC	532
NADPHcytP450r	ACCCCTGCAAAGTCAAACC	CATTGTCTCCCACTTCTTTGC	122
dsNADPHcytP450r	CTTTGAACTTGGGCTTGGAG	TGCTTTCCTGGTCAGTGTTG	473
dsNADPHcytP450r_T7	TAATACGACTCACTATAGGGAGA CTTTGAACTTGGGCTTGGAG	TAATACGACTCACTATAGGGAGAT GCTTTCCTGGTCAGTGTTG	519
dsKan	TGCTCCTGCCGAGAAAGTAT	AGAACTCGTCAAGAAGGCGAT	470
dsKanT7	TAATACGACTCACTATAGGGAGA TGCTCCTGCCGAGAAAGTAT	TAATACGACTCACTATAGGGAGAA GAACTCGTCAAGAAGGCGAT	516
RPL13A	AAGTAGGCTGGAAGTACCG	CCTTCGTGATTCTCTTCAAC	108
β-actin	TGTTTGAGACCTTCAACTCC	GAGCGTAACCTTCGTAGATG	144
EF1 αfactor	CTGGTGACTCCAAGAACAAC	ATCCAGCACAGGTGTGTATC	110
nAChR Degenerate	TTYATHACNAAYGGNGARTGG	CATRAACATDATRCARTTRAA	342

2.4 Polymerase Chain Reaction

Synthesized cDNA was used as a template for subsequent PCR reactions to confirm the efficacy of each primer pair in amplifying only the target sequence. PCR was conducted with the T100™ Thermal Cycler (Bio-rad) using reactions containing 25 µL PCR Master Mix (2X) (Thermo Fisher Scientific), 1 µL forward and reverse primers, 2 µL template cDNA and nuclease free water up to 50 µL volume. Cycling conditions were initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at 59 °C (*ace-1*), 60 °C (*ace-2*), 60 °C (*para*), 59 °C (*nAChR*), 58 °C (*NADPHcytP450r*), 56 °C (*RPL13A*), 54 °C (*EF1* αfactor), 54 °C (*β-actin*) for 30 s and extension at 72 °C for 15 s, with a final extension step at 72 °C for 5 min.

For degenerate PCR, reactions contained 25 µL PCR Master Mix (2X) (Thermo Fisher Scientific), 3 µL forward and reverse degenerate primers, 2 µL template and nuclease free water up to 50 µl volume. Various annealing temperatures were used and reaction conditions were initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at (48.0, 47.7, 47.3, 46.7, 45.9, 45.2 and 44.7 °C) for 30 s and extension at 72 °C for 20 s with a final extension step at 72 °C for 10 min. Agarose gel electrophoresis identified multiple bands, so the 342 bp band corresponding to an annealing temperature of 46.7 °C was selected and re-amplified via band-stab PCR (Bjourson and Cooper, 1992) through a 1/40 dilution in Diethyl pyrocarbonate (DEPC) water.

2.5 Sequence verification of degenerate target sites

To check for correct amplification, PCR products were purified with the QIAquick PCR purification kit (Qiagen) per manufacturer's instructions. Purified products were ligated into the pCR®2.1 vector and subsequently transformed into competent *E. coli* using the TA Cloning™ Kit (ThermoFisher) (per manufacturer's instructions). After plating the transformation on LB agar (40 mg/ml X-Gal, 100 µg/ml ampicillin and 100 mM Isopropyl β-d-1-thiogalactopyranoside, IPTG), white colonies (selected by blue-white screening) were grown in LB broth. Plasmids were purified with the QIAprep Spin Miniprep Kit (Qiagen) per manufacturer's instructions and ECORI digestion, followed by agarose gel electrophoresis, was used to identify the presence of the insert, which was subsequently sequenced (DBS genomics, Durham University). Sequenced inserts were aligned with NCBI identified sequences using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and *in silico*

translation via ExPASy (https://web.ExPASy.org/traslate/) determined the amino acid sequence.

2.6 dsRNA synthesis

Template sequences for use in subsequent dsRNA synthesis were amplified via PCR using specific primers (Table 2.1) and were transformed into *E. coli* and sequenced as described in section 2.5. Purified plasmids were used as a template for PCR, using T7 promoter primers (Table 2.1) which enable the generation of a dsRNA template with a binding site for T7 RNA polymerase. PCR conditions were initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing at (56 °C for *ace-1*, 57 °C for *para*, 58 °C for *nAChR*, 57 °C for *NADPHcytP450r* and 59 °C for Kan) for 30 s and extension at 72 °C for 30 s with a final extension step at 72 °C for 10 min. dsRNA was synthesized from the T7 dsRNA template using the MEGAscript™ RNAi Kit (Ambion®). Reactions contained 1 µg T7 dsRNA template, 2 µl 10X T7 reaction buffer, 2 µl of each ribonucleotide solution (ATP, CTP, GTP, and UTP) and 2 µl T7 enzyme mix. Reactions were incubated at 37°C overnight, then 75°C for 5 min, then cooled to RT and agarose gel electrophoresis subsequently confirmed the synthesis of dsRNA specific to *S. littoralis ace-1*, *para*, *nAChR*, *NADPHcytP450r* and the bacterial kanamycin resistance gene (which will now be referred to as ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and ds*Kan*). Nuclease digestion was performed to remove DNA and ssRNA then dsRNA was purified, eluted in 10 mM Tris-HCl, 1mM EDTA and stored at -20°C per MEGAscript™ RNAi Kit manufacturer's instructions. Due to time constraints, further dsRNA was synthesised by agroRNA (http://genolution.co.kr/agrorna/service-overview/) which was provided in liquid form and subsequently stored at -20°C.

2.7 Reverse transcription quantitative PCR (RT-qPCR)

2.7.1 Primer efficiencies

Prior to expression studies, the efficiency of primer pairs designed to amplify sections of the *S. littoralis ace-1*, *ace-2*, *para*, *nAChR*, *NADPHcytP450r*, *RPL13A, β-actin* and *EF1 α-factor* was determined via RT-qPCR. RNA extraction (5 individuals per replicate) and cDNA synthesis were conducted as described in section 2.2. A 1in10 dilution series of target cDNA (with three technical replicates) was prepared and each dilution was used as a template in subsequent 2-step RT-qPCR reactions using a Rotor-gene Q (Qiagen) real-time PCR system with 2X sensifast SYBR no-rox mix (Bioline) and gene specific primers (Table 2.1), following

manufacturer's instruction. Cycling conditions were polymerase activation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. Subsequent melt-curve analyses confirmed that the amplified products were specific via a single peak for each amplification. Amplification efficiency of each primer pair was determined using the slope of the line generated after plotting mean CT value against the logarithm of a 10-fold cDNA serial dilution via the Thermofisher qPCR primer efficiency calculator (Thermofisher, 2023b).

2.7.2 Endogenous gene expression

Endogenous expression of each *S. littoralis* specific gene was determined in larval instars 1-6, pupae and adults via RT-qPCR analysis. RNA was extracted from each life stage with ~20, 16, 12, 8, 4 and 2 insects per sample for *S. littoralis* instars 1-6, respectively, and 2 individuals per sample for RNA extractions from *S. littoralis* pupa and adults. cDNA was synthesised as described in section 2.2, then RT-qPCR was conducted as described in section 2.7.1. Each life stage was analysed in duplicate with three technical replicates for each and relative transcript quantity was determined using the Pfaffl $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001) with gene of interest expression normalised against the *RPL13A* reference gene. Fold change was calculated relative to expression at the 1st larval instar stage.

2.7.3 Relative expression studies

For relative expression studies, RNA was extracted from representative insects from each experimental group with the number of individuals per sample as described in section 2.7.2, depending on the life stage being investigated. Subsequently, 1 µg RNA was used to synthesise cDNA as described in section 2.2. In each case, two biological replicates, each split into three technical replicates, were used to determine relative transcript levels which were normalised against the *RPL13A* reference gene and were calculated relative to a calibrator sample (defined in each figure legend throughout experimental chapters). Data was analysed as described in section 2.7.2 and the specific time point at which gene expression was determined is highlighted throughout each experimental chapter. Additionally, as data are represented as relative expression, error bars are not used as it is only reasonable to use these when displaying raw CT values.

2.8 Delivering dsRNA to S. littoralis

2.8.1 Delivery via feeding

To determine the effect of feeding *S. littoralis* specific dsRNA to fourth instar larvae, 40 µl of a 0.2 µg/µl dsRNA solution (in DEPC water) specific to ds*ace-1*, ds*para*, ds*nAChR* was applied to the surface of a small section of artificial diet (the weight of which was previously determined by average diet consumption of larvae at the same instar stage) and left to percolate throughout at room temperature (RT). For insects given a combination of ds*ace-1* + ds*nAChR*, 20 µl of a 0.2 µg/µl solution containing dsRNA specific to each gene was added simultaneously. Controls were insects reared on artificial diet ± 40 µl of 0.2 µg/µl ds*Kan* percolated throughout. Each insect was provided with the same weight of artificial diet and were reared in plastic chamber pots (25 mm X 25 mm) under conditions described in section 2.1. After 2 days, insects were transferred to artificial diet for the remainder of the trial which was replaced every 2 days (until pupation) to avoid insect starvation and diet contamination and desiccation. Three biological replicates containing 15 insects were used for each treatment. Insects were monitored each day and survival, pupation and emergence were recorded. Insects were left under experimental conditions for a length of time past the average time taken for an untreated culture of insects to emerge. Gene expression was determined via 48 after experiments began as described in section 2.7.3.

To determine the effect of continuously feeding dsRNA, neonate larvae were reared on artificial diet ± 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r*, 4 µg ds*NADPHcytP450r* or 1 µg ds*ace-1* + 1 µg ds*nAChR* or 2 µg ds*ace-1* + 2 µg ds*nAChR* percolated throughout. For those provided with 1 µg dsRNA, 10 µl of a 0.1 µg/µl dsRNA solution was applied to the surface of a small section of artificial diet (the weight of which was previously determined by average diet consumption of larvae at the same instar stage) and left to percolate throughout at RT. For those provided with 2 µg and 4 µg dsRNA, 10 µl of a 0.2 µg/µl and 0.4 µg/µl dsRNA solution was applied to the diet, respectively. For those provided with 1 µg + 1 µg ds*ace-1* + ds*nAChR* in combination, 5 µl of 0.2 µg/µl of each was applied to the diet and for those provided with 2 µg + 2 µg ds*ace-1* + ds*nAChR* in combination, 5 µl of 0.4 µg/µl was applied to the diet. Insects were reared in plastic chamber pots (25 mm X 25 mm) under conditions described in sections 2.1 and diets were replaced every two days but weight of diet increased to accommodate larval size (predetermined by average diet consumption by insects at the same larval stages) and

insects were transferred to artificial diet after 9 days. Trials consisted of three groups each with 15 insects. Insect weight was recorded 6 and 9 days after experiments started and survival was monitored every day for 12 consecutive days. Pupation and emergence were recorded and insects were left under experimental conditions for a length of time past the average time taken for an untreated culture of insects to emerge. Pupal weight was recorded 24 h after pupation to avoid variation due to moisture content which is high in freshly-formed pupae (Gong *et al*, 2021) and gene expression was determined after 9 days of continuous feeding (as described in section .7.3).

To deliver dsRNA via gavage feeding, the mouths of 5th instar larvae were gently opened and dsRNA was delivered directly to the gut via 1700 series gastight syringes with N termination (Hamilton). Insects received either 8 µg ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r*, ds*Kan* or 4 µg each of ds*ace-1* and ds*nAChR*. Those gavaged with 8 µg dsRNA received 5 µl of a 1.6 µg/µl dsRNA solution (in DEPC water) and those gavaged with 4 µg ds*ace-1* + 4 µg ds*nAChR* received 2.5 µl each of 1.6 µg/µl ds*ace-1* and ds*nAChR*. Post-gavage, insects were monitored for 30 min then were transferred to plastic chamber pots (25 mm X 25 mm) containing artificial diet (prepared as described in section 2.1). Trials consisted of three groups each with 10 insects. Pupation and emergence were recorded and gene expression was determined 48-h post gavage (as described in section 2.7.3).

2.8.2 Delivery via injection

The nanoject II microinjector (Drummond Scientific) was used to directly inject naked dsRNA into the haemolymph of *S. littoralis* larvae (Figure 2.1a) under a dissecting microscope. To facilitate this, an injection needle was produced by pulling glass capillary tubes with the P-1000 next-generation micropipette puller (Sutter) (Figure 2.1b). Prior to filling with gene specific dsRNA, the needle was back-filled with immersion oil. For injection at the fourth larval instar stage, larvae were transferred to ice for 2 min, then naked dsRNA was directly injected into the haemolymph at increasing concentrations (0.5, 1, 2, 4 and 8 µg/larva) and non-injected insects and those injected with 8 µg ds*Kan* were used as controls. dsRNA concentration was adjusted to ensure that the same volume of liquid was injected into all insects. Larvae were monitored for 30 min, transferred to plastic chamber pots (25 mm X 25 mm) containing artificial diet then were incubated at conditions described in section 2.1. Three biological replicates were used, each with 15 insects. Survival was monitored every

day for 5 consecutive days and insects were left to pupate and emerge. Gene expression analyses were conducted 48 h post injection (as described in section 2.7.3). Pupal weight was recorded 24 h after pupation to avoid variation due to moisture content which is high in freshly-formed pupae (Gong *et al*, 2021). The amount of artificial diet consumed was recorded by removing residual diet from the plastic chamber pot and weighing.

The same protocols were repeated for 3rd instar haemolymph injections except those injected with ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and ds*Kan* all received 8 µg dsRNA and those injected with ds*ace-1* + ds*nAChR* received 4 µg of both. To determine the time taken to pupate and emerge, insects were monitored every day for 25 consecutive days after injection. As with insects injected at the fourth instar stage, insects were left under experimental conditions past the average time taken for an untreated culture of insects to emerge.

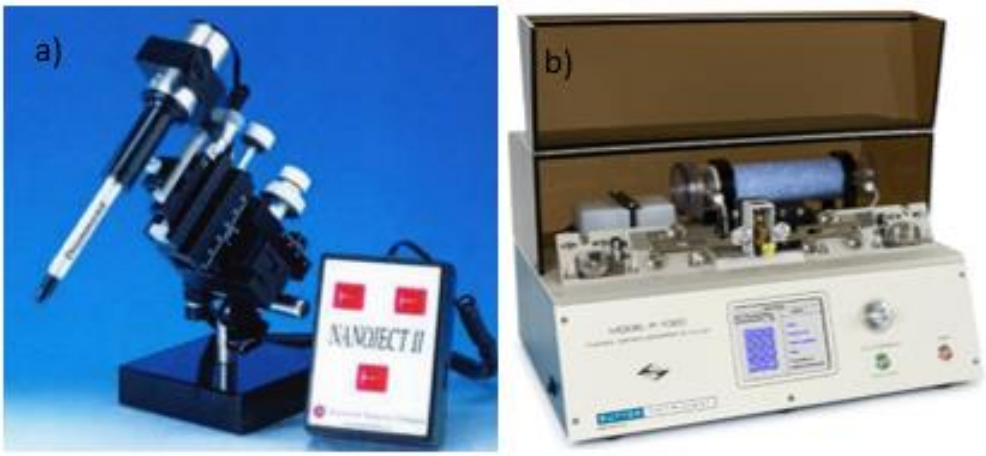

Figure 2.1 Equipment used to directly inject into the haemolymph of *S. littoralis* larvae. a) P-1000 next generation micropipette puller, b) nanoject ii microinjector. Sources: Drummond Scientific (2023) and Sutter (2023).

2.8.3 Delivery via egg soaking

Masses of freshly laid *S. littoralis* eggs (~50/mass) were soaked in 200 µl of 100 ng/µl ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* (diluted in phosphate-buffered saline, PBS, pH 7) or 100 µl each of 100 ng/µl ds*ace-1*+ds*nAChR* as in Wang *et al* (2011). To facilitate this, egg masses were left on the pieces of card that adult females had oviposited on and were transferred to Eppendorf tubes containing the specific solution. After 2 h, egg masses were transferred to plastic chamber pots and incubated as described in section 2.1. Controls were untreated egg masses and those soaked in 200 µl PBS (pH 7) and. Three biological replicates,

each containing ~50 eggs/mass, were used and successful larval hatching was subsequently recorded.

2.9 Increasing pesticide susceptibility through dsNADPHycytp450r pre-exposure

To determine the LC_{50} for deltamethrin against 4th instar larvae, insects previously reared on optimal artificial diet had 0.5 µl 2.5, 5, 10, 20, 40 and 80 mg/ml deltamethrin (diluted in pure hexane) applied topically to the head, corresponding to 1.25, 5, 10, 20 and 40 µg/larva. The recommended field doses of deltamethrin have been cited between 6.25 and 35 g/hectare of sprayed pure solution (usually at 25 g/L) diluted from 500- to 3000-fold (Chowdhury et al. 2001; Sharma et al. 2018). Consequently, insects are generally exposed to approximatively 8 to 50 mg/L deltamethrin (Malbert-Colas et al, 2020). Hexane was used because deltamethrin displays low solubility in polar solvents such as water (Massot *et al*, 2021) and because hexane has no or very little toxicity effects on 4th instar *S. littoralis* in comparison to other solvents such as acetone or ethanol (Malbert-Colas *et al*, 2020). Controls were insects with 0.5 µl pure hexane applied topically to the head and untreated insects reared on artificial diet. LC_{50} was determined by plotting deltamethrin concentration against % mortality and fitting an exponential line of best fit. To determine the effects of ds*NADPHcytP450r* exposure on insecticide susceptibility, *S. littoralis* larvae were reared from neonate on artificial diet containing 2 µg or 4 µg ds*NADPHcytP450r* for 9 days (as described in section 2.8.1). Subsequently, 0.5 µl 2.5, 5, 10, 20, 40 and 80 mg/ml deltamethrin (diluted in pure hexane) was applied topically to the larval head. Controls were insects previously reared from neonate on artificial diet containing 4 µg ds*Kan* with 0.5 µl 2.5, 5, 10, 20, 40 and 80 mg/ml deltamethrin applied topically to the head. For each treatment, 3 biological replicates, each with 10 insects, were used. Insects were transferred to plastic chamber pots containing artificial diet, as described previously, mortality was assessed 24 h later LC_{50} was determined as described previously.

To determine the LC_{50} for chlorpyrifos against 4th instar larvae, insects previously reared on optimal artificial diet had 0.5 µl 0.687, 1.375, 2.75, 5.5, 11, 22, 44, 88, 176 and 352 mg/l chlorpyrifos (Sigma) (diluted in pure hexane) applied topically to the head. Controls were insects with 0.5 µl pure hexane applied topically to the head and insects reared on artificial diet. Each group comprised of 10 insects each with three biological replicates. Pupation and pupal weight were determined as described previously.

2.10 Extraction of *S. littoralis* haemolymph and midgut juice

S. littoralis 5th instar larvae were anaesthetized on ice prior to abdominal proleg incision. Haemolymph was drained via Hamilton syringe and added to phenylthioureia (PTU) to prevent oxidation, then was immediately chilled on ice. Midguts were extracted from ten 5th instar larvae by applying pressure to the head prior to cutting the final posterior segment. The midgut was removed with tweezers (avoiding residual food) and immediately chilled on ice. The physiological pH of both tissues was determined with Whatman indicator paper with three experimental replicates. Both haemolymph and midgut were centrifuged at 14,000 × *g* for 20 min and the resulting supernatants were used for the *ex vivo* dsRNA stability assays.

2.11 Naked dsRNA stability in *S. littoralis* haemolymph and midgut juice

To determine dsRNA stability in *S. littoralis* haemolymph and midgut juice, 1 µg ds*ace-1* (1 µg/µl) was incubated at 37**°** for 30 min in either 10 µl pure or diluted haemolymph and midgut juice. Pure haemolymph was diluted to 1/50 and 1/100 in PBS pH 6.8 and pure midgut juice was diluted to 1/50 and 1/100 in PBS pH 8.8. Controls consisted of 10 µl pure or diluted (1/50 and 1/100) haemolymph or midgut juice, 1 µg ds*ace-1* incubated in 10 µl PBS 6.8, 8.8 or DEPC water or RNAseA. After incubation, samples were electrophoresed on 1.5% agarose gel (120 v, 50 min) with EtBr staining.

2.12 Formation and confirmation of dsRNA-chitosan-TPP complexes

dsRNA-CS-TPP complexes were formed at 100 ng dsRNA/µl via sonication. In preparation, commercially available chitosan (Sigma) was solubilised in 1% acetic acid solution (2 mg/ml), tripolyphosphate was solubilised in DEPC-treated water (1 mg/ml) and each gene specific dsRNA, as well as ds*Kan*, were diluted in DEPC water (1 µg/µl*).* dsRNA-CS-TPP complexes were formed by adding 1.2 ml of tripolyphosphate (TPP) (1 mg/ml) and 1 ml dsRNA (1 µg/µl), simultaneously in a dropwise manner (over a 15 min time period), to 3 ml of chitosan solution (2 mg/ml) and 4.8 ml DEPC water, with stirring magnet agitation at RT. Agitation continued for 30 min at RT, after which each solution was incubated for 15 min at RT in a Soniprep 150 ultrasonic disintegrator at 80 amp. To confirm dsRNA-CS-TPP complex formation*,* 5 µL of each dsRNA-CS-TPP were incubated at RT for 30 min in 10 µL of PBS pHs 6.8 and 8.8. After incubation, samples were electrophoresed on 1.5% agarose gel (120 v, 50 min) with EtBr staining.

2.13 dsRNA-chitosan-TPP stability in *S. littoralis* haemolymph and midgut juice

To determine the stability of dsRNA when complexed with CS-TPP, 2 µl ds*ace-1*-CS-TPP (containing 0.1 µg/µl dsRNA) was incubated at 37° for 30 min in either 10 µl pure or diluted haemolymph and midgut juice. Pure haemolymph was diluted to 1/20, 1/50 and 1/100 in PBS pH 6.8 and pure midgut juice was diluted to 1/20, 1/50 and 1/100 in PBS pH 8.8. Controls consisted of 10 µl pure haemolymph or midgut juice with 2 µl DEPC water, 2 µl chitosan incubated in PBS pH 6.8 and 8.8, 2 µl ds*ace-1*-CS-TPP incubated in PBS pH 6.8 and 8.8 and 2 µl ds*ace-1* incubated in 10 µl RNAseA. After incubation, samples were electrophoresed on a 1.5% agarose gel (120 v, 50 min) with EtBr staining.

2.14 Delivery of naked dsRNA and dsRNA-CS-TPP complexes via artificial diet to 4th instar and neonate larvae through a single feeding event

To determine the effect of feeding dsRNA-CS-TPP to fourth instar larvae, 80 µl ds*ace-1*-CS-TPP, ds*para*-CS-TPP, ds*nAChR*-CS-TPP or ds*NADPHcytP450r*-CS-TPP (8 µg dsRNA per insect) was left to percolate throughout artificial diet. Those provided with naked dsRNA received artificial diet with 80 µl of 0.1 µg/µl ds*ace-1*, ds*nAChR*, ds*NADPHcytP450r*, ds*para* percolated throughout. Controls were insects fed with artificial diet ± 80 µl of 0.1 µg/µl ds*Kan*, 80 µl ds*Kan*-CS-TPP and artificial diet with an equivalent chitosan concentration. Insects were fed once with artificial diet containing dsRNA or dsRNA-CS-TPP and were reared in plastic chamber pots (25 mm X 25 mm) under conditions described in section 2.1. After 2 days, insects were transferred to artificial diet for the remainder of the trial which was replaced every 2 days (until pupation). Three biological replicates containing 15 insects were used for each treatment, insects were monitored each day and survival, pupation and emergence were recorded. Insects were left under experimental conditions for a length of time past the average time taken for an untreated culture of insects to emerge. Gene expression was determined 48 h after experiments began (as described in section 2.7.3). The same process was repeated with neonate larvae.

2.15 Immune system priming through dsKan pre-injection

Each 5th instar larva was injected (as described in section 2.8.2) with DEPC water or 5 µg ds*Kan* followed by 10 µg either ds*Kan*, ds*ace-1*, ds*nAChR*, ds*ace-1*+ds*nAChR* (5 µg of both) or ds*para* 2 h later as in Fan *et al*, 2020a. Insects were monitored after both injections for 30 min to ensure that injection trauma did not cause mortality. Gene expression was investigated via RT-qPCR 24 h after the second injection and data was analysed, as described in section 2.7.3, with all values relative to expression of insects injected with DEPC water followed by 10 µg ds*Kan*.

2.16 *Bombus terrestris* rearing

Adult *B. terrestris* were collected from 'natupol' colonies provided by Koppert UK. Bees were provided with pollen from Koppert UK and kept in constant darkness at 25 °C ± 2 °C.

2.17 *B. terrestris* artificial diet bioassays

To assess the biosafety of each *S. littoralis* specific dsRNA, bees were first collected using a modified vacuum (Figure 2.2a) in which they were anaesthetised with CO_2 then randomly allocated to a group. Each bee was placed individually in ventilated plastic chambers (65 mm × 35 mm) with a syringe containing 50% (w/v) sucrose solution prepared with DEPC water inserted between chamber 'slots' (Figure 2.2b). Individual feeding chambers were placed adjacent to each other to account for *B. terrestris* eusociality (Figure 2.2c) and were incubated in constant darkness at 34 ˚C and 75-80 % RH. Overall, eight treatments were used; each with 3 biological replicates and 9 insects per replicate. *B. terrestris* were fed for two consecutive days with 40 µl 50 % sucrose solution containing 200 ng/µl ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* or 20 µl each of 200 ng/µl ds*ace-1* and ds*nAChR*. Controls were insects provided with 40 µl 50 % sucrose solution ± 200 ng/µl ds*Kan* and those provided with 40 µl 50 % sucrose solution containing esfenvalerate pesticide (625 ng/µl). After 2 days, bees were reared on 50 % sucrose solution until the end of the trial and mortality was recorded for 6 consecutive days.

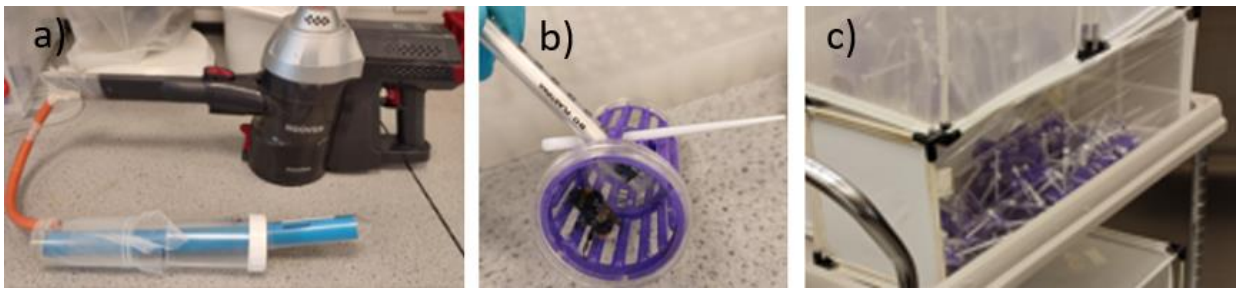

Figure 2.2 Experimental setup for *B. terrestris* bioassay. a) modified vacuum used to transfer anaesthetised bees, b) individual rearing chambers, c) bioassay cages used to ensure bees were placed adjacent to each other.

2.18 dsRNA stability in *B. terrestris* midgut juice

Adult *B. terrestris* were anaesthetized with CO_2 and midguts were extracted and processed to obtain midgut juice as described in section 2.10. To determine dsRNA stability in *B. terrestris* midgut juice, 1 µg ds*ace-1* (1 µg/µl) was incubated at 37° for 30 min in 10 µl *B. terrestris* pure midgut juice or 1/10, 1/20, 1/50 and 1/100 midgut juice dilutions (diluted in PBS pH 8.8). Controls were pure midgut juice (alone), 1/10 midgut juice, 1/20 midgut juice, 1/50 midgut juice 1/100 midgut juice. Controls consisted of 1 µg ds*ace-1* incubated in PBS pH 8.8, DEPC water and RNAseA. All samples were incubated at 37° for 30 min. After incubation, samples were electrophoresed on 1.5% agarose gel (120 v, 50 min) with EtBr staining.

2.19 Bioinformatics analyses

2.19.1 Similarity between full-length S. littoralis specific dsRNA and the genome of non-target species

NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) determined homology between full length ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and ds*Kan* against the entire human and *B. terrestris* genomes.

2.19.2 Similarity between component siRNAs and the corresponding gene in NTOs

To determine possible off-target effects of component siRNA molecules on a range of NTOs, full length ds*ace-1*, ds*para*, ds*nAChR*, dsNADPHcytP450 and ds*Kan* were *in silico* cleaved into 19-mers by the E-RNAi algorithm (https://www.dkfz.de/signaling/e-rnai3/). Subsequently, each specific dsRNA was manually cleaved into all possible n-mers ranging from 16-27 bp and ViroBlast (https://indra.mullins.microbiol.washington.edu/viroblast/viroblast.php)

identified exact sequence matches between every component n-mer for each gene and the specific gene in a range of NTOs, the sequences for which were obtained from NCBI.

2.19.3 Phylogenetic analyses of the relationship between S. littoralis and a range of NTOs

Phylogenetic relationships between *S. littoralis* and all NTOs included in each specific bioinformatics analysis were determined using the NCBI taxonomy browser (https://www.ncbi.nlm.nih.gov/Taxonomy/CommonTree/wwwcmt.cgi) and the resulting PHYLIP tree was visualised with iTol (https://itol.embl.de/).

2.20 Statistical analyses

Insect mortality was analysed using SigmaPlot for Windows 10. Survival curves were produced using the Kaplan-Meier survival analysis and the log-rank test was used to determine significance between curves. One-way ANOVA (with subsequent post-hoc Tukey's test) was conducted using Minitab to determine daily differences in the mortality inflicted by specific treatments.

Chapter 3. Targeting essential central nervous system and detoxification genes in *Spodoptera littoralis* as a novel control strategy

3.1 Abstract

Spodoptera littoralis is a highly polyphagous lepidopteran pest capable of damaging a range of crops. Chemical insecticides routinely used to control this insect have detrimental effects on the environment and non-target organisms, suggesting the necessity for further control strategies. RNA interference (RNAi) can be triggered via the introduction of sequence specific dsRNA, leading to post transcriptional down-regulation of gene expression and insect mortality, depending on gene choice. Thus, dsRNA-based biopesticides or genetically engineered plants expressing dsRNA offer highly specific pest control methods without the environmental concerns surrounding most chemical insecticides. Lepidopteran pests are generally recalcitrant to RNAi due to nucleases present in the saliva, midgut and haemolymph although some success has been reported through various administration methods. The present study aimed to reduce *S. littoralis* survival through targeting the essential central nervous system genes, acetylcholinesterase 1 (*ace-1*), the nicotinic acetylcholine receptor (*nAChR*) and the voltage-gated sodium channel (*para*), which are also the targets of major insecticides used to control this insect. Furthermore, the NADPH cytochrome P450 reductase (*NADPHcytP450r*) gene, responsible for cytochrome P450 (*P450r*) functionality, was targeted as a means of reducing the activity of the cytp450 monooxygenases, with the intention of reducing the LC_{50} value for a range of insecticides against this insect. Oral dsRNA delivery to 4th instar larvae via artificial diet and direct midgut delivery of dsRNA to 5th instar larvae via gavage feeding did not significantly reduce ($P > 0.05$) gene expression, survival, pupation or adult emergence suggesting the possible presence of dsRNA degrading nucleases in the midgut and haemolymph of this insect. Continuous feeding for 9 days of 1 µg *ace-1*, 2 µg *ace-1*, 1 µg *nAChR*, 2 µg *nAChR*, 1 µg *ace-1* + 1 µg *nAChR*, 2 µg *ace-1* + 2 µg *nAChR*, 1 µg *para* or 2 µg *para* specific dsRNA to neonate larvae significantly reduced larval weight ($P < 0.05$) by 30 %, 13 %, 13 %, 43 %, 40 %, 24 % and 24 %, respectively, which may suggest reduced midgut nuclease activity in earlier larval instars. Direct haemolymph injection of increasing concentrations of dsRNA (0.5, 1, 2, 4 and 8 µg) specific to the *ace-1*, *para* and *nAChR* genes did not significantly reduce gene expression or survival in 4th instar larvae, further suggesting the possible presence of dsRNA degrading nucleases in the haemolymph. However, direct haemolymph injection of *ace-1* and *para* specific dsRNA into 4th instar larvae led to significant reductions in pupation and emergence ($P < 0.05$) and developmental delays were reported upon injection of gene specific dsRNA into 3rd instar larvae. Furthermore, delivery of *ace-1*, *nAChR* and *NADPHcytP450r* specific

dsRNA through egg soaking led to significantly reduced larval hatching, suggesting their non-cholinergic roles. Additionally, pre-exposure to *NADPHcytP450r* specific dsRNA reduced the LC_{50} of deltamethrin against *S. littoralis* suggesting the possible role of *P450s* in deltamethrin detoxification in this insect whilst highlighting the efficacy of targeting this gene as a method of reducing recommended field concentrations for this insecticide. Overall, this study highlights the efficacy of targeting four genes as a novel *S. littoralis* control strategy whilst also suggesting the need for optimised delivery techniques.

3.2 Introduction

Insect pests cost the global economy an estimated $70 billion annually (FAO, 2023). They damage crops both directly and indirectly via feeding or acting as vectors for various plant diseases. *Spodoptera littoralis* is a highly polyphagous lepidopteran insect capable of damaging a plethora of nutritionally and economically important crops (Khalil *et al*, 2023). Current *S. littoralis* control methods, such as the use of chemical insecticides, damage the environment and non-target organisms (NTOs) (Aktar *et al*, 2009) and various *Spodoptera* spp have developed resistance to all insecticide groups (Hilliou *et al*, 2021). Thus, novel *S. littoralis* control strategies are required.

RNA interference (RNAi), a natural method of gene regulation, can be triggered via the introduction of sequence specific dsRNA into a chosen organism. The consequent post transcriptional down-regulation of gene expression (Hammond *et al*, 2001) can lead to significant phenotypic changes and insect mortality (Sharma *et al*, 2021), depending on gene choice. Thus, dsRNA-based biopesticides or genetically engineered plants expressing dsRNA offer highly specific pest control methods without the environmental concerns associated with most chemical insecticides. Laboratory experiments generally expose insects to dsRNA via three main methods, oral feeding (via an artificial diet or gavage), direct haemolymph injection or egg soaking. Achieving RNAi via oral feeding is advantageous as it gives an indication as to how a species will respond to dsRNA in the field but direct injection offers 'proof of concept' in species considered recalcitrant to orally induced RNAi.

Insects differ in their susceptibility to RNAi, with Coleoptera generally considered the most susceptible and Lepidoptera the least. RNAi in the coleopteran *T. castaneum* is often systemic, long-lived and heritable (Horn *et al*, 2022) through both oral delivery (Cao *et al*, 2018) and direct haemolymph injection (Knorr *et al*, 2021). Conversely, RNAi is often difficult to achieve in many Lepidoptera, likely due to dsRNA degradation by nucleases present in the saliva, haemolymph and midgut (Singh *et al*, 2017) alongside the extremely alkaline, and thus hostile environment for dsRNA, provided by the midgut (Christiaens *et al*, 2018a). Despite this, successful RNAi-mediated gene knockdown has been reported in many lepidopteran species (Poreddy *et al*, 2017 and Zhang *et al*, 2015), however, the effect is often transient and localised (Zhang *et al*, 2015) and higher dsRNA concentrations are often necessary to elicit the intended response in Lepidoptera compared to Coleoptera. For

example, Ivashuta *et al* (2015) reported no significant effects on mortality or phenotype when *S. frugiperda* or *Helicoverpa zea* (Corn earworm) were fed with dsRNA specific to the *V-ATPase* gene, although the concentration was > 1000-fold higher than the LC_{50} for the coleopteran *D. virgifera virgifera* (Baum *et al*, 2007). However, as methods are available to increase dsRNA stability in the presence of nucleases, with the intent of increasing RNAi efficiency via oral feeding, (Christiaens *et al*, 2018a; Yang *et al*, 2022), proof of concept studies remain vitally important.

The insect central nervous system (CNS) is the target of many commercial chemical insecticides (Ffrench-Constant *et al*, 2016) most of which paralyse insects, leading to feeding cessation and death. Organophosphates and carbamates irreversibly inactivate acetylcholinesterase (*AChE*) (Haddi *et al*, 2017), an enzyme that terminates nerve impulses by hydrolysing the neurotransmitter acetylcholine (ACh) (Johnson and Moore 2000), leading to ACh accumulation and overstimulation of nicotinic receptors (Colović *et al*, 2013). Pyrethroids block neuronal transmission through the prevention of voltage-gated sodium channel (VGSC) closure (Silver *et al*, 2018) whereas the mode of action of spinosyns and neonicotinoids is to conformationally change *nAChR*s on post-synaptic muscle cells (Aditya and Rattan, 2012), thus inhibiting the binding of Ach and terminating signal transmission.

Insects can develop genetic resistance to insecticides via point mutations in the genes encoding target enzymes (as described in section 1.7.1). They can also exhibit metabolic resistance through the upregulation of the expression of detoxification enzymes, which can both sequester and degrade insecticides either by enzyme upregulation or structural changes (Hirata *et al*, 2017). Cytochrome P450 monooxygenases (*P450s*) (Ye *et al*, 2022) are the major detoxifying enzymes and most insects have > 100 (Feyereisen, 1999), with no singular P450 responsible for insecticide detoxification both within and between species (Zhao *et al*, 2020; David *et al*, 2013; Hu *et al*, 2014).

The present study investigates the potential of RNAi-mediated knockdown of the genes encoding acetylcholinesterase (*ace-1*), VGSC (*para*) and the nicotinic receptor (*nAChR*) in *S. littoralis* via oral feeding (through an artificial diet and gavage), direct haemolymph injection and egg soaking. We hypothesise that successful post-transcriptional silencing of these genes will induce mortality, similar to that achieved by current chemical insecticides. Reductions in

gene expression and mortality after administration of dsRNA specific to *ace-1*, *nAChR* and *para* have been reported in various insects (Salim *et al*, 2017; Majidiana *et al*, 2019; Garber *et al*, 2012). Thus, similar results in the present study would highlight the use of RNAi technology targeting these genes as a novel, environmentally friendly *S. littoralis* control strategy.

As several insects have a second acetylcholinesterase gene (Tmimi *et al*, 2018), the expression of the *S. littoralis ace-2* gene is also investigated, with the hypothesis that *ace-2* gene expression may increase as a result of reduced *ace-1* expression, such as the compensatory effect reported in *B. mori* by Cao *et al* (2012). The NADPH cytochrome P450 reductase gene (*NADPHcytP450r*), which is required for electron transfer from NADPH to cytochrome P450 and therefore for *P450* functionality (Zhu *et al*, 2012), was also targeted via RNAi as a means of targeting all *P450s* simultaneously. The aim of which is to determine the collective role of *P450s* in *S. littoralis* insecticide detoxification, with the hypothesis that reduced *NADPHcytp450*r expression will lead to increased sensitivity to a range of commercial insecticides.

The non-cholinergic roles of the *ace-1*, *nAChR* and *para* genes have been reported previously. For example, Salim *et al* (2017) reported that injecting *ace-1* specific dsRNA into 6th instar *S. litura* led to significantly reduced pupation and adult emergence, Majidiana *et al* (2019) reported reduced weight of pre-pupae and pupae upon injection of *nAChR* specific dsRNA into *T. absoluta* larvae and Garber *et al* (2012) reported that mutants of *Drosophila melanogaster* with lower sodium channel abundance exhibited significantly lower adult eclosion rates and reduced adult longevity. Thus, in the present study, various parameters including larval weight, pupal weight, pupation rate and adult emergence are investigated after administration of *ace-1*, *nAChR* and *para* specific dsRNA. These parameters are also investigated post *NADPHcytP450r* specific dsRNA administration as, alongside detoxification capabilities, *P450s* are also involved in insect growth and development (Ye *et al*, 2022).

3.3 Results

3.3.1 Amplification of nAChR sequence fragments via degenerate primers

As sequence data was not initially available for the *S. littoralis nAChR* gene, degenerate primers were designed using amino acid sequence alignments of the *nAChR* gene from closely related species (Figure 3.1). The forward priming site FITNGEW and the reverse priming site FNCIMFM, giving a predicted amplicon size of 342 bp, were chosen. Degenerate RT-PCR ran across a gradient of annealing temperatures amplified the correct size band (342 bp) along with an unexpected ~ 650 bp band (Figure 3.2). Band-stab PCR, using the 342 bp band corresponding to annealing temperature 46.7 °C, was used in a subsequent PCR reaction to amplify only the expected 342 bp single band (Figure 3.3). After ligation, *E. coli* transformation and restriction digestion, the correct band was identified in two colonies (Figure 3.4). Subsequently, sequencing revealed the nucleotide sequence and i*n silico* translation confirmed the expected amino acid sequence (results not shown). This sequence was initially intended for use in RACE PCR, to retrieve more of the *S. littoralis nAChR* sequence for dsRNA synthesis, but sequences for the *nAChR* gene were subsequently found in Roy *et al* (2016) supplementary material. Degenerate primers were also designed for the *S. littoralis para* gene (results not shown) but sequences from Roy *et al* (2016) were again used.

```
Drosophila_melanogaster     QHCEMKFGSWTYDGNQLDLVLNSEDGGDLSDFITNGEWYLLAMPGKKNTIVYACCPEPYV   240
Tuta_absoluta               QHCDMKFGSWTYDGNQLDLVLKDENGGDLSDFITNGEWYLIGMPGKKNTISYACCPEPYV   221
Plutella_xylostella         QHCDMKFGSWTYDGNQLDLVLKDENGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYV   218
Cydia_pomonella             QHCDMKFGSWTYDGNQLDLVLKDENGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYV   210
Chilo_suppressalis          QHCDMKFGSWTYDGNQLDLVLKDEAGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYV   218
Bombyx_mori                 QHCDMKFGSWTYDGNQLDLILKDEAGGDLSDFITNGEWYLIGMPGKKNTITYACCPEPYV   218
                            ***:***************:*:.* ***************:.******** *********

Drosophila_melanogaster     DITFTIQIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   300
Tuta_absoluta               DVTFTIRIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   281
Plutella_xylostella         DVTFTIMIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   278
Cydia_pomonella             DVTFTIMIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   270
Chilo_suppressalis          DVTFTIMIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   278
Bombyx_mori                 DVTFTIMIRRRTLYYFFNLIVPCVLISSMALLGFTLPPDSGEKLTLGVTILLSLTVFLNL   278
                            *:**** **************************************************

Drosophila_melanogaster     VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPPWIKSVFLQW   360
Tuta_absoluta               VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPQWIKSVFLQW   341
Plutella_xylostella         VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPQWIKSVFLQW   338
Cydia_pomonella             VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPQWIKSVLLQW   330
Chilo_suppressalis          VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPQWIKSVFLQW   338
Bombyx_mori                 VAETLPQVSDAIPLLGTYFNCIMFMVASSVVLTVVVLNYHHRTADIHEMPQWIKTVFLQW   338
                            ************************************************ ***:*:***
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Figure 3.1 Amino acid sequence alignments used to design degenerate primers to amplify a section of the *S. littoralis nAChR* sequence. Sequences from five lepidopteran and one dipteran species were aligned and regions of the highest conservation (boxed regions) were used to design degenerate primers. Alignments were made using Clustal Omega.

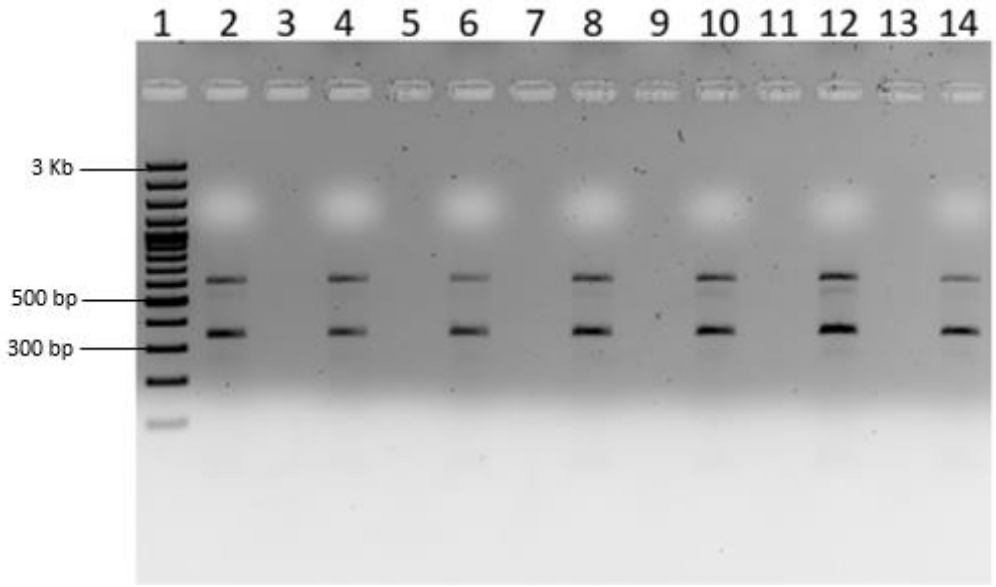

Figure 3.2 RT-PCR amplification of a section of *nAChR* using degenerate primers on a 1.6 % (w/v) agarose gel with EtBr staining (110 volts, 1 hr). Lane 1 = ogeneruler 100 bp+, lanes 2, 4, 6, 8, 10, 12 and 14 = *S. littoralis nAChR* at annealing temperatures of 48.0, 47.7, 47.3, 46.7, 45.9, 45.2 and 44.7 °C, respectively. Lanes 3, 5, 7, 9, 11 and 13 = empty.

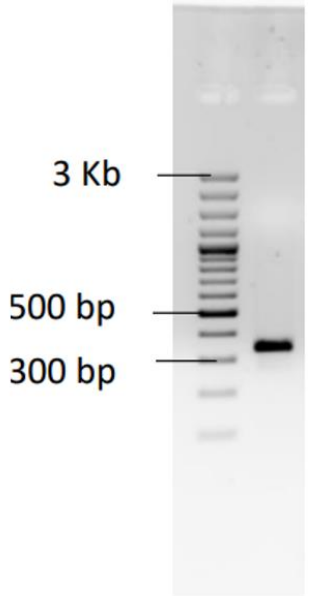

Figure 3.3 PCR amplification of a section of *nAChR* using degenerate primers on a 1.6 % (w/v) agarose gel (120 v, 1 hr). PCR amplified a single band using band-stab PCR from a previous agarose gel. Lane 1 = ogeneruler 100 bp+, lane 2 = *S. littoralis nAChR*.

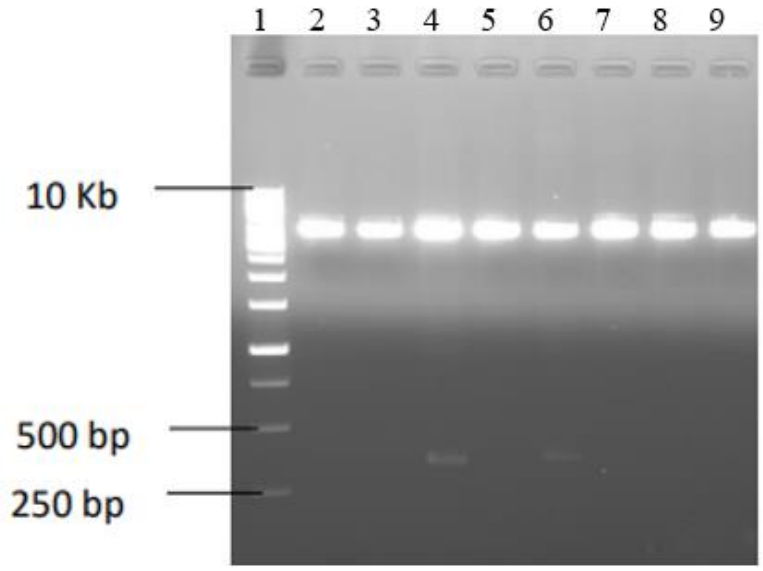

Figure 3.4 Electrophoresis of cloned *S. littoralis nAChR* fragments after ECORI restriction digestion on a 1.2 % (w/v) agarose gel (120 v, 1 hr). PCR amplified a section of *nAChR* which was subsequently ligated into a plasmid and transformed into *E. coli*. Extracted plasmids were analysed via ECORI digestion to check for the correct insert. Lane 1 = ogeneruler 1kB, lanes 2-9 = *S. littoralis nAChR* fragments.

3.3.2 Determining primer efficiency

Prior to expression studies, the efficacy of primer pairs designed to amplify sections of the *S. littoralis ace-1*, *ace-2*, *para*, *nAChR*, *NADPHcytP450r* and the housekeeping genes *RPL13A, β-actin* and *EF1 αfactor* were determined. Amplification efficiency of each pair of gene specific primers was determined using the slope of the line generated after plotting mean CT value against the logarithm of a 10-fold cDNA serial dilutions (Figure 3.5). Primer efficiencies were 99.98 %, 99.98 %, 99.74 %, 108.98%, 102.66 %, 104.91 %, 96.31 % and 104.76 % for *ace-1*, *ace-2*, *para*, *nAChR, NADPHcytP450r*, *RPL13A, β-actin* and *EF1 αfactor*, respectively.

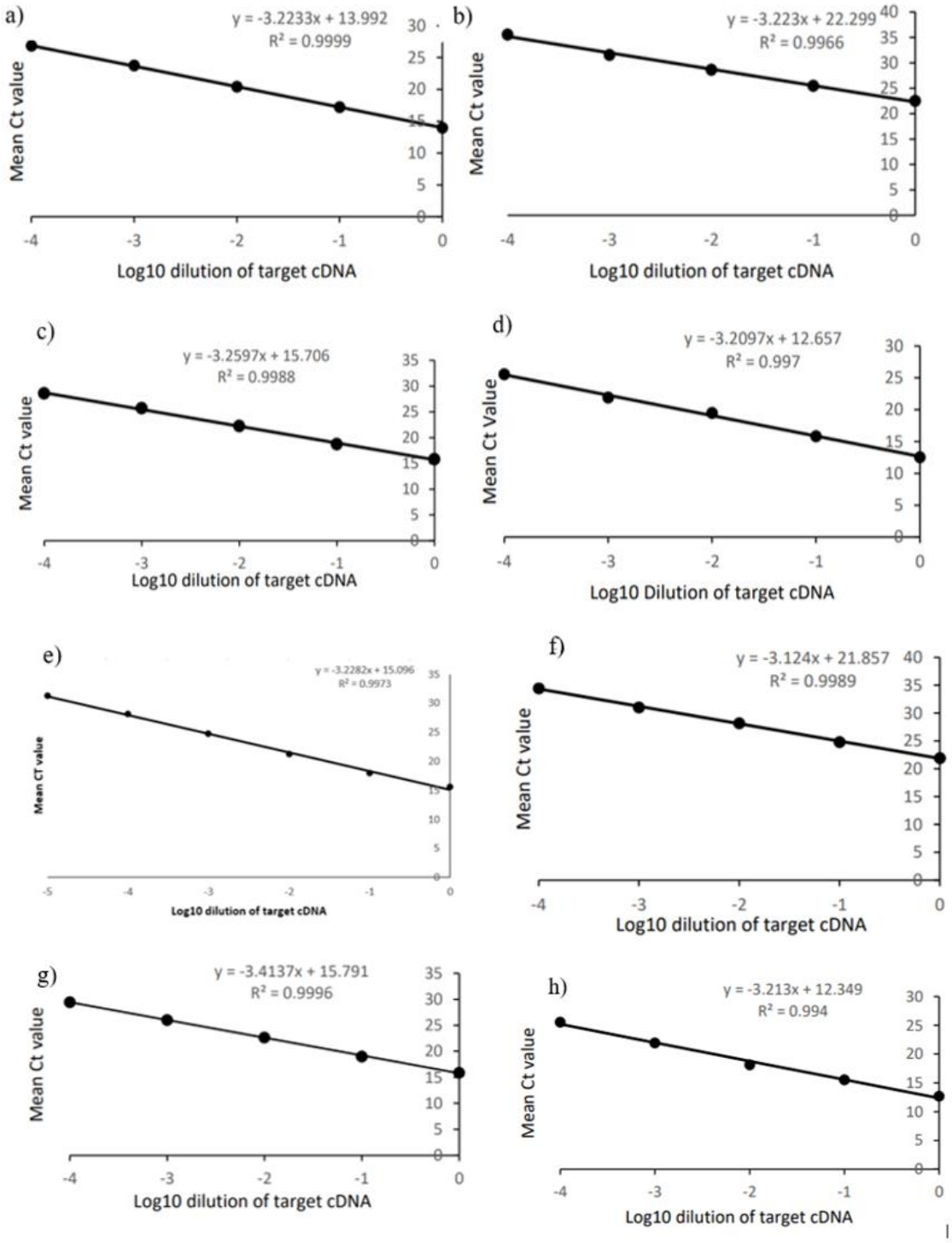

Figure 3.5 Efficiencies for *S. littoralis* primer pairs determined by RT-qPCR. cDNA was synthesised from RNA extracted from 4th instar larvae and a 10-fold cDNA dilution series was used to calculate primer efficiency a) *ace-1* b) *ace-2* c) *para* d) *nAChR* e) *NADPHcytP450r* f) *RPL13A* g) *β -actin* h) *EF1 αfactor*.

3.3.3 Baseline gene expression

To assess baseline gene expression prior to RNAi and to determine the most appropriate life stage to target in subsequent experiments, the expression of the *ace-1*, *ace-2*, *para*, *nAChR* and *NADPHcytP450r* genes was investigated at different developmental stages (larval instars 1-6, pupae and adult) using RT-qPCR. *RPL13A* was used as the reference gene as the expression of *β-actin* and *EF1 αfactor* did not remain stable across life stages.

All five genes were expressed at all investigated developmental stages. Expression of *ace-1* was highest in the 1st larval instar stage with expression at all other stages significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) in comparison (Figure 3.6). Expression of *ace-1* was 0.22-, 0.20-, 0.20-, 0.25-, 0.15-, 0.34- and 0.21-fold for instars 2, 3, 4, 5, 6, pupae and adult, respectively, relative to expression in 1st instar larvae. Expression at all other life stages was not significantly different from one another ($P > 0.05$; ANOVA, Tukey post-hoc). Expression of *ace-2* was also highest in the 1st larval instar stage but expression at every other stage was not significantly lower ($P > 0.05$; ANOVA, Tukey post-hoc) (Figure 3.6). Expression of *ace-2* was 0.63-, 0.64-, 0.76-, 0.76-, 0.68-, 0.74- and 0.88-fold for instars 2, 3, 4, 5, 6, pupae and adult, respectively, relative to that of 1st instar larvae.

Expression of *para* was highest in the 1st larval instar stage with expression for instars 2, 3, 4, 5, 6, pupae and adult 0.21-, 0.14-, 0.13-, 0.06-, 0.05-, 0.24- and 0.04- fold that of expression in instar 1 (Figure 3.7). After instar 1, *para* expression was highest in instar 2 and pupae and lowest in the adult stage, although expression was not significantly different from that of other instars ($P > 0.05$; ANOVA, Tukey post-hoc).

Expression of *nAChR* was 1.24, 1.69, 1.56, 1.78, 1.48, 1.12 and 1.96-fold, relative to expression in instar 1, for instars 2, 3, 4, 5, 6, pupae and adult, respectively, (Figure 3.8). Despite being highest in the adult stage and lowest in the 1st instar stage, expression was not significantly different across any of the investigated developmental stages ($P > 0.05$; ANOVA, Tukey post-hoc). *NADPHcytP450r* expression was 1.29, 1.27, 0.28, 028, 1.14, 0.36 and 1.49-fold relative to that of instar 1 (Figure 3.9) and expression in larval instars 1, 2, 3, 6 and the adult stage was significantly higher than that of instars 4, 5, and the pupal stage ($P < 0.05$; ANOVA, Tukey post-hoc).

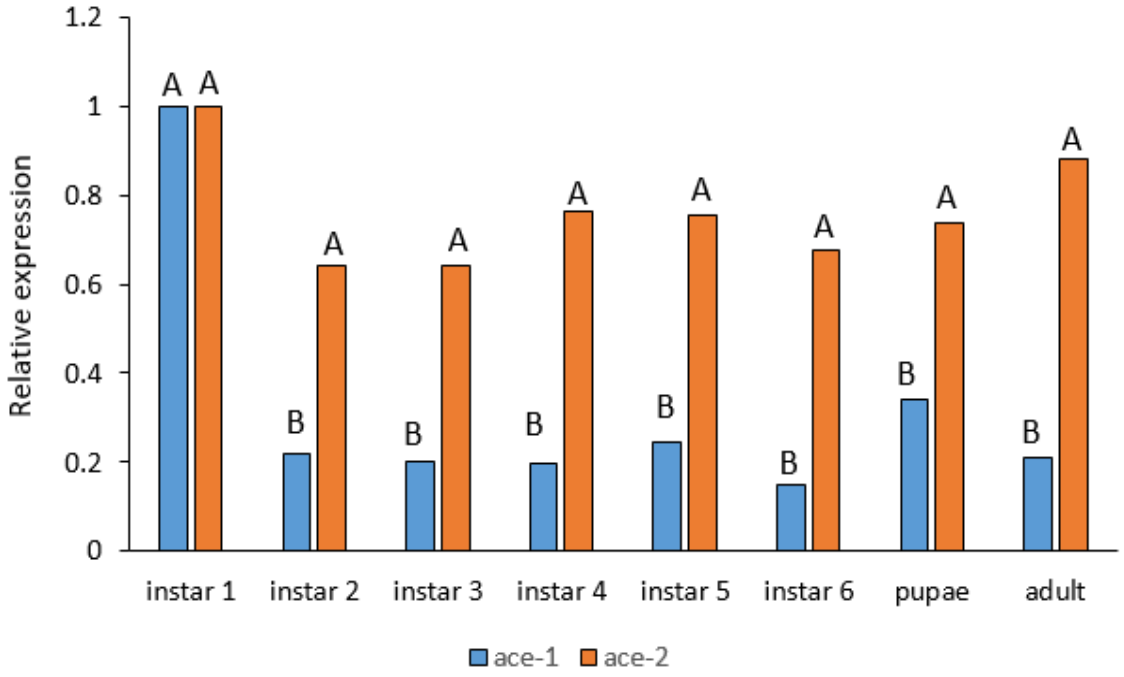

Figure 3.6 Expression of the *S. littoralis ace-1* and *ace-2* genes across developmental stages. cDNA was synthesised from RNA extracted from each life stage and RT-qPCR amplified each gene using a 1/1000 cDNA dilution. *ace-1* expression values are an average of three biological replicates and *ace-2* values are an average of two biological replicates, all are relative to expression at the 1st larval instar stage and are normalised against the *RPL13A* housekeeping gene. Different letters denote significant differences in mean dCt. Blue and orange bars show relative expression of *ace-1* and *ace-2*, respectively.

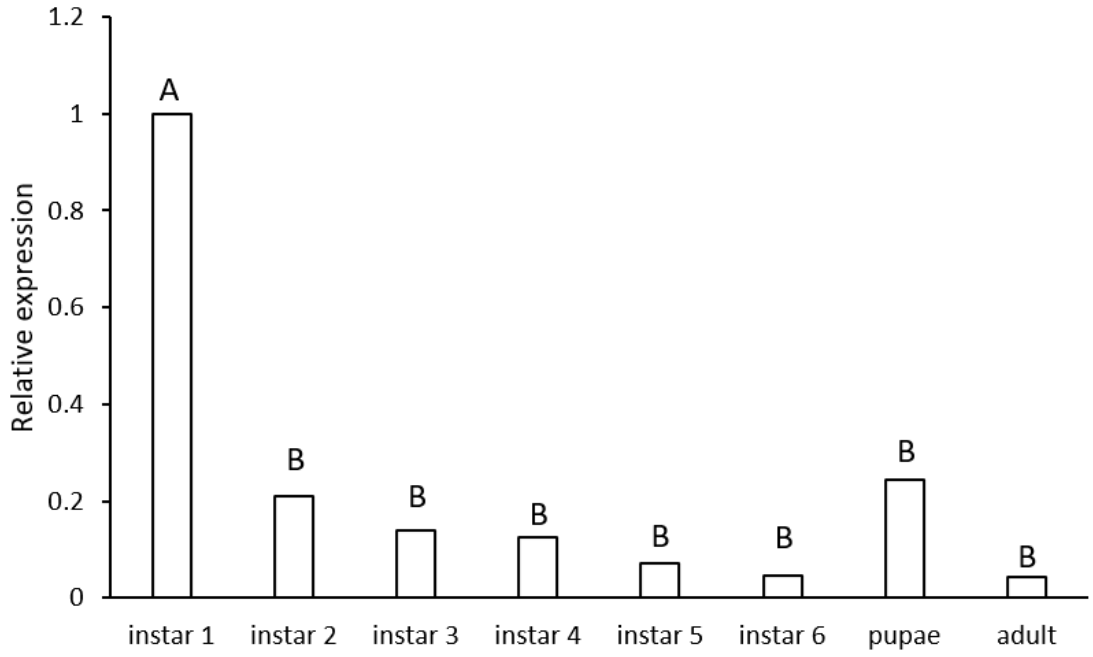

Figure 3.7 Expression of the *S. littoralis para* gene across developmental stages. cDNA was synthesised from RNA extracted from each life stage and RT-qPCR amplified each gene using a 1/1000 cDNA dilution. All expression values are an average of two biological replicates, are relative to expression at the 1st larval instar stage and are normalised against the *RPL13A* housekeeping gene. Different letters denote significant differences in mean dCt.

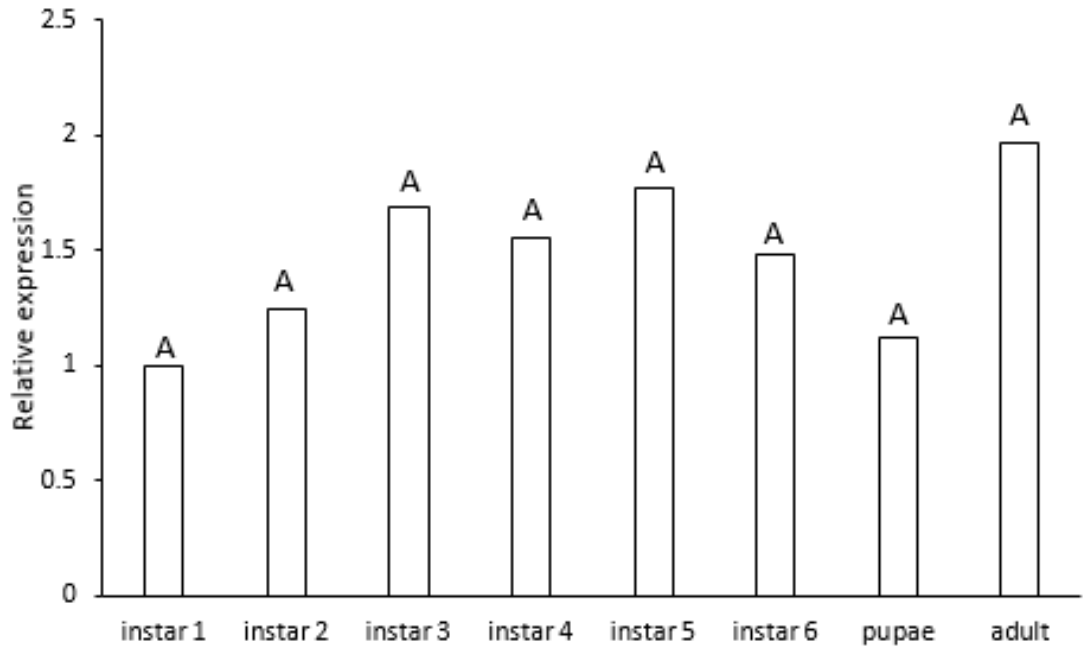

Figure 3.8 Expression of the *S. littoralis nAChR* gene across developmental stages. cDNA was synthesised from RNA extracted from each life stage and RT-qPCR amplified each gene using a 1/1000 cDNA dilution. All expression values are an average of two biological replicates, are relative to expression at the 1st larval instar stage and are normalised against the *RPL13A* housekeeping gene. Different letters denote significant differences in mean dCt.

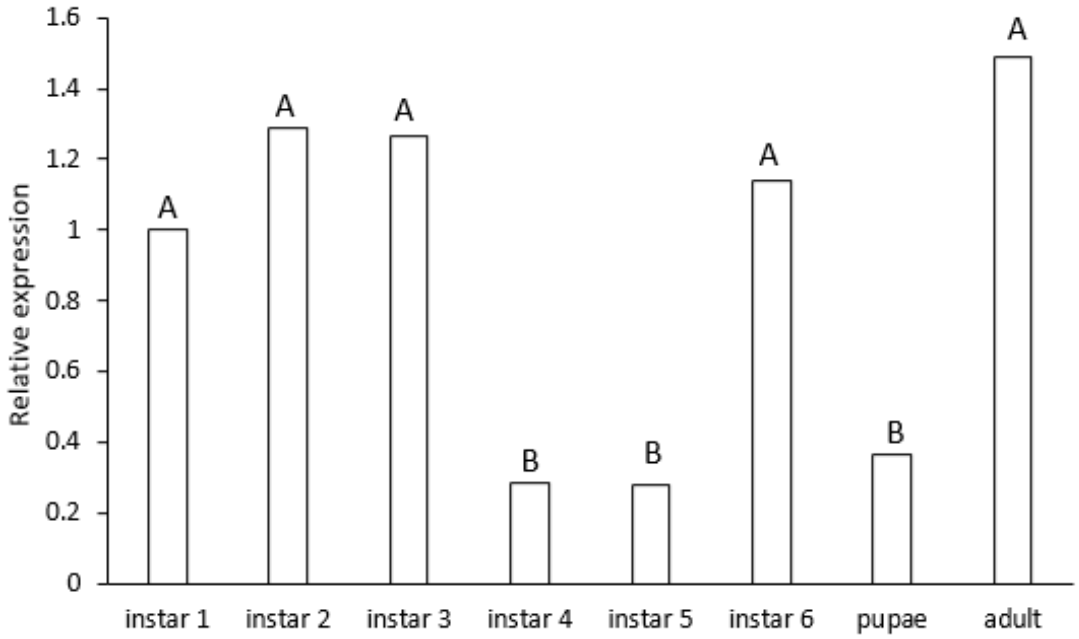

Figure 3.9 Expression of the *S. littoralis NADPHcytP450r* gene across developmental stages. cDNA was synthesised from RNA extracted from each life stage and RT-qPCR amplified each gene using a 1/1000 cDNA dilution. All expression values are an average of two biological replicates, are relative to expression at the 1st larval instar stage and are normalised against the *RPL13A* housekeeping gene. Different letters denote significant differences in mean dCt.

3.3.4 Amplification of dsRNA template sequences

Regions of the *S. littoralis ace-1*, *para*, *nAChR* and *NADPHcytP450r* genes were amplified via RT-PCR for use in subsequent dsRNA synthesis experiments. Gene specific primers amplified the expected 485 bp, 499 bp, 486 bp and 473 bp bands corresponding to *ace-1*, *para*, *nAChR* and *NADPHcytP450r*, respectively (Figure 3.10).

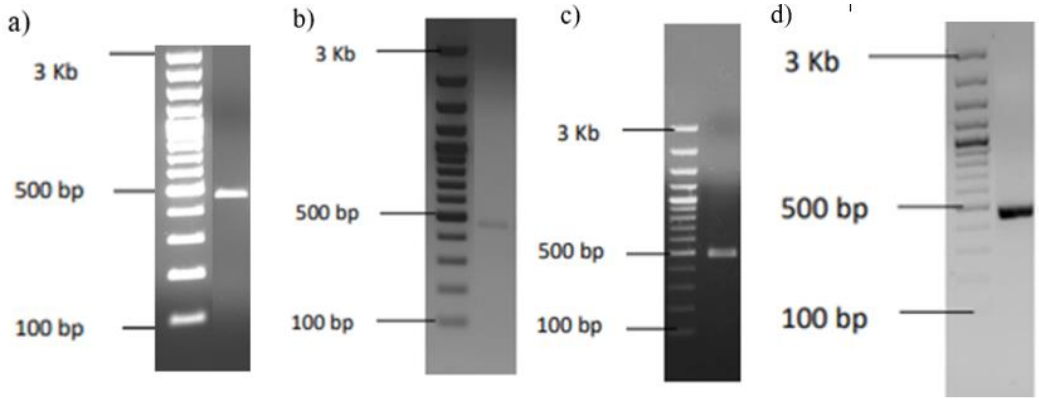

Figure 3.10 Electrophoresis of the *S. littoralis* (a) *ace-1*, (b) *para*, (c) *nAChR,* d) *NADPHcytP450r* genes on 1.2% (w/v) agarose gels (120 V, 1 h 10 min) with EtBr staining. RT-PCR amplified the dsRNA target region from cDNA synthesised from RNA extracted from 4th instar larvae (*ace-1*) or adults (*para*, *nAChR*, *NADPHcytP450r*). Lane 1 = ogeneruler 100bp+, lane 2 = dsRNA synthesis template.

3.3.5 Confirmation of dsRNA template sequences

To confirm amplification of the correct dsRNA template region, the 485 bp, 499 bp, 486 bp and 473 bp regions corresponding to the *ace-1*, *para*, *nAChR* and *NADPHcytP450r* genes were cloned into plasmid vectors and transformed into *E. coli*, then restriction enzyme digestion was used to identify correctly transformed plasmids (results not shown) and the inserts were subsequently sequenced. Clustal Omega sequence alignment of the *ace-1* sequences showed 5 misincorporated bases (Figure 3.11a) and *in vitro* translation using ExPASy confirmed these also changed the amino acid sequence (Figure 3.11b). Alignment of the *para* sequences identified one nucleotide base change compared to the expected sequence (Figure 3.12a) which also changed one amino acid base (Figure 3.12b). Alignment of the *nAChR* sequences identified one nucleotide base change (Figure 3.13a), which changed one amino acid base (Figure 3.13b). Alignment of the *NADPHcytP450r* sequences identified one nucleotide base change (Figure 3.14a), but this did not change the amino acid sequence (Figure 3.14b). In an attempt to avoid misincorporated bases, a high fidelity polymerase was subsequently used to amplify template sequences (results not shown) but these were not confirmed by sequencing. Due to time constraints, dsRNA specific to the *S.*

littoralis ace-1, *para*, *nAChR*, *NADPHcytP450r* genes was ordered from agroRNA (http://genolution.co.kr/service-overview/) along with dsRNA specific to the bacterial kanamycin resistance gene which was used as a non-target control in all RNAi experiments.

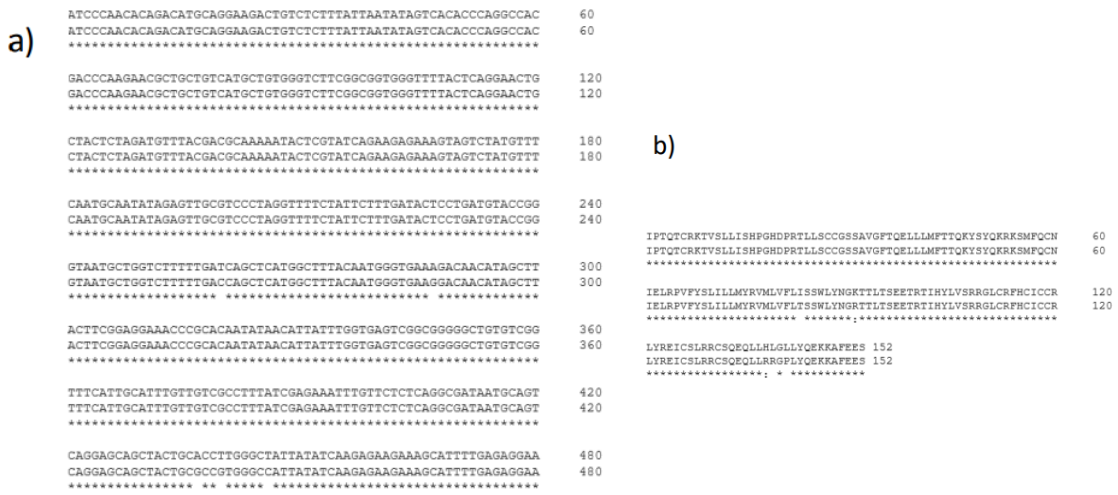

Figure 3.11 a) Alignment of *S. littoralis ace-1* dsRNA template nucleotide sequences created with Clustal Omega. Top line = *S. littoralis ace-1* sequence from NCBI, Bottom line = plasmid insert sequence. b) Alignment of *ace-1* amino acid sequences. Nucleotide sequences were *in vitro* translated using ExPASy. Top line = *S. littoralis ace-1* sequence, Bottom line = plasmid insert sequence, * denotes a match, space = not a match.

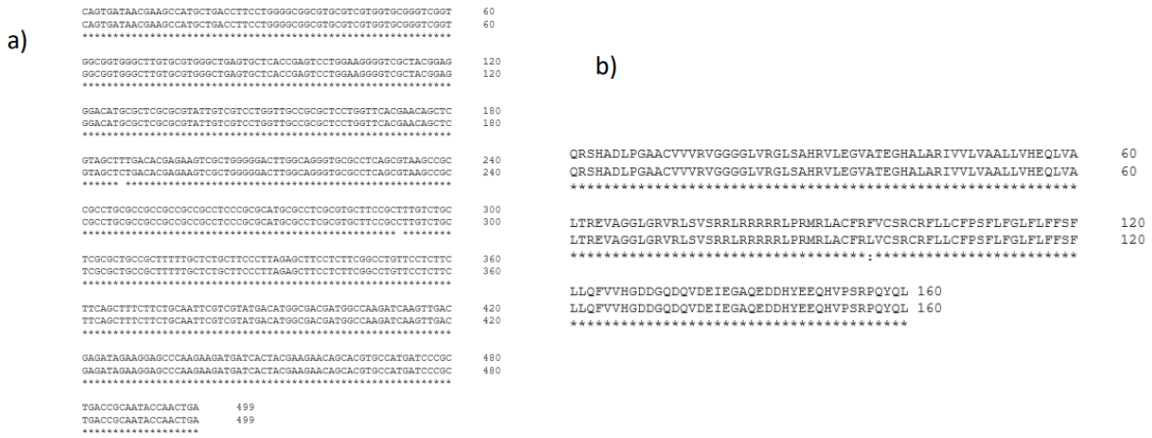

Figure 3.12 a) Alignment of *S. littoralis para* dsRNA template nucleotide sequences created with Clustal Omega. Top line = *S. littoralis para* sequence from NCBI, Bottom line = plasmid insert sequence. b) Alignment of *para* amino acid sequences. Nucleotide sequences were *in vitro* translated using ExPASy. Top line = *S. littoralis para* sequence, Bottom line = plasmid insert sequence, * denotes a match, space = not a match.

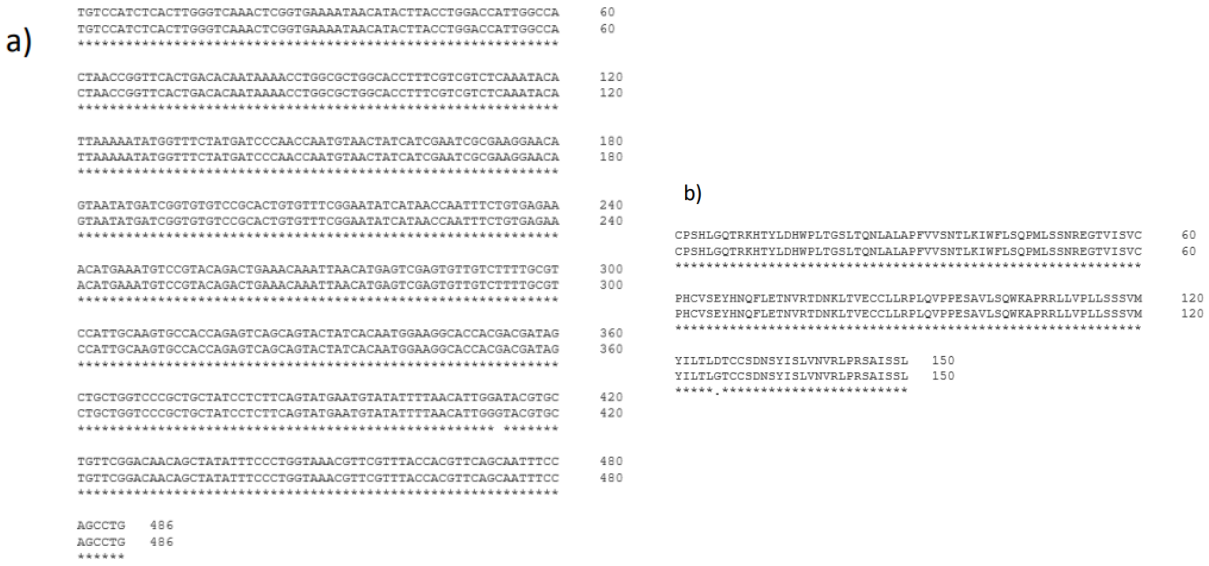

Figure 3.13 a) Alignment of *S. littoralis nAChR* dsRNA template nucleotide sequences created with Clustal Omega. Top line = *S. littoralis nAChR* sequence from NCBI, Bottom line = plasmid insert sequence. b) Alignment of *nAChR* amino acid sequences. Nucleotide sequences were *in vitro* translated using ExPASy. Top line = *S. littoralis nAChR* sequence, Bottom line = plasmid insert sequence, * denotes a match, space = not a match.

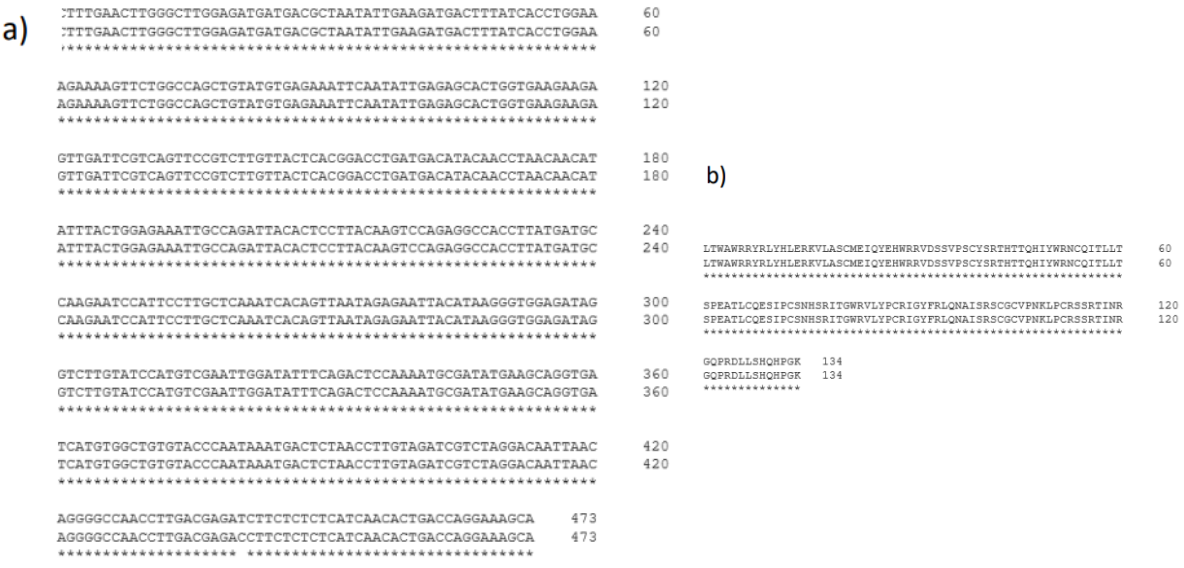

Figure 3.14 a) Alignment of *S. littoralis NADPHcytP450r* template dsRNA nucleotide sequences created with Clustal Omega. Top line = *S. littoralis NADPHcytP450r* sequence from NCBI, Bottom line = plasmid insert sequence. b) Alignment of *NADPHcytP450r* amino acid sequences. Nucleotide sequences were *in vitro* translated using ExPASy. Top line = *S. littoralis NADPHcytP450r* sequence, Bottom line = plasmid insert sequence, * denotes a match, space = not a match.

3.3.6 dsRNA synthesis

Although gene specific dsRNA was ordered from agroRNA due to time constraints, the *in vitro* dsRNA synthesis technique was practiced. Prior to dsRNA synthesis, PCR amplified each dsRNA template, with additional T7 tags incorporated in the primers, using decreasing concentrations of plasmids transformed with each gene specific insert. As an example, a 530 bp band, corresponding to the 484 bp *ace-1* specific dsRNA template sequence with 23 bp T7 promoter regions appended to the 5' end of both the forward and reverse primers, was amplified (Figure 3.15a) and the expected 530 bp *ace-1* specific dsRNA was subsequently synthesised using the Megascript RNAi kit (Figure 3.15b).

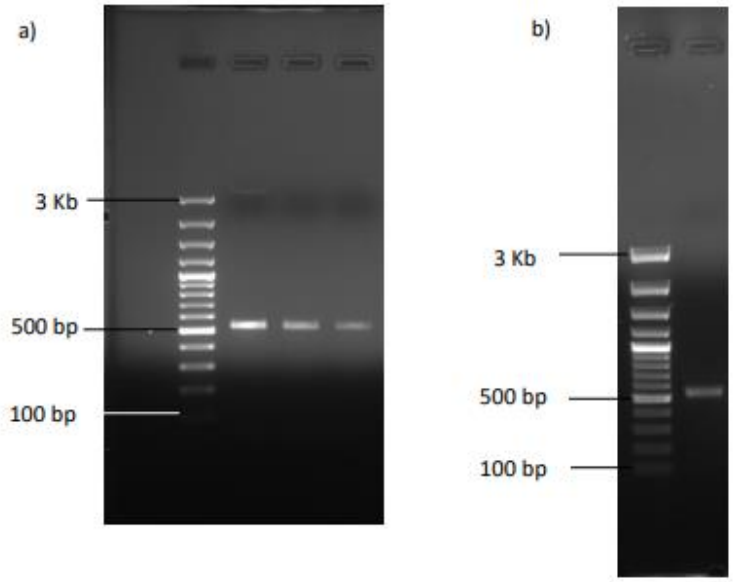

Figure 3.15 Electrophoresis of the *S. littoralis ace-1* specific dsRNA target region after amplification from a transformed *E. coli* plasmid on a 1.6% (w/v) agarose gel with EtBr staining. PCR was used to add T7 tags to the insert and decreasing concentrations of plasmid were used for amplification. Lane 1 = ogeneruler 100 bp+, lanes 2-4 = *ace-1* amplification from 10 ng, 6 ng, 4 ng plasmid. b) Electrophoresis of dsRNA specific to the *S. littoralis ace-1* gene synthesised by the Megascript RNAi kit on a 1.6% gel. Lane 1 = ogeneruler 100 bp+, lane 2 = *ace-1* dsRNA.

3.3.7 Defining gene up/down regulation for subsequent gene expression

As relative expression (RE) values can vary from one repetition to another (Genomique, 2023) and because only one reference gene was used in the present study, only RE values ± two-fold that of the calibrator sample are considered biologically significant in subsequent gene expression analyses. This threshold is summarised in Figure 3.16, where values within the red area are not classed as a biologically significant change in gene expression but values within the green areas are.

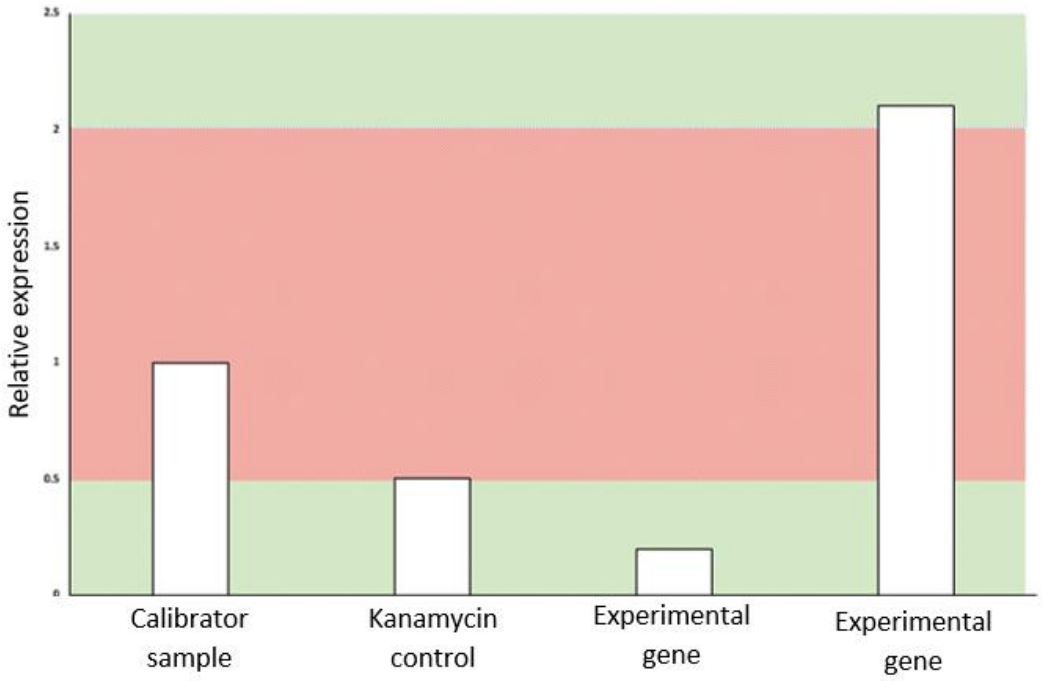

Figure 3.16 Clarification of gene expression up/down regulation for subsequent analyses in the present study. All values are relative to expression of the calibrator sample (shown as Y = 1) and expression values ± two times that of the calibrator sample are considered biologically significant. Therefore, values within the red area are not classed as a significant change in gene expression relative to the calibrator sample, but values within the green areas are.

3.3.8 Delivery of dsRNA via artificial diet to 4th instar larvae via a single feeding event

To determine the effect of feeding gene specific dsRNA via an artificial diet on survival, pupation, adult emergence and gene expression, fourth instar larvae were reared on artificial diet with 8 µg dsRNA specific to the *ace-1* (ds*ace-1*), *para* (ds*para*) *nAChR* (ds*nAChR*) genes or a combination of 4 µg + 4 µg *ace-1* + *nAChR* (ds*ace-1* + ds*nAChR*) percolated throughout. Controls were insects reared on artificial diet ± 8 µg dsRNA specific to the kanamycin resistance gene (ds*Kan*).

No mortality was recorded when insects were reared on artificial diet by day 5 (Figure 3.17) and mortality for insects reared on artificial diet containing any *S. littoralis* specific dsRNA was 2-7% compared to 2% for the control group reared on artificial diet containing ds*Kan*. Kaplan-Meier log-rank survival analysis identified no significant difference in survival ($P > 0.05$; ANOVA, Tukey post-hoc) between insects reared on artificial diet ± ds*Kan* or any *S. littoralis* specific dsRNA. Regarding pupation, 82-86 %, of those reared on artificial diet containing any *S. littoralis* specific dsRNA pupated compared to 87 % of those reared on artificial diet alone and 84 % of those reared on artificial diet + ds*Kan* (Figure 3.18) and there

was no significant difference (P > 0.05; ANOVA, Tukey post-hoc) in pupation between insects in any group. For those reared on artificial diet containing any *S. littoralis* specific dsRNA, 83-87 % of those that pupated emerged as adults compared to 87 % and 88 % of those reared on artificial diet alone or containing ds*Kan*, respectively (Figure 3.19), respectively, and there was no significant difference (P > 0.05; ANOVA, Tukey post-hoc) between the instance of adult emergence for any group. Thus dsRNA, irrespective of the target gene, had no significant effects on mortality, pupation or subsequent adult emergence when delivered via artificial diet to 4th instar larvae.

Gene expression was investigated via RT-qPCR after feeding 4th instar larvae on artificial diet containing ds*Kan* ds*ace-1*, ds*nAChR*, ds*para* or ds*ace-1* + ds*nAChR*, with expression compared to insects reared on artificial diet. The expression of *ace-1* was 0.91, 0.96 and 1.10 when insects were reared on artificial diet containing ds*Kan*, ds*ace-1* or ds*ace-1* + ds*nAChR*,respectively), thus was not significantly different than when insects were reared on artificial diet (Figure 3.20a). The expression of *nAChR* was 0.93, 0.94 and 1.02 when insects were reared on artificial diet containing ds*Kan*, ds*ace-1* or ds*ace-1* + ds*nAChR*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.20b). The expression of *para* was 1.04 and 0.78 when insects were reared on artificial diet containing ds*Kan* or ds*para*, respectively), thus was not significantly different than when insects were reared on artificial diet (Figure 3.20c). These results show that there was no knockdown in gene expression, irrespective of the target gene, when dsRNA was fed via artificial diet to 4th instar larvae.

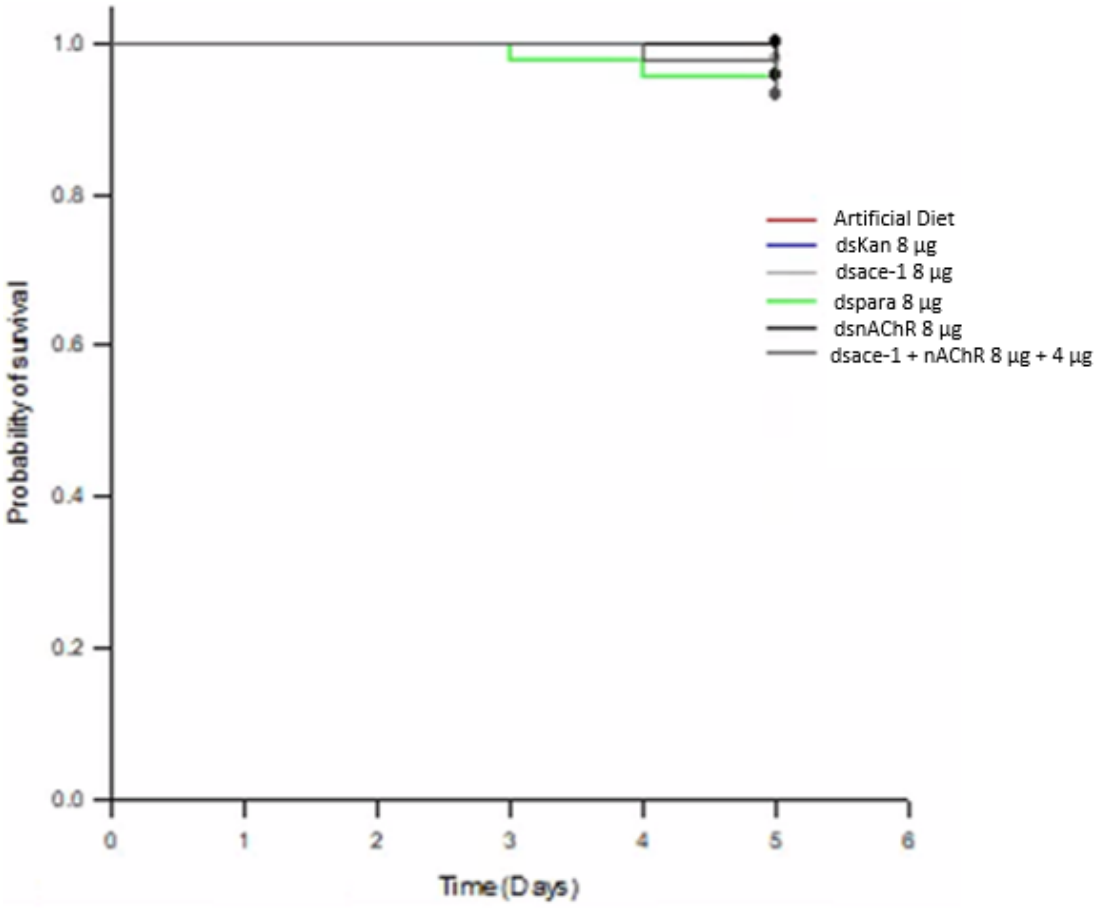

Figure 3.17 Survival of *S. littoralis* 4th instar larvae after ingesting artificial diet with 8 µg ds*ace-1*, ds*nAChR*, ds*para* or 4 µg ds*ace-1* + 4 µg ds*nAChR* percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan* were used as a control (n=45). Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial.

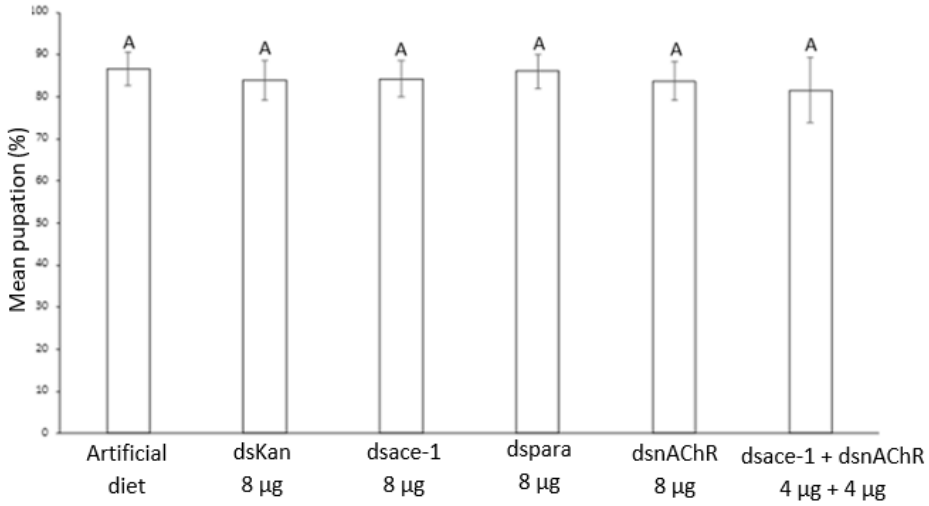

Figure 3.18 *S. littoralis* pupation after 4th instar larvae ingested artificial diet with 8 µg ds*ace-1*, ds*nAChR*, ds*para* or 4 µg ds*ace-1* + 4 µg ds*nAChR* percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan* were used as a control. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

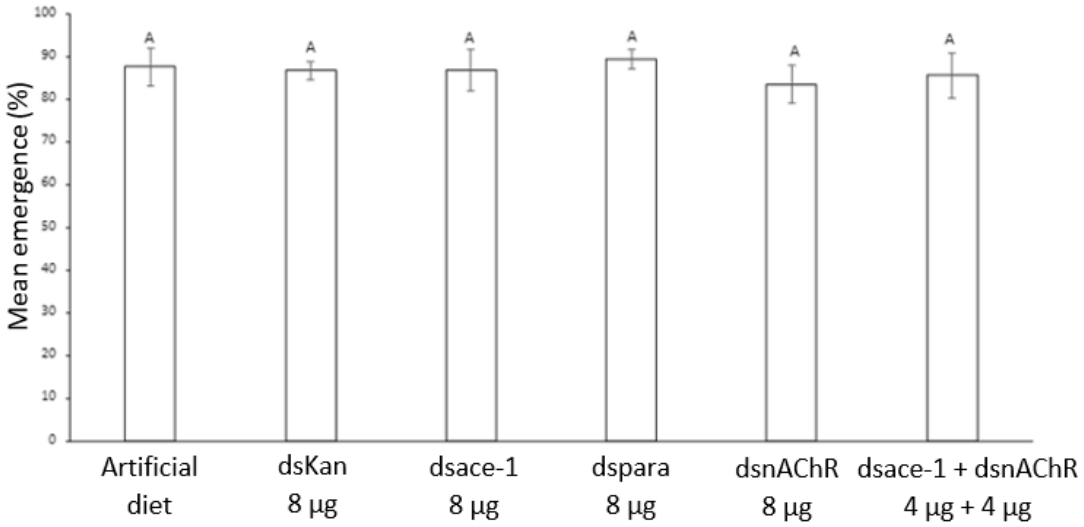

Figure 3.19 *S. littoralis* adult emergence after 4th instar larvae ingested artificial diet with 8 µg ds*ace-1*, ds*nAChR*, ds*para* or a combination of 4 µg ds*ace-1* + 4 µg ds*nAChR* percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan* were used as a control. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of pupating insects, error bars denote standard error of the mean and different letters denote significant differences.

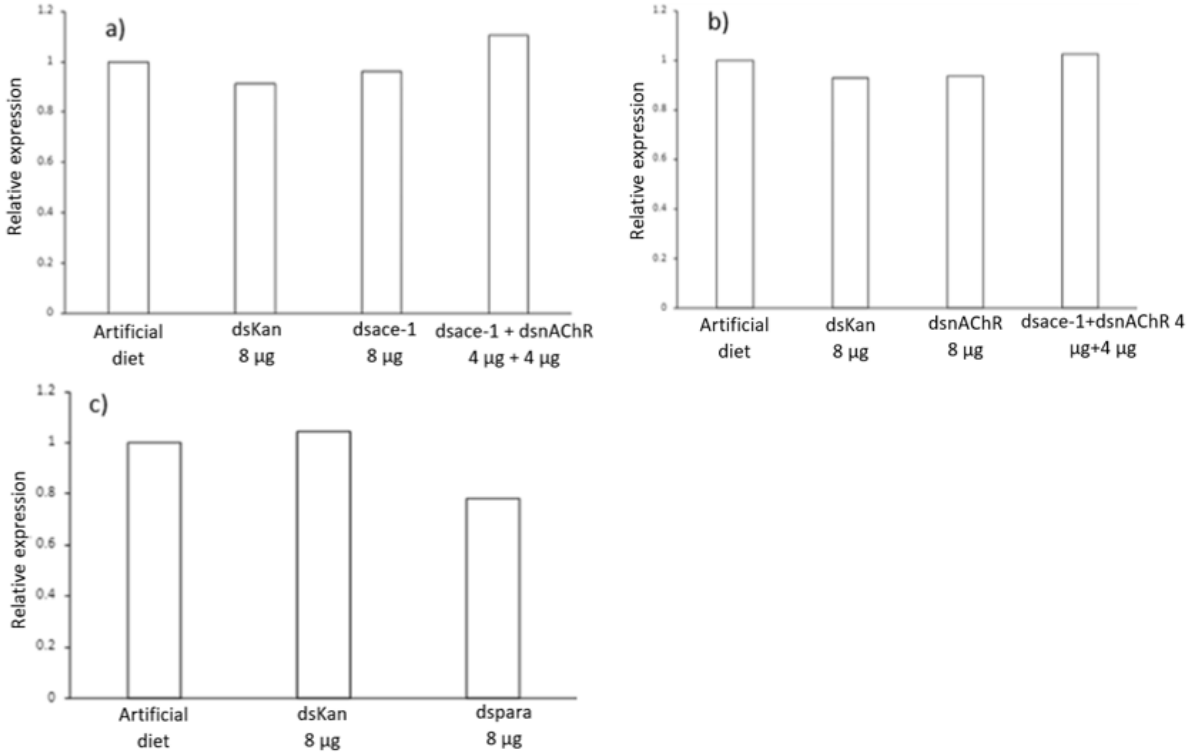

Figure 3.20 *S. littoralis* gene expression after 4th instar *S. littoralis* larvae were fed on artificial diet containing 8 µg ds*ace-1*, ds*nAChR* and ds*para* or 4 µg *dsace-1*+ 4 µg *dsnAChR*. Controls were insects reared on artificial diet ± 8 µg ds*Kan*. a) *ace-1* expression, b) *nAChR* expression, c) *para* expression. Relative transcript levels were determined 48 h later and RT-qPCR data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the reference gene and all values are relative to expression of insects reared on artificial diet (shown as Y=1).

3.3.9 Delivery of dsRNA via artificial diet via continuous feeding to neonate larvae

To determine the effect of continuously feeding gene specific dsRNA via an artificial diet on insect weight, survival, pupation, pupae weight and gene expression, *S. littoralis* neonate larvae were reared on artificial diet with 1 µg or 2 µg ds*ace-1*, ds*nAChR*, ds*para*, 2 µg or 4 µg ds*NADPHcytP450r* or 1 µg or 2 µg of both ds*ace-1* + ds*nAChR* (in combination) percolated throughout. Controls were insects reared on artificial diet ± 4 µg dsKan. Examples of larval sizes after continuous feeding on; i) artificial diet ± ds*Kan*, ds*ace-1*, ds*nAChR* or ds*ace-1* + ds*nAChR* after 6 and 9 days are shown in Figures 3.21a and 3.21b, ii) artificial diet ± ds*para* for 6 and 9 days are shown in Figures 3.22a and 3.22b, iii) artificial diet ± ds*NADPHcytP450r* after 6 and 9 days are shown in Figures 3.23a and 3.23b. Overall, continuously feeding dsRNA specific to any gene reduced larval development and growth.

On day 6, the average weight of insects reared on artificial diet or artificial diet containing ds*Kan*, 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg + 1 µg ds*ace-1* + ds*nAChR*, 2 µg + 2 µg ds*ace-1* + ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r* and 4 µg ds*NADPHcytP450r* was 232 mg, 227 mg, 157 mg, 154 mg, 150 mg, 132 mg, 93 mg, 83 mg, 157 mg, 163 mg, 212 mg and 193 mg, respectively) (Figure 3.24). The average weight of insects reared on artificial diet ± ds*Kan* were not significantly different from each other but both were significantly higher than the average weights of insects from any other group. However, the average weight of insects reared on artificial diet containing ds*Kan* was not significantly different from those reared on artificial diet containing 2 µg ds*NADPHcytP450r*. The average weight of insects reared on artificial diet containing 1 µg + 1 µg ds*ace-1* + ds*nAChR* or 2 µg + 2 µg ds*ace-1* + ds*nAChR* was significantly lower than that of insects reared on any other diet, including those reared on artificial diet containing either ds*ace-1* or ds*nAChR*, individually, at any concentration.

On day 9, the average weight of insects reared on artificial diet or artificial diet containing ds*Kan*, 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg + 1 µg ds*ace-1* + ds*nAChR*, 2 µg + 2 µg ds*ace-1* + ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r* and 4 µg ds*NADPHcytP450r* was 415 mg, 409 mg, 323 mg, 292 mg, 335 mg, 333 mg, 178 mg, 165 mg, 317 mg, 320 mg, 414 mg and 418 mg, respectively (Figure 3.24). The average weight of insects reared on artificial diet ± ds*Kan* was not significantly different from each other or insects reared on artificial diet containing 2 µg or 4 µg ds*NADPHcytP450r*. Furthermore, the average weight of insects reared on artificial diet containing 1 µg or 2 µg of both ds*ace-1* +

ds*nAChR* (in combination) was significantly lower than that of insects reared on any other diet, including those reared on artificial diet containing either ds*ace-1* or ds*nAChR*, individually, at either concentration. Additionally, despite the initial impact on larval size, the dsRNA did not inhibit feeding as average weight significantly increased between days 6 and 9 for insects reared on every diet (results not shown) and percentage weight increase between days 6 and 9 for insects reared on artificial diet or artificial diet containing ds*Kan*, 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg + 1 µg ds*ace-1* + ds*nAChR*, 2 µg + 2 µg ds*ace-1* + ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r* and 4 µg ds*NADPHcytP450r* was 44 %, 45 %, 51 %, 47 %, 55 %, 60 %, 48 %, 50 %, 50 %, 49 %, 49 % and 54 %, respectively.

After 9 days of continuous feeding, insects were transferred to basal artificial diet. Survival was recorded from days 1-12. For insects reared on artificial diet or artificial diet containing ds*Kan*, 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg + 1 µg ds*ace-1* + ds*nAChR*, 2 µg + 2 µg ds*ace-1* + ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r* and 4 µg ds*NADPHcytP450r* survival was reduced by 13 %, 11 %, 16 %, 24 %, 20 %, 24 %, 27 %, 31 %, 18 %, 22 %, 13 % and 16 %, respectively (Figure 3.25). Although Kaplan-Meier log-rank survival analysis identified that there was no significant difference ($P = 0.131$) between overall survival of insects reared on any diet, ANOVA with subsequent Tukey post-hoc test identified that by day 6, survival of insects reared on artificial diet with 2 µg + 2 µg ds*ace-1* + ds*nAChR* incorporated was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than the survival of insects reared on any other diet which remained the case until day 12.

After continuously feeding *S. littoralis* neonate larvae for 9 days, 82-92 %, of insects reared on artificial diet containing any *S. littoralis* specific dsRNA successfully pupated compared to 82 % and 80 % for those reared on artificial diet alone or artificial diet containing ds*Kan*, respectively and there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) between the instance of pupation between any group (Figure 3.26). Additionally, average pupal weight for insects reared on artificial diet containing any *S. littoralis* specific dsRNA was 298-305 mg, compared to 304 mg and 299 mg for those reared on artificial diet alone or artificial diet containing ds*Kan* and there was no significant difference between the weight of pupae formed from insects reared on any diet (Figure 3.27). Thus, continuous feeding of dsRNA specific to any gene did not significantly impact pupation or pupal weight.

Gene expression was investigated via RT-qPCR after 9 days of rearing *S. littoralis* neonate larvae on artificial diet containing ds*Kan*, ds*ace-1*, ds*nAChR*, ds*ace-1* + ds*nAChR*, ds*para* or ds*NADPHcytP450r* with expression compared to insects reared on optimal artificial diet. The expression of *ace-1* was 0.90, 1.08 and 1.24 when insects were reared on artificial diet containing ds*Kan*, 2 µg ds*ace-1* or 2 µg + 2 µg ds*ace-1* + ds*nAChR*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.28a). The expression of *nAChR* was 1.31, 1.39 and 1.27 when insects were reared on artificial diet containing kanamycin, ds*Kan*, 2 µg ds ds*nAChR* or 2 µg + 2 µg ds*ace-1* + ds*nAChR*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.28b). The expression of *para* was 1.55, 1.45 and 1.55 when insects were reared on artificial diet containing ds*Kan*, 1 µg ds*para* and 2 µg ds*para*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.28b). The expression of *NADPHcytP450r* was 0.91, 0.74 and 0.79 when insects were reared on artificial diet containing ds*Kan*, 2 µg ds*NADPHcytP450r* or 4 µg ds*NADPHcytP450r*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.28d). Overall, continuously feeding dsRNA specific to any gene for 9 days did not significantly reduce gene expression.

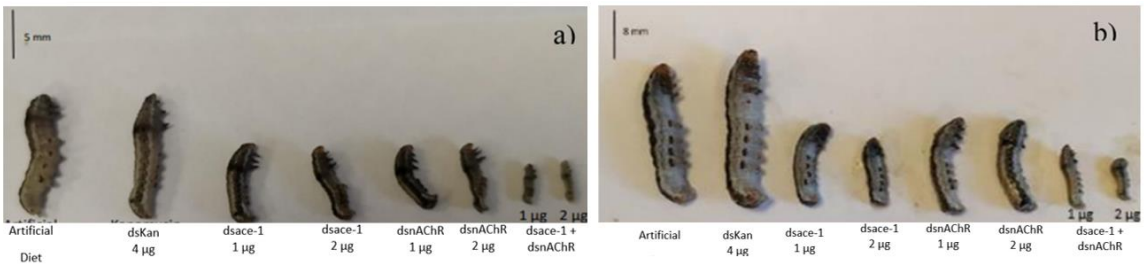

Figure 3.21 Examples of the phenotypic differences between *S. littoralis* larvae after continuous feeding of ds*ace-1*, ds*nAChR* or ds*ace-1+nAChR* (in combination) for a) 6 days b) 9 days. Insects were fed from neonate on artificial diet containing gene specific dsRNA and were frozen prior to imaging. Controls were insects continuously fed with artificial diet ± dsKan.

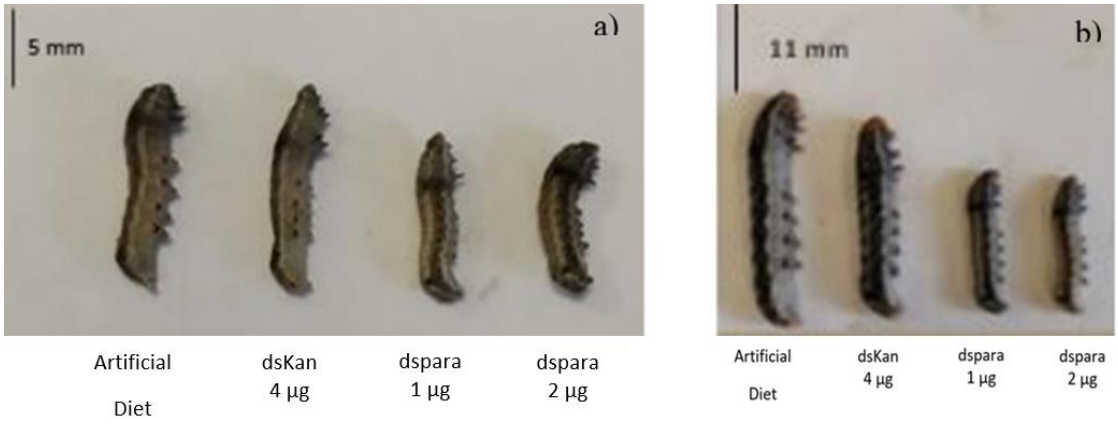

Figure 3.22 Examples of the phenotypic differences between *S. littoralis* larvae after continuous feeding of ds*para* for a) 6 days b) 9 days. Insects were fed from neonate on artificial diet containing gene specific dsRNA and were frozen prior to imaging. Controls were insects continuously fed with artificial diet ± dsKan.

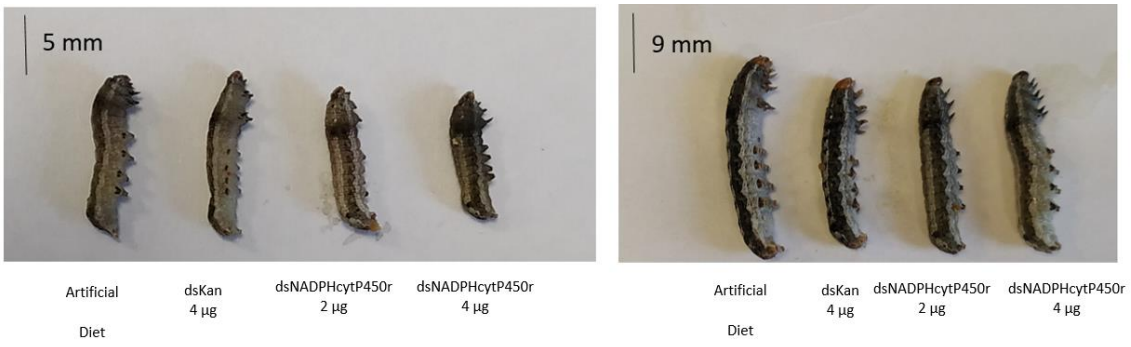

Figure 3.23 Examples of the phenotypic differences between *S. littoralis* larvae after continuous feeding of ds*NADPHcytP450r* for a) 6 days b) 9 days. Insects were fed from neonate on artificial diet containing gene specific dsRNA and were frozen prior to imaging. Controls were insects continuously fed with artificial diet ± dsKan.

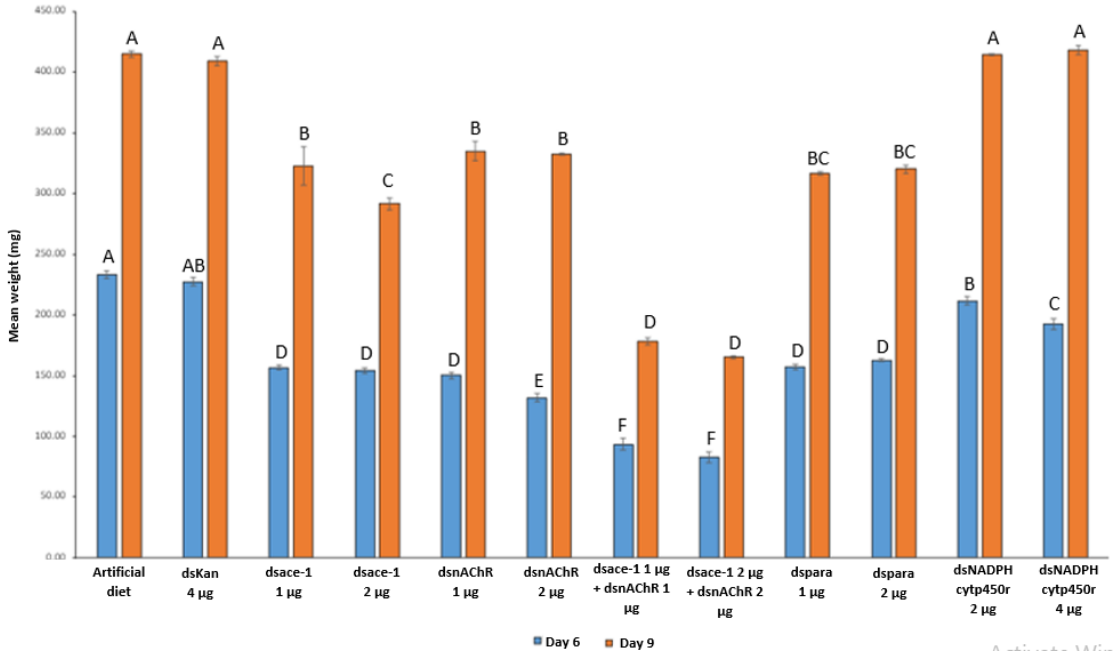

Figure 3.24 *S. littoralis* larval weight after continuous feeding of gene specific dsRNA for 6 and 9 days. Insects were fed from neonate on artificial diet containing gene specific dsRNA and those reared on artificial diet ± 4 µg ds*Kan* were used as a control. Values are the average of 3 biological replicates (n=15), error bars denote standard error of the mean and different letters denote significant differences and are specific to differences between weights of insects reared on each diet rather than between days. Outliers either 1.5*IQR below or above Q1 and Q3, respectively, were removed. Blue and orange bars represent mean weight of larvae on days 6 and 9, respectively.

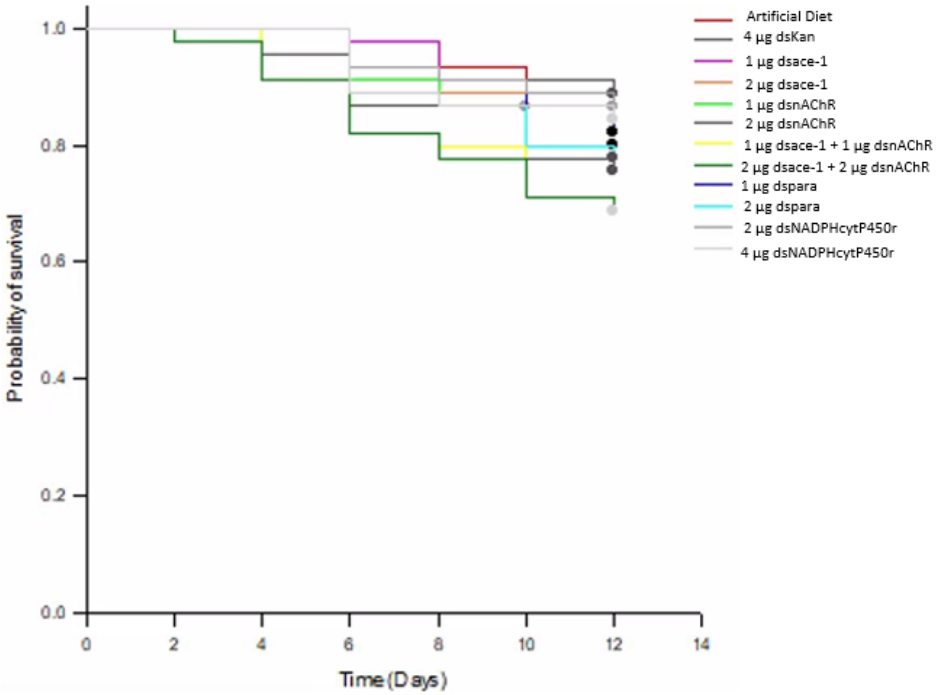

Figure 3.25 Survival of *S. littoralis* larvae reared on artificial diet containing *dsace-1*, ds*nAChR*, ds*para*, ds*NADPHcytP450r* or ds*ace-1* + ds*nAChR* (n=45). Controls were insects reared on artificial diet ± 4 µg ds*Kan* (n=45). Insects reared on artificial diet with dsRNA incorporated were fed continuously for 9 days and then transferred to artificial diet.

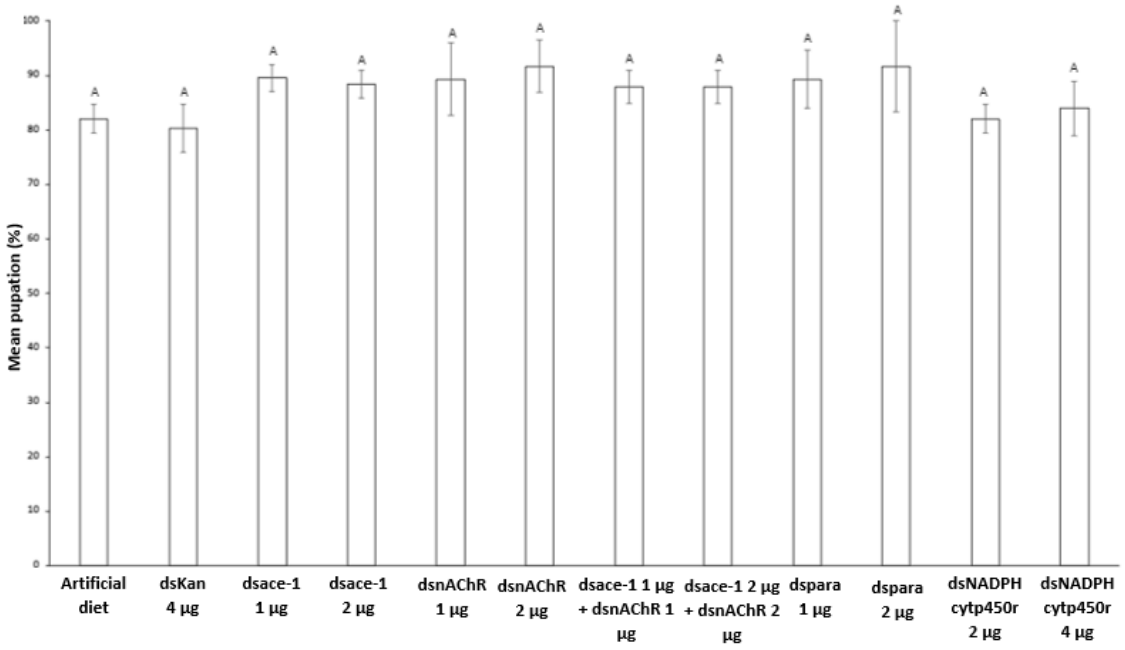

Figure 3.26 *S. littoralis* pupation after larvae were fed from neonate on artificial diet containing ds*ace-1*, ds*nAChR*, ds*para*, ds*NADPHcytP450r* or ds*ace- 1*+ *nAChR*. Controls were insects reared on artificial diet ± or 4 µg ds*Kan*. Insects reared on artificial diet ± dsRNA were fed continuously for 9 days and then transferred to artificial diet. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

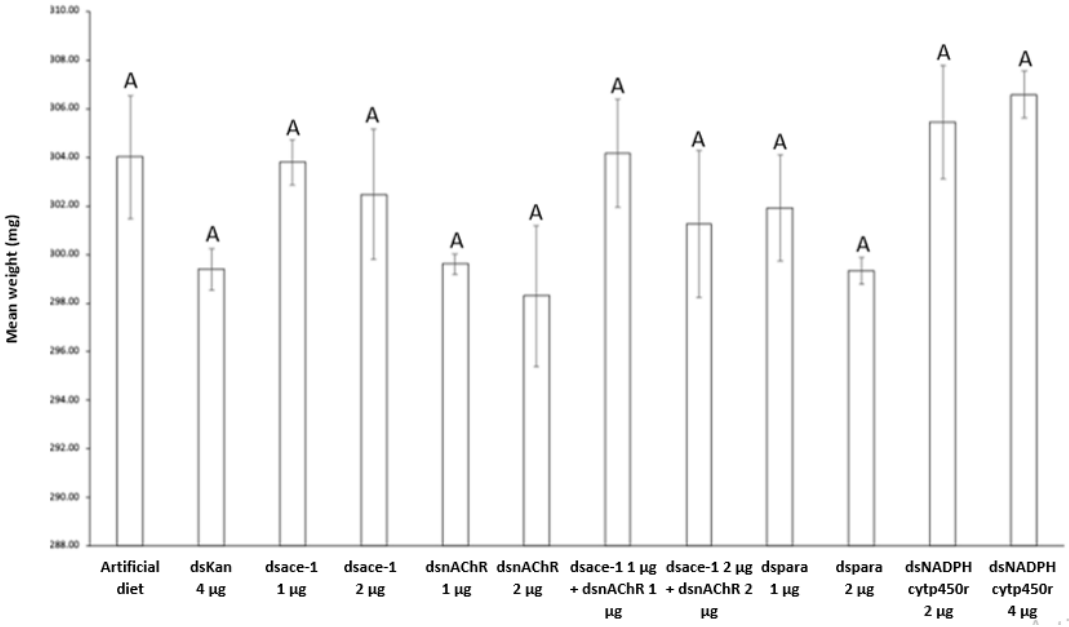

Figure 3.27 Weight of *S. littoralis* pupae after larvae were fed continuously from neonate on artificial diet containing ds*ace-1*, ds*nAChR*, ds*para*, ds*NADPHcytP450r* or ds*ace-1* + ds*nAChR* (n=45). Controls were insects reared on artificial diet ±4 µg ds*Kan*. Insects reared on artificial diet ± dsRNA were fed continuously for 9 days and then transferred to artificial diet. Values are the average of 3 biological replicates (n=15), error bars denote standard error of the mean and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively, were removed.

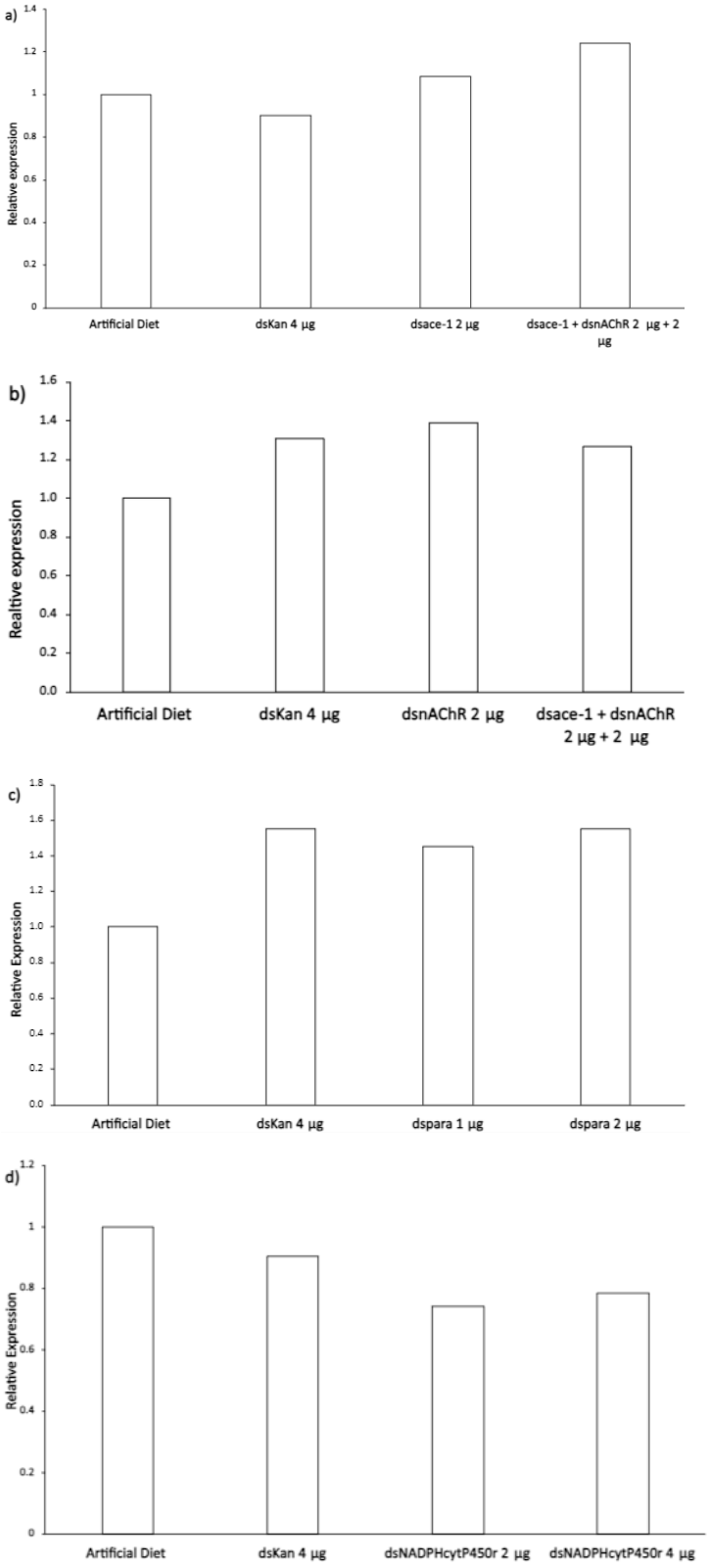

Figure 3.28 *S. littoralis* gene expression after larvae were fed for 9 days from neonate on artificial diet containing ds*ace-1*, ds*nAChR*, ds*para* ds*NADPHcytP450r* or ds*ace-1* + ds*nAChR*. Controls were insects reared on artificial diet ± 4 µg ds*Kan*. a) *ace-1* expression, b) *para* expression, c) *nAChR* expression, d) *NADPHcytP450r* expression. RT-qPCR data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to expression of insects reared on artificial diet (shown as Y=1).

3.3.10 Delivery of dsRNA via gavage feeding to 5th instar larvae via a single feeding event

Successful pupation was determined after delivering *S. littoralis* specific dsRNA to 5th instar larvae via gavage feeding. For insects reared on artificial diet or gavage fed with 8 µg ds*Kan*, ds*ace-1*, ds*nAChR*, ds*ace-1* + ds*nAChR* (4 µg of both), ds*para* or ds*NADPHcytP450r*, 90 %, 97 %, 80 %, 80%, 63 %, 77 %, 83 % of insects pupated, respectively (Figure 3.29). Furthermore, there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) in the instance of pupation for insects reared on artificial diet or gavage fed with ds*Kan*, ds*ace-1*, ds*nAChR*, ds*para* or ds*NADPHcytP450r*. However, significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated after being gavage fed with 4 µg ds*ace-1* + 4 µg ds*nAChR* compared with those reared on artificial diet.

The average pupal weight of insects gavage fed with dsRNA targeted to any *S. littoralis* specific dsRNA was 291- 318 mg compared to 305 mg when reared on artificial diet alone or 302 mg for those gavage fed with dsKan and there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) between the average weight of pupae formed from insects reared on artificial diet or those gavage fed with dsRNA specific to any gene specific (Figure 3.30). However, the average weight of pupae from insects gavage fed with 4 µg ds*ace-1* + 4 µg ds*nAChR* was significantly lower than those gavage fed with ds*ace-1* ($P < 0.05$; ANOVA, Tukey post-hoc), 291 and 318 mg, respectively. When gavage fed with dsRNA specific to *S. littoralis*, 71-88 % of insects that pupated emerged as adults, compared to 81 % for those fed with artificial diet alone or 73 % for those gavage fed with dsKan (Figure 3.31). Additionally, there was no significant difference in the number of adults emerging when insects were gavage fed with any *S. littoralis* gene specific dsRNA compared to when reared on artificial diet or gavage fed with ds*Kan*. Thus, whilst gavage feeding ds*ace-1*, ds*nAChR*, ds*para* or ds*NADPHcytP450r* to 5th instar larvae did not significantly affect pupal weight, ds*ace-1* and ds*nAChR* delivered in combination did. However, gavage delivery of any *S. littoralis* specific dsRNA did not significantly impact adult emergence.

The effect on gene expression after rearing 5th instar *S. littoralis* on artificial diet or gavage feeding with 8 µg ds*Kan*, ds*ace-1*, ds*nAChR*, ds*para*, ds*NADPHcytP450r* or 4 µg ds*ace-1* + 4 µg ds*nAChR* was investigated 48 h post-gavage via RT-qPCR, with all expression levels compared to insects reared on optimal artificial diet. The expression of *ace-1* was 1.25, 0.87 and 1.26 when insects were gavage fed with ds*Kan*, ds*ace-1* or ds*ace-1* + ds*nAChR*, respectively, thus was not significantly different than when insects were reared on artificial

diet (Figure 3.32a). The expression of *nAChR* was 1.18, 1.04 and 1.11 when insects were gavage fed with ds*Kan*, ds*nAChR* or ds*ace-1* + ds*nAChR*, thus was not significantly different than when insects were reared on artificial diet (Figure 3.32b). The expression of *para* was 1.39 and 0.89 when insects were gavage fed with ds*Kan* or ds*para*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.32c). The expression of *NADPHcytP450r* was 1.05 and 1.17 when insects were gavage fed with ds*Kan* or ds*NADPHcytP450r*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.32d). Overall, these results show that dsRNA delivery via gavage to 5th instar larvae had no significant effects on expression of the target genes.

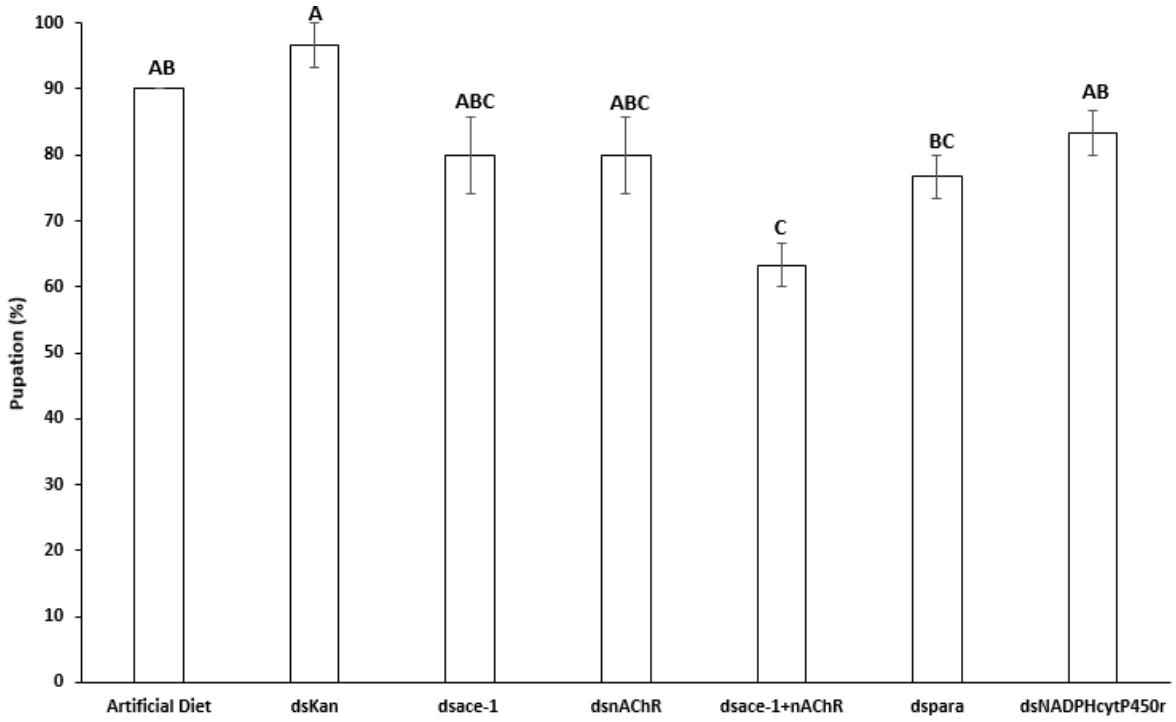

Figure 3.29 *S. littoralis* pupation after 5th instar larvae were gavage fed once with *S. littoralis* specific dsRNA. Each insect received 8 µg dsRNA, except the group gavage fed with *ace-1* + *nAChR* which received 4 µg of both. Controls were insects reared on an artificial diet and insects gavage fed with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=10) and different letters denote significant differences.

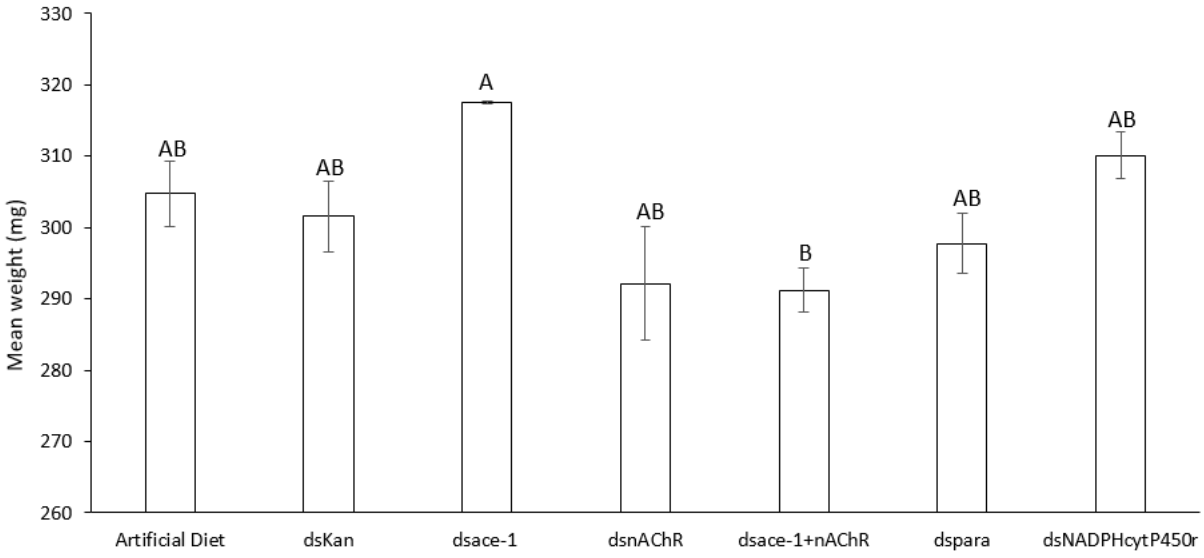

Figure 3.30 Weight of *S. littoralis* pupae after 5th instar larvae were gavage fed once with *S. littoralis* specific dsRNA. Each insect received 8 µg dsRNA, except the insects gavage fed with *ace-1* + *nAChR* which received 4 µg of both. Controls were insects fed on artificial diet and insects gavage fed with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=10) and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively, were removed.

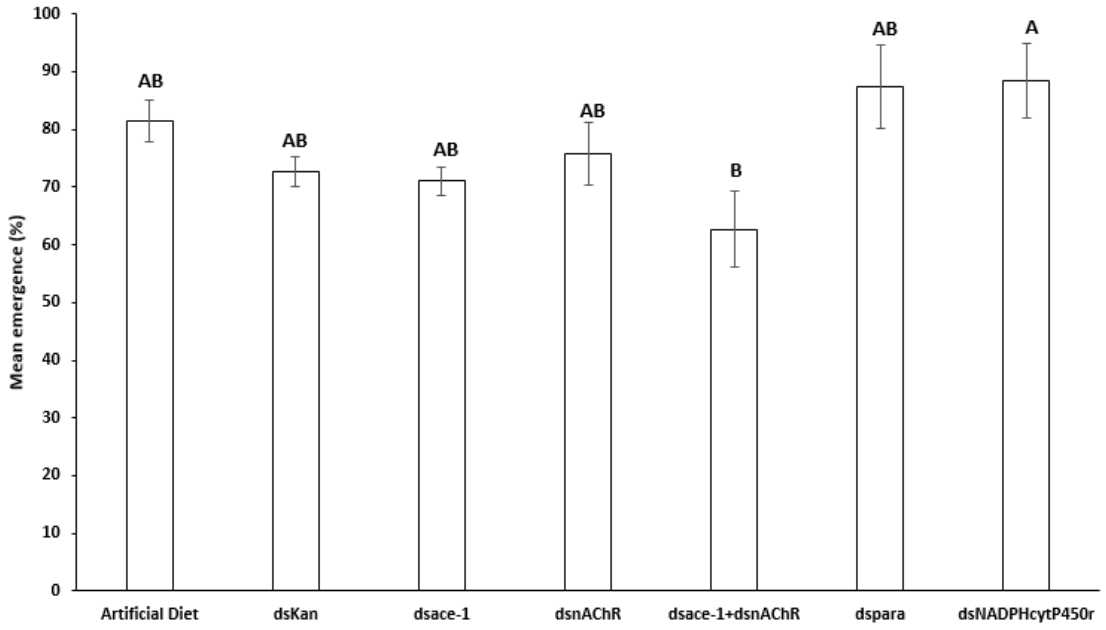

Figure 3.31 *S. littoralis* adult emergence after 5th instar larvae were gavage fed once with *S. littoralis* specific dsRNA. Each insect received 8 µg dsRNA, except insects gavage fed with *ace-1* + *nAChR* which received 4 µg of both. Controls were insects reared on artificial diet and insects gavage fed with 8 µg ds*Kan*. Values are the average of 3 biological replicates and are given as percentages of those insects which pupated, error bars denote standard error of the mean of 3 groups (n=10) and different letters denote significant differences.

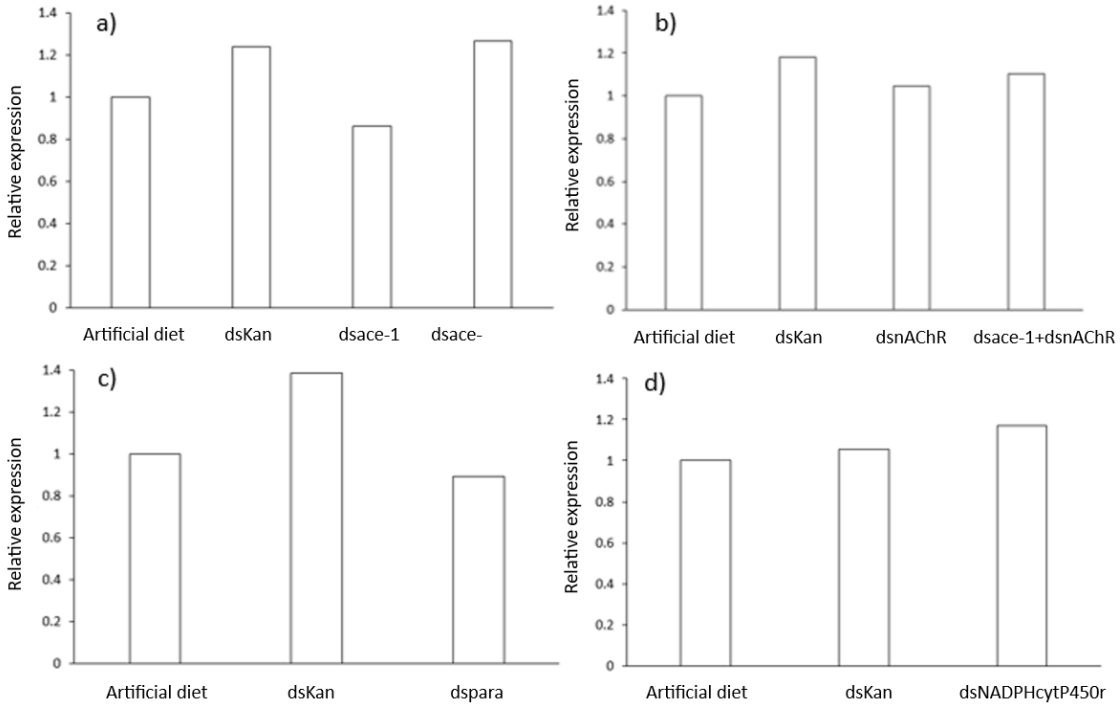

Figure 3.32 *S. littoralis* gene expression after 5th instar *S. littoralis* larvae were gavage fed once with *S. littoralis* specific dsRNA. Each insect received 8 µg dsRNA, except insects gavage fed with *ace-1* + *nAChR* which received 4 µg of both. Controls were insects fed on an artificial diet and insects gavaged with 8 µg ds*Kan*. a) *ace-1* expression, b) *nAChR* expression, c) *para* expression, d) *NADPHcytP450r* expression. RT-qPCR data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to expression of insects reared on artificial diet (shown as Y=1).

3.3.11 Delivery of dsRNA via direct haemolymph injection into 4th instar larvae

Injection of dsace-1 in 4th instar larvae

Survival was recorded after injecting 4th instar *S. littoralis* larvae with increasing concentrations of ds*ace-1*. When injected with 0.5 - 8 µg ds*ace-1*, survival was reduced by 9-18 % compared to 0 % when not injected and 16 % when injected with 8 µg ds*Kan* (Figure 3.33) and Kaplan-Meier log-rank survival identified no significant difference between survival curves. Additionally, 58 – 76 % of insects injected with 0.5 - 8 µg ds*ace-1* pupated compared to 82 % when not injected and 84 % when injected with 8 µg ds*Kan* (Figure 3.34) and there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) between pupation of non-injected insects and those injected with 0.5 µg, 1 µg and 2 µg ds*ace-1* but significantly fewer ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 4 µg ds*ace-1* compared to non-injected insects. Furthermore, 55 – 77 % of insects injected with 0.5 - 8 µg ds*ace-1* emerged as adults compared to 84 % when not injected and 82 % when injected with 8 µg ds*Kan* (Figure 3.35) and emergence was not significantly different ($P > 0.05$; ANOVA, Tukey post-hoc) between any groups. Additionally, average pupal weight

for insects injected with 0.5 - 8 µg ds*ace-1* ranged from 276 – 296 mg compared to 313 mg when not injected and 310 mg when injected with 8 µg ds*Kan* (Figure 3.36) and only the average weight of pupae from insects injected with 4 µg ds*ace-1* was significantly reduced compared to insects that were not injected or that were injected with 8 µg ds*Kan*.

The mean weight of artificial diet consumed by insects injected with 0.5 - 8 µg ds*ace-1* (3 biological replicates of 15 insects) was between 262 – 350 mg between 0 and 2 days post-injection compared to 274 mg for non-injected insects and 339 mg for those injected with 8 µg ds*Kan* (Figure 3.37). Between days 3-5 post-injection, the mean weight of diet consumed by insects injected with 0.5 - 8 µg ds*ace-1* was between 705 – 947 mg compared to 707 mg for non-injected insects and 915 mg for those injected with 8 µg ds*Ka*n (Figure 3.37). Between days 0 and 2, the mean weight of diet consumed by insects injected with 2 µg, 4 µg, 8 µg ds*ace-1* or 8 µg ds*Kan* was higher than that consumed by non-injected insects or those injected with 0.5 µg or 1 µg ds*ace-1*, but did not differ significantly ($P > 0.05$). Between days 3 and 5, the mean weight of diet consumed by insects injected with 2 µg, 4 µg, 8 µg ds*ace-1* or 8 µg ds*Kan* was higher than that consumed by non-injected insects or those injected with 0.5 µg or 1 µg ds*ace-1*. Although insects injected with 2 µg or 4 µg ds*ace-1* consumed significantly more food than those reared on artificial diet, there was no significant difference with those injected with 8 µg ds*Kan* ($P < 0.05$; ANOVA, Tukey post-hoc).

The effect on gene expression after injecting 4th instar *S. littoralis* larvae with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*ace-1* or 8 µg ds*Kan* was investigated 48 h post-injection via RT-qPCR, with all expression levels compared to non-injected insects reared on artificial diet. The expression of *ace-1* was 1.17, 0.65, 1.16, 0.85, 1.01 and 0.82-fold when insects were injected with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*ace-1* or 8 µg ds*Kan*, respectively, thus was not significantly different than when insects were not injected (Figure 3.38). The expression of *ace-2* was 1.39, 0.92, 1.09, 1.07, 1.11 and 0.76-fold when insects were injected with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*ace-1* or 8 µg ds*Kan*, respectively, thus was not significantly different than when insects were not injected (Figure 3.39).

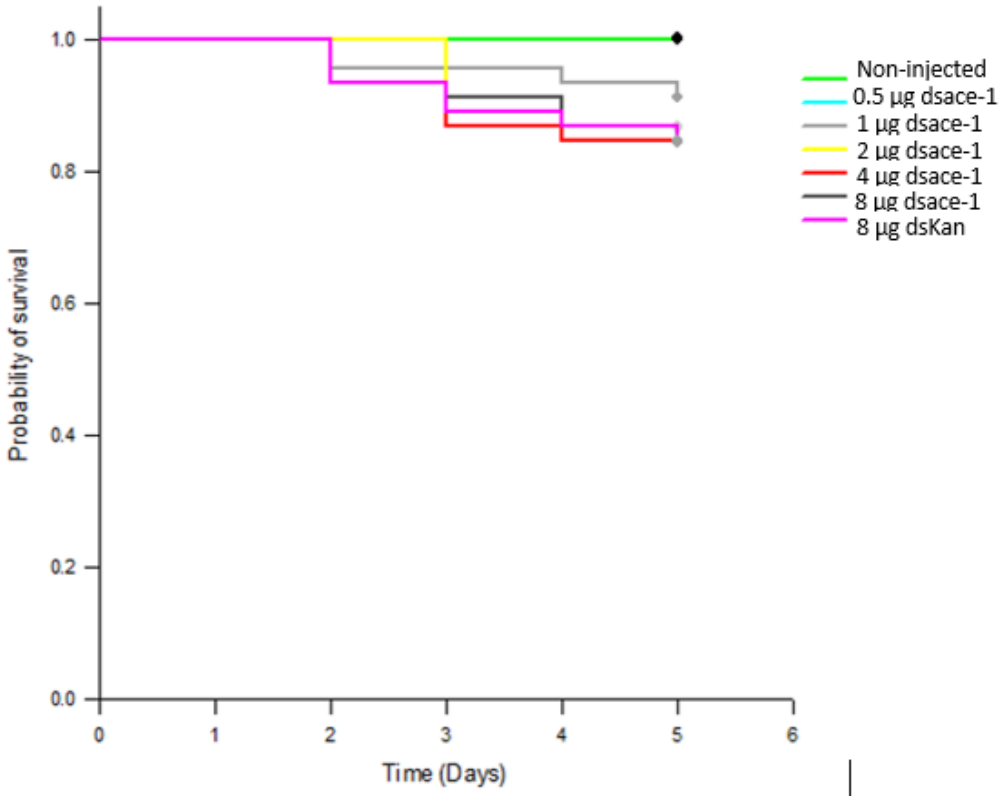

Figure 3.33 Survival of 4th *S. littoralis* larvae after direct haemolymph injection of increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan* (n=45). All insects were transferred to artificial diet post-injection.

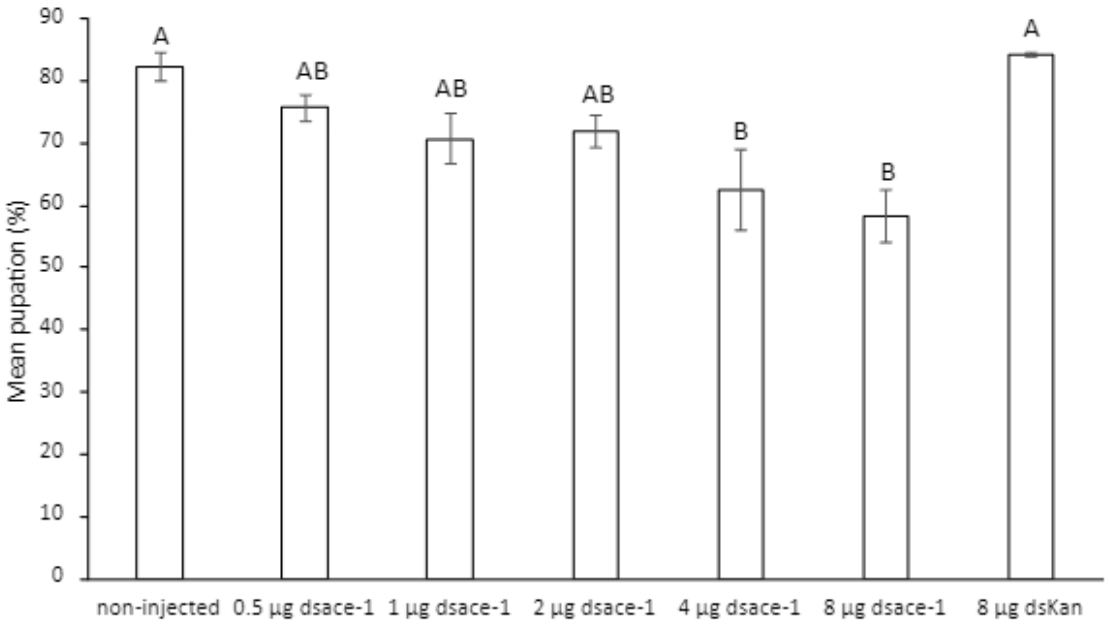

Figure 3.34 *S. littoralis* pupation after 4th instar larvae were injected once with ds*ace-1*. Controls were non-injected and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

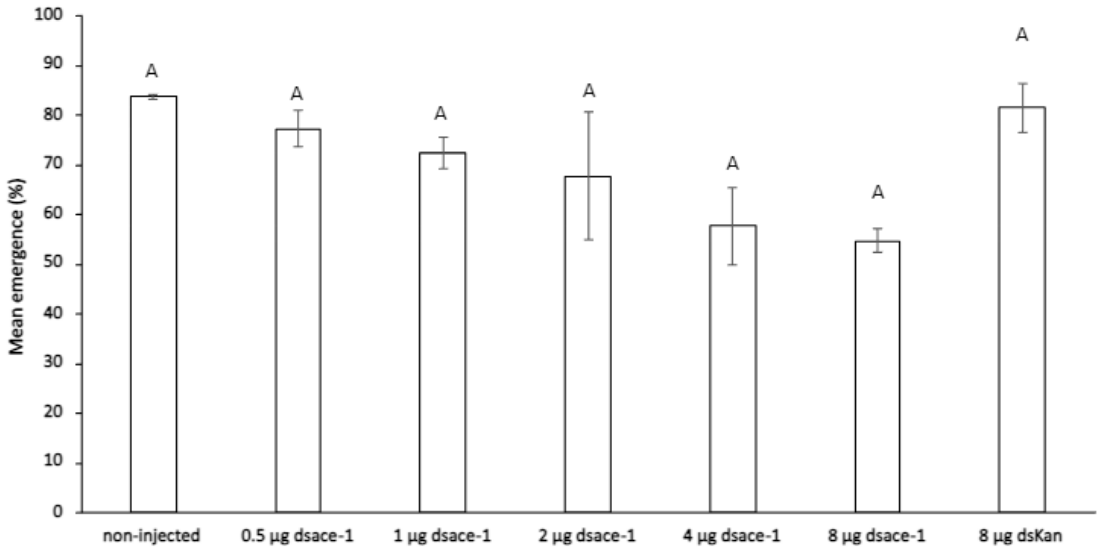

Figure 3.35 *S. littoralis* adult emergence after 4th instar larvae were injected once with increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of those insects which pupated, error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences.

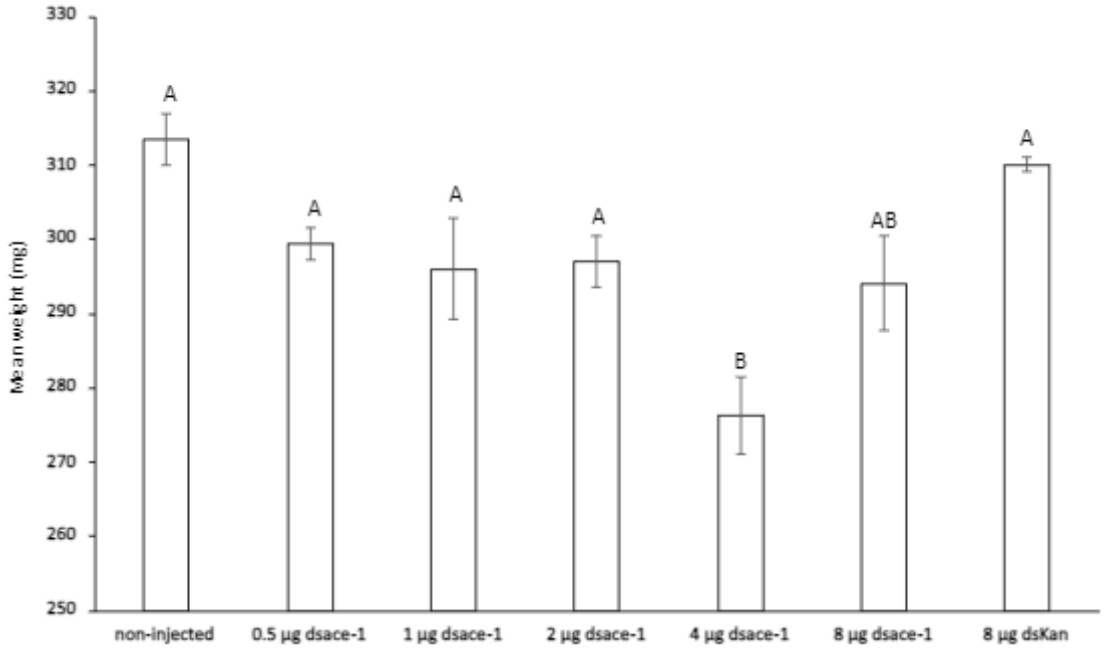

Figure 3.36 Weight of *S. littoralis* pupae after 4th instar larvae were injected once with increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively were removed.

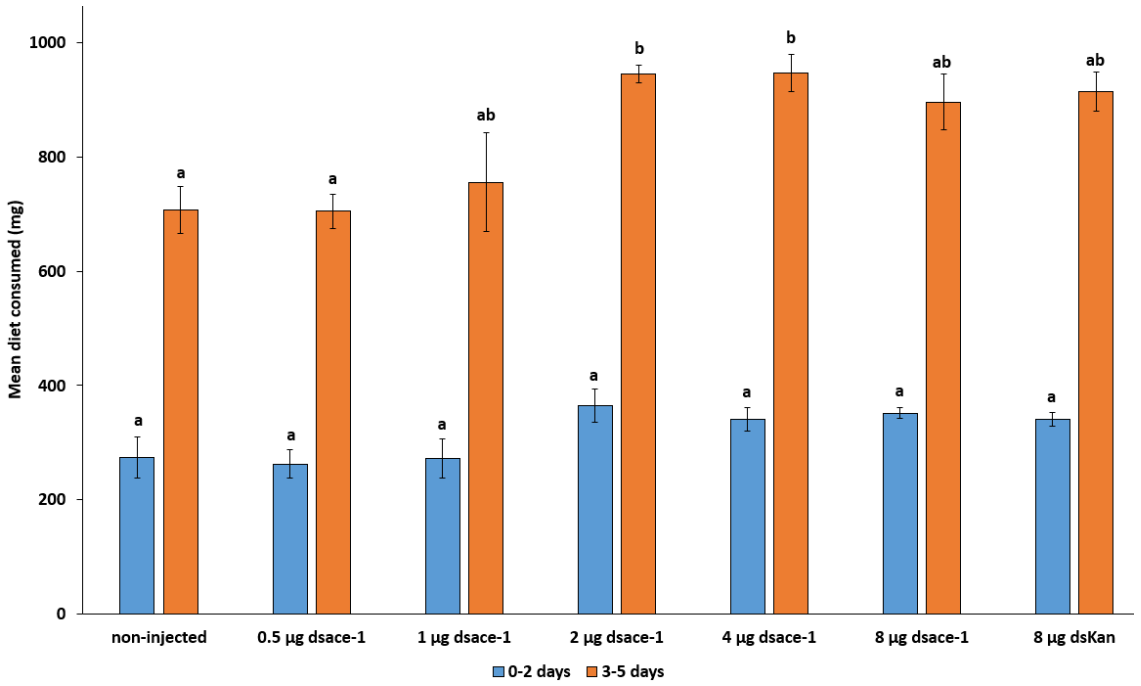

Figure 3.37 Weight of artificial diet consumed by *S. littoralis* 4th instar larvae between 0 and 2 days and 3 and 5 days post-injection with increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences (lower case letters denote differences in mean diet consumed between each time point and capital letters denote differences between ?) (P = 0.05 ANOVA, Post-hoc Tukey test). Outliers either 1.5*IQR below or above Q1 and Q3, respectively, were removed. Blue and orange bars show mean diet consumed between days 0-2 and days 3-5 respectively.

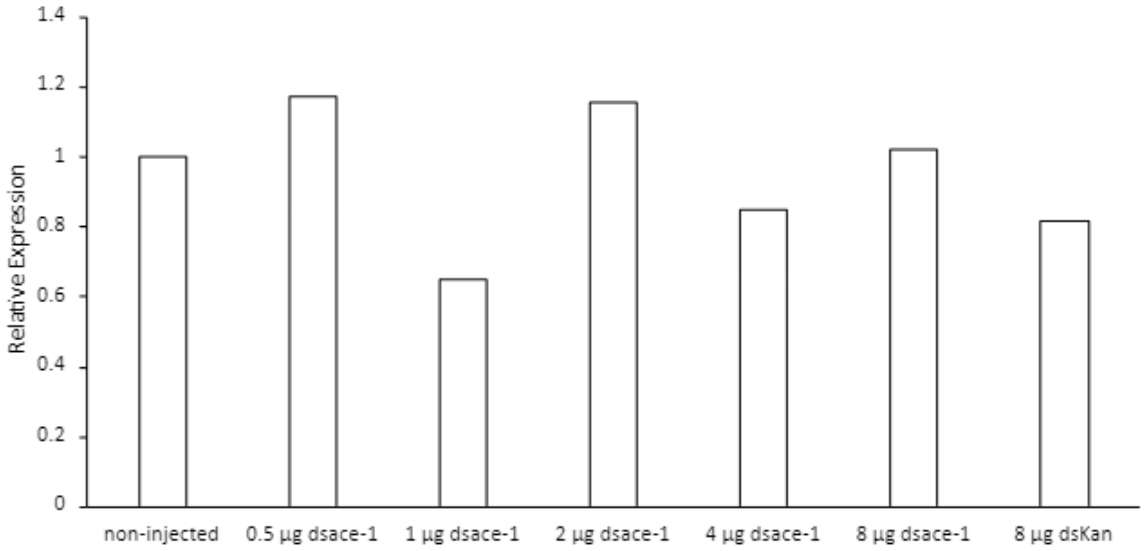

Figure 3.38 *S. littoralis ace-1* gene expression after 4th instar larvae were injected once with increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Expression was determined 48 h post-injection via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to gene expression of *S. littoralis* reared on artificial diet (shown as Y=1).

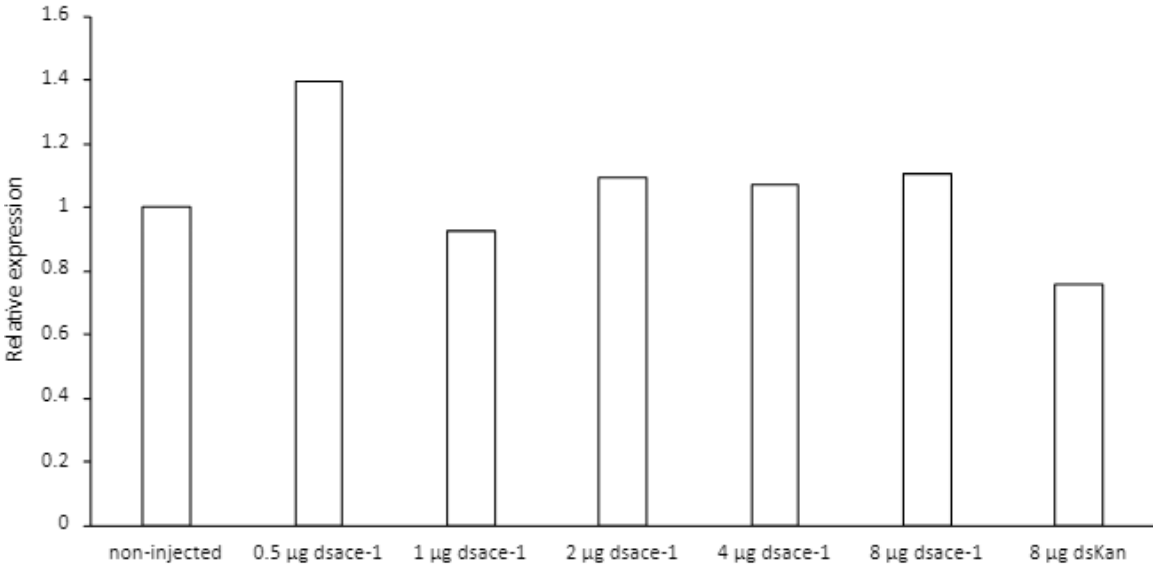

Figure 3.39 *S. littoralis ace-2* gene expression after 4th instar larvae were injected once with increasing concentrations of ds*ace-1*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Expression was determined 48 h post-injection via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to gene expression of *S. littoralis* reared on artificial diet (shown as Y=1).

Injection of dspara in 4th instar larvae

Survival was recorded after injecting 4th instar *S. littoralis* larvae with increasing concentrations of ds*para*. For those injected with 0.5 - 8µg ds*para*, survival was reduced 9 – 18 % compared to 0 % and 18 % for non-injected insects and those injected with 8 µg ds*Kan*, respectively (Figure 3.40) and Kaplan-Meier log-rank survival analysis identified no significant difference between survival curves. Furthermore, 51 – 69 % of insects injected with 0.5 – 8µg ds*para* pupated compared to 82 % for both those insects that were not injected and those injected with 8µg ds*Kan* (Figure 3.41).There was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) between pupation of non-injected insects and those injected with 0.5 µg, 1 µg, 2 µg ds*para* and 8 µg ds*Kan* but significantly fewer ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 4 µg and 8 µg ds*para* compared to those who were not injected. Additionally, 62 – 74 % of insects injected with 0.5 – 8µg ds*para* emerged as adults compared to 81 % and 84 % when insects were not injected or were injected with 8 µg ds*Kan*, respectively (Figure 3.42) and the instance of emergence was not significantly different ($P > 0.05$; ANOVA, Tukey post-hoc) between any groups. Thus, injection of ds*para* into 4th instar larvae did not significantly affect survival, pupation or emergence.

The effect on gene expression after injecting 4th instar *S. littoralis* larvae with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*para* or 8 µg ds*Kan* was investigated 48 h post-injection via RT-qPCR, with all expression levels compared to insects reared on artificial diet. The expression of *para* was 0.95, 1.22, 1.04, 1.10, 0.95 and 1.00-fold when insects were injected with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*para* or 8 µg ds*Kan*, respectively, thus was not significantly different than when insects were reared on artificial diet (Figure 3.43).

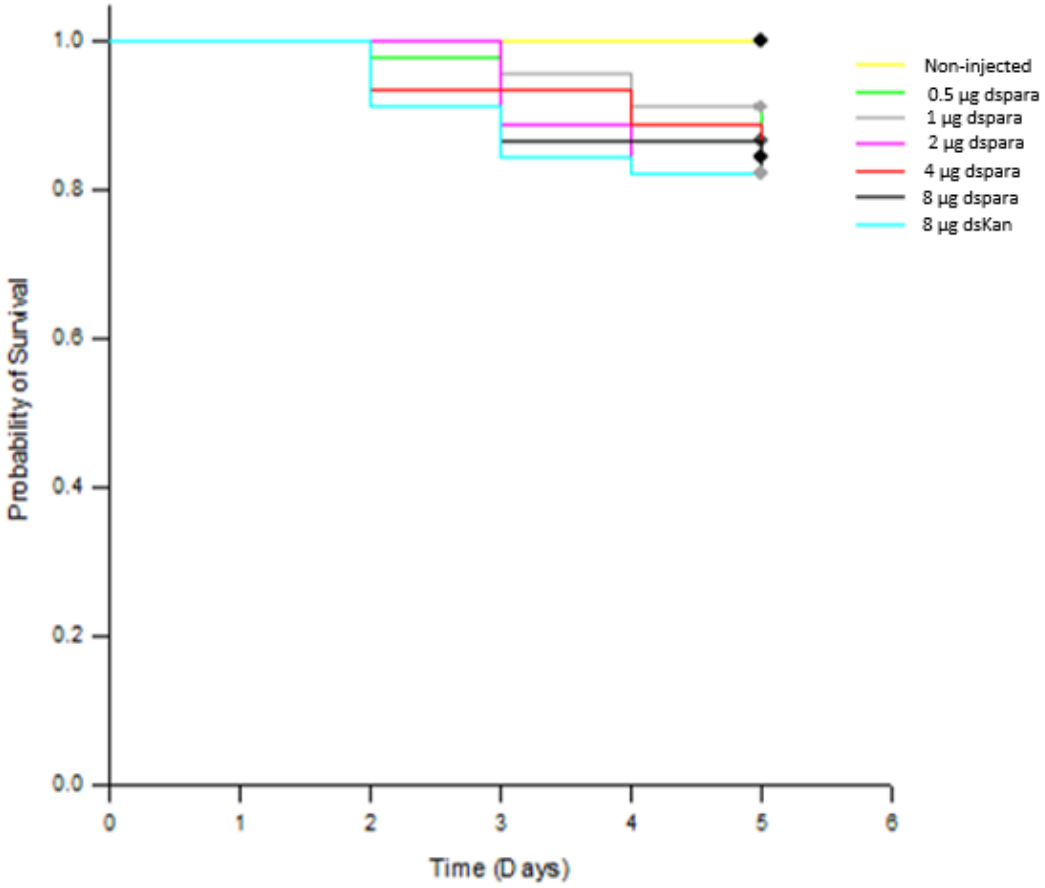

Figure 3.40 Survival of 4th *S. littoralis* larvae after direct haemolymph injection of increasing concentrations of ds*para*. Controls were non-injected insects and insects injected with 8 µg ds*Kan* (n=45). All insects were transferred to artificial diet post-injection.

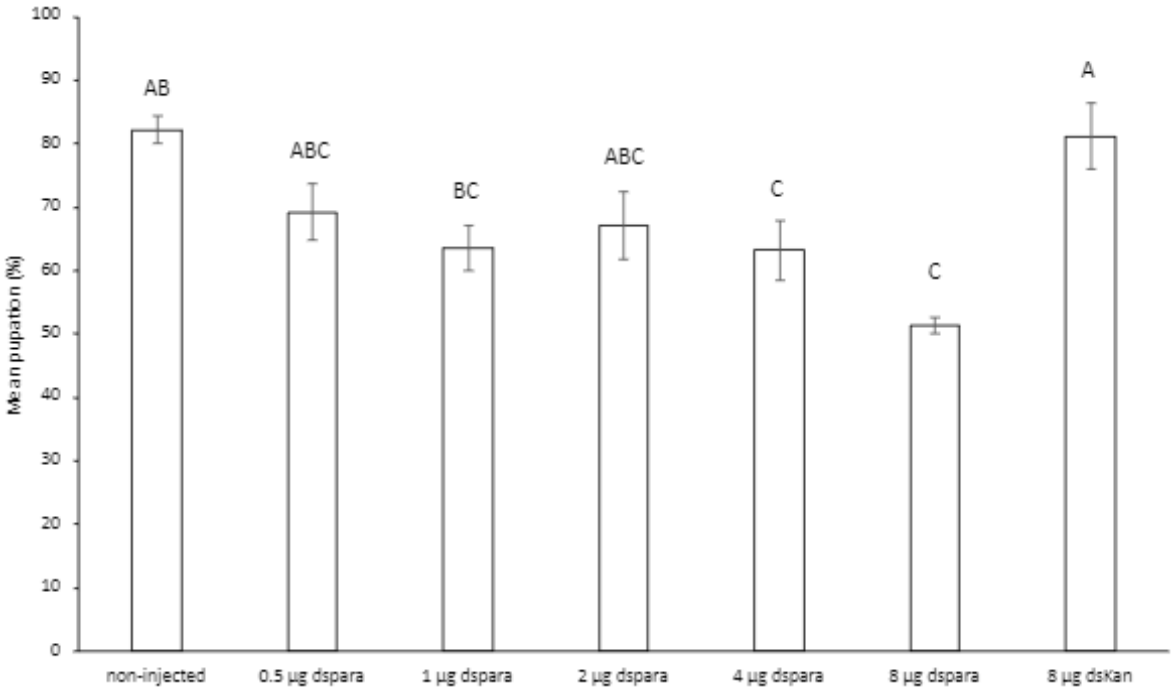

Figure 3.41 *S. littoralis* pupation after 4th instar larvae were injected once with *dspara*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

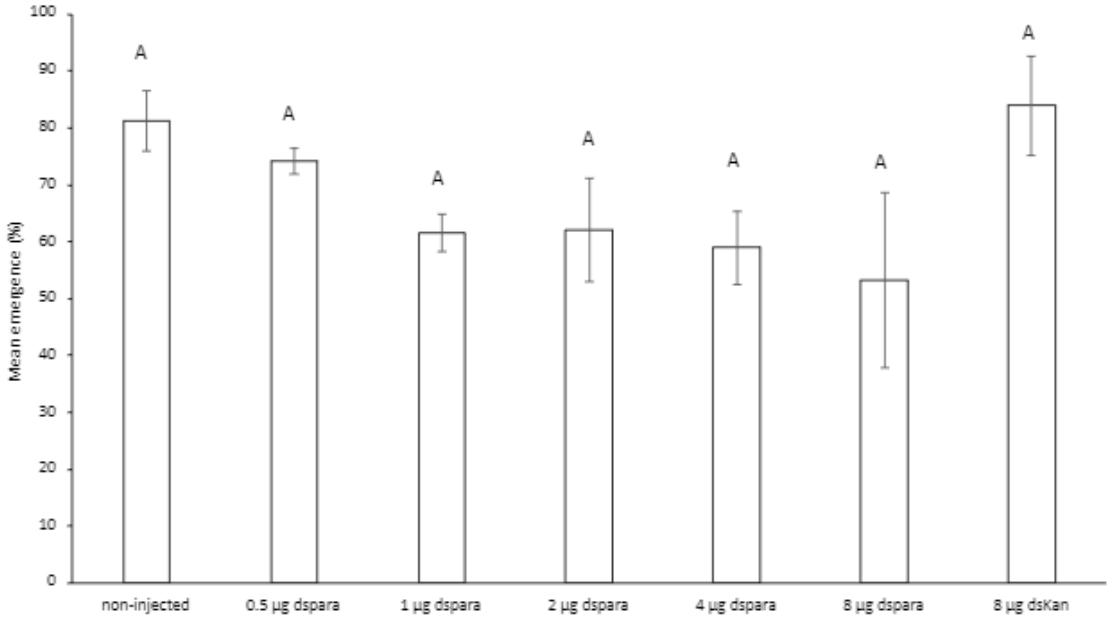

Figure 3.42 *S. littoralis* adult emergence after 4th instar larvae were injected once with *S. littoralis para* specific dsRNA. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of those insects which pupated, error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences.

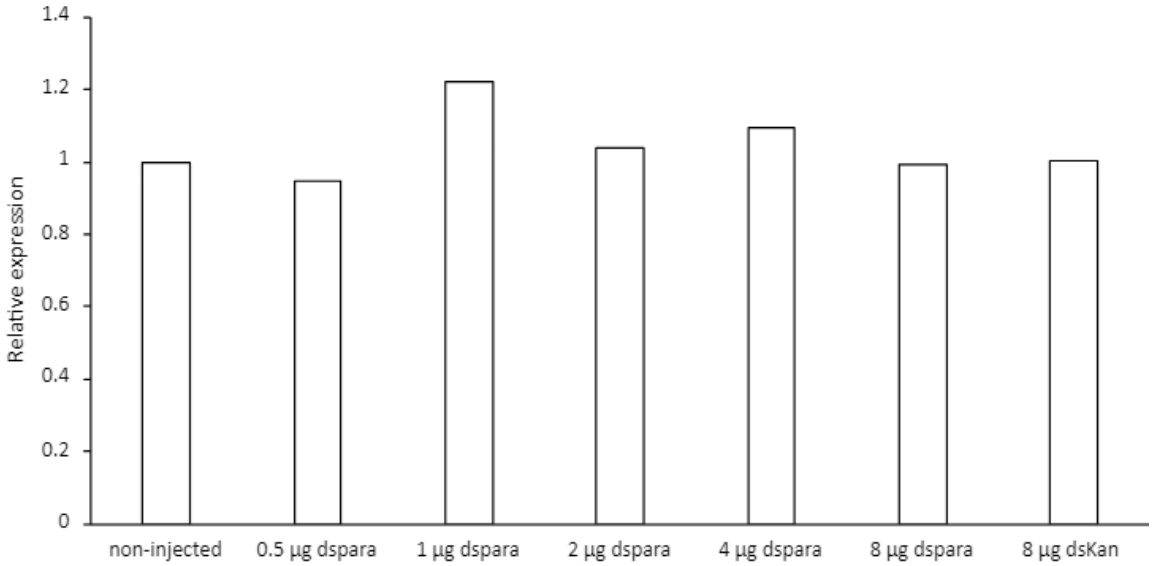

Figure 3.43 *S. littoralis para* gene expression after 4th instar *S. littoralis* larvae were injected once with increasing concentrations of ds*para*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Expression was determined 48 h post-injection via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to gene expression in *S. littoralis* reared on AD (shown as Y=1).

Injection of dsnAChR in 4th instar larvae

Survival was recorded after injecting 4th instar *S. littoralis* larvae with increasing concentrations of ds*nAChR*. When insects were injected with 0.5 - 8 µg ds*nAChR,* survival was reduced by 11 – 16 % compared to 0 % and 18 % when insects were not injected or injected with 8 µg ds*Kan*, respectively (Figure 3.44) and Kaplan-Meier log-rank survival analysis identified no significant difference between survival curves. Furthermore, 63 % - 90 % of insects pupated when they were injected with 0.5 µg - 8 µg ds*nAChR* compared to 84 % and 86 % when insects were not injected or were injected with 8 µg ds*Kan,* respectively (Figure 3.45) and there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) between pupation of non-injected insects and those injected with any concentration of ds*nAChR*. Additionally, 83 – 89 % of insects injected with 0.5 µg - 8 µg ds*nAChR* emerged as adults compared to 84 % and 88 % when insects were not injected or were injected with 8 µg ds*Kan*, respectively (Figure 3.46) and emergence was not significantly different ($P > 0.05$; ANOVA, Tukey post-hoc) between any group.

The effect on gene expression after injecting 4th instar *S. littoralis* larvae with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*nAChR* or 8 µg ds*Kan* was investigated 48 h post-injection via RT-qPCR, with all expression levels compared to non-injected insects. The expression of *nAChR* was

0.81, 0.75, 1.24, 1.19, 0.95 and 0.96-fold when insects were injected with 0.5 µg, 1 µg, 2 µg, 4 µg, 8 µg ds*nAChR* or 8 µg ds*Kan*, respectively, thus was not significantly different than when insects were not injected (Figure 3.47). Therefore, as for ds*para*, injecting ds*nAChR* into 4th instar larvae had no effect on survival, pupation, adult emergence or gene expression.

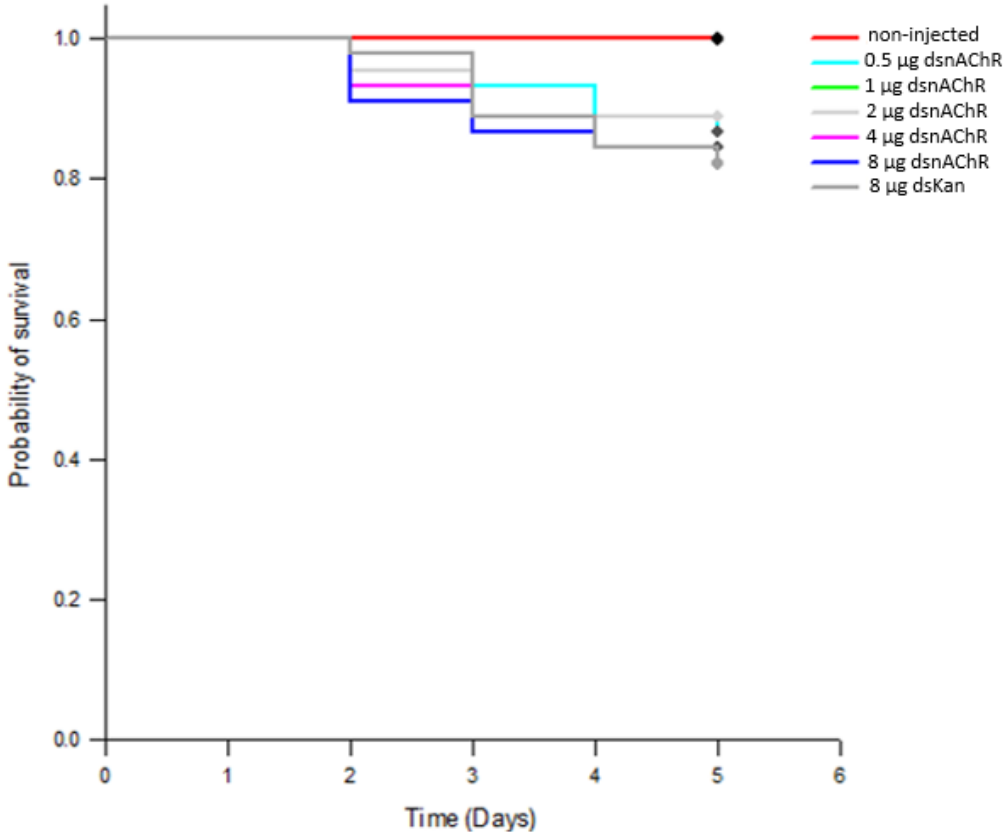

Figure 3.44 Survival of 4th *S. littoralis* larvae after direct haemolymph injection of increasing concentrations of ds*nAChR* gene. Controls were non-injected insects and insects injected with 8 µg ds*Kan* specific dsRNA (n=45). All insects were transferred to artificial diet post-injection.

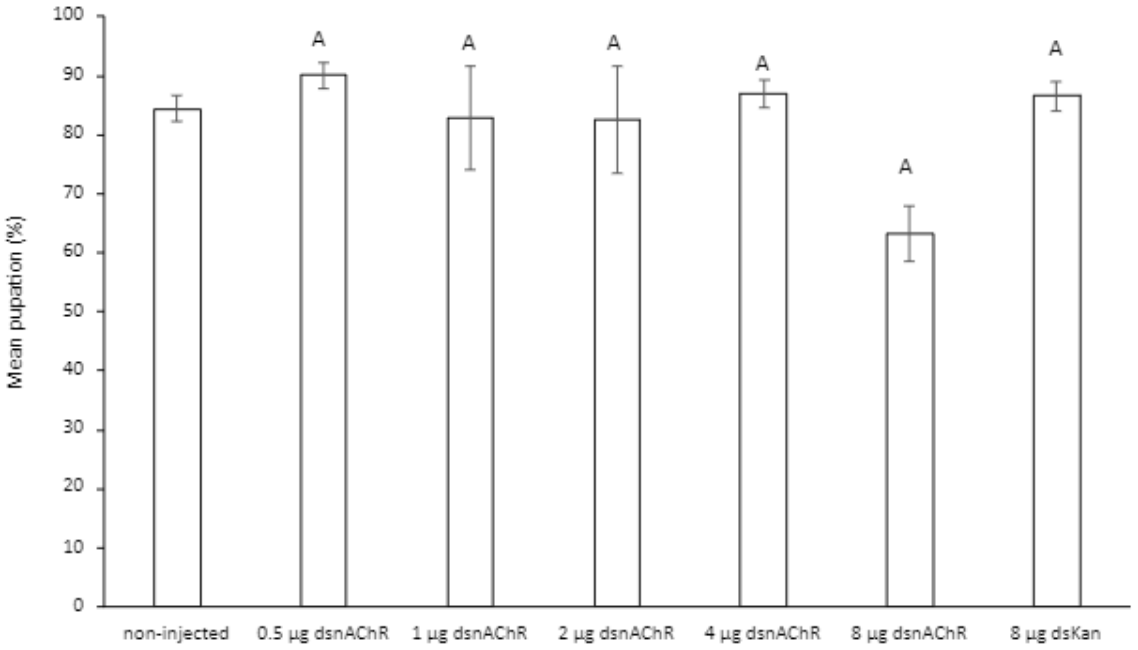

Figure 3.45 *S. littoralis* pupation after 4th instar larvae were injected once with ds*nAChR* specific dsRNA. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

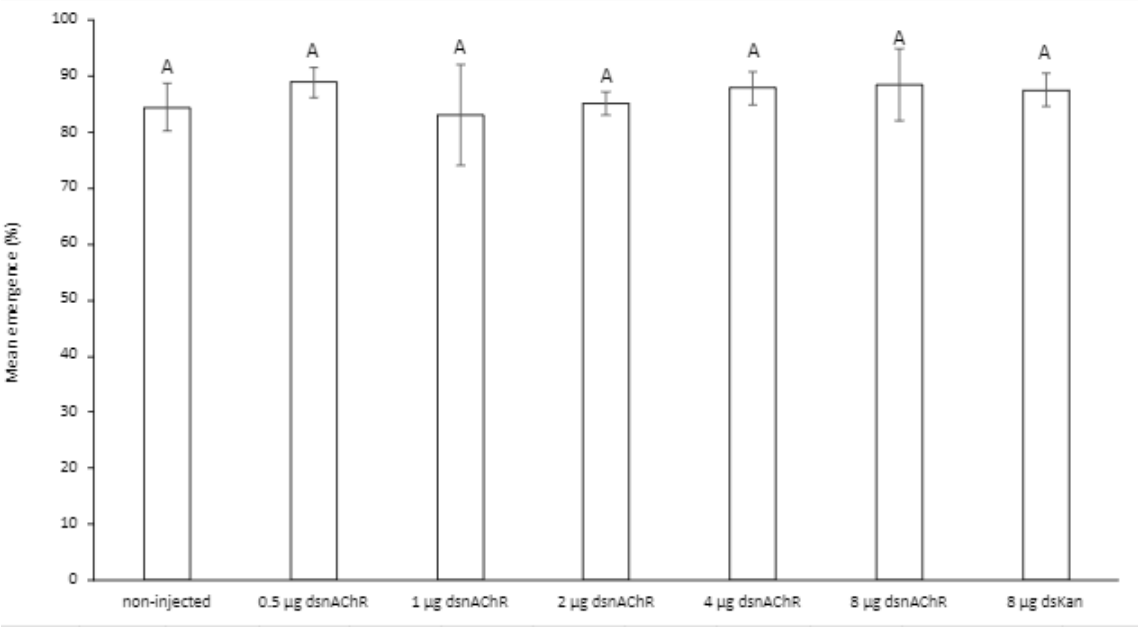

Figure 3.46 *S. littoralis* adult emergence after 4th instar larvae were injected once with ds*nAChR*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of insects that pupated, error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences.

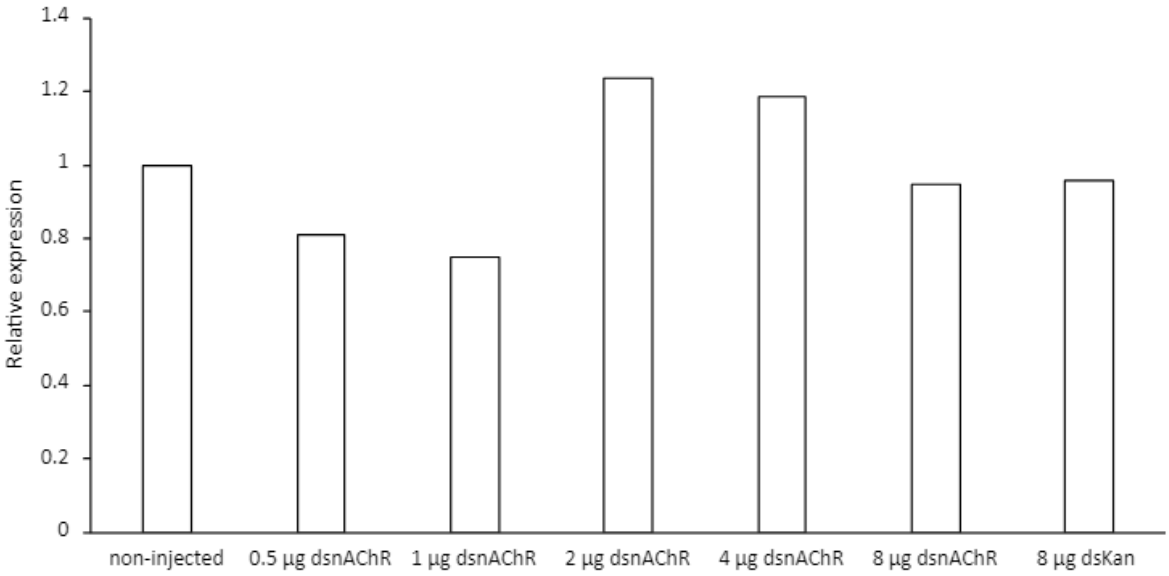

Figure 3.47 *S. littoralis nAChR* gene expression after 4th instar *S. littoralis* larvae were injected once with increasing concentrations of ds*nAChR*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Expression was determined 48 h post-injection via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to gene expression in *S. littoralis* reared on AD (shown as Y=1).

3.3.12 Delivery of dsRNA via a single direct haemolymph injection to 3rd instar larvae

Survival was recorded after injecting 3rd instar *S. littoralis* larvae with 8 µg of *S. littoralis* specific dsRNA. For non-injected insects and those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* survival was reduced by 0 %, 24 %, 27 %, 31 %, 29 %, 31 % and 29 %, respectively (Figure 3.48). Kaplan-Meier log-rank survival analysis identified that the survival of insects injected with 8 µg of any *S. littoralis* specific dsRNA or 8 µg ds*Kan* was significantly lower than non-injected insects. By day 2, survival of insects injected with 8 µg ds*para* was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than non-injected insects and by day 3, survival of insects injected with any *S. littoralis* specific dsRNA as well as ds*Kan* was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than non-injected insects and this remained the case until the end of the trial.

The mean weight of artificial diet consumed by non-injected insects or those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* was 654 mg, 656 mg, 411 mg, 307 mg, 315 mg, 378 mg and 381 mg, respectively (Figure 3.49). The mean weight of diet consumed by insects injected with 8 µg of any *S. littoralis* specific dsRNA was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc)

than both the non-injected group and those injected with 8 µg ds*Kan*. Furthermore, insects injected with 4 µg ds*ace-1* + 4 µg *nAChR* consumed significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) than those injected with 8 µg ds*ace-1*.

Regarding pupation after 3rd instar *S. littoralis* larvae were injected with 8 µg of *S. littoralis* specific dsRNA, for non-injected insects and those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r*, 82 %, 82 %, 81 %, 74 %, 63 %, 74 % and 72 % of insects pupated, respectively (Figure 3.50). Pupation was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than non-injected insects and those injected with 8 µg ds*Kan* when insects were injected with 4 µg ds*ace-1* + 4 µg *nAChR*.

The time taken for 3rd instar larvae to pupate after *S. littoralis* specific dsRNA injection into the haemolymph was recorded. On day 6, 37 %, 36 %, 16 %, 13 %, 0 %, 0 % and 8 % of non-injected insects and those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* had pupated, respectively (Figure 3.51), this increased to 51 %, 54 %, 26 %, 13 %, 25 %, 17 % and 35 % by day 7 and 70 %, 54 %, 48 %, 65 %, 56 %, 30 % and 57 % by day 8. On day 9, 84 %, 75 %, 59 %, 70 %, 65 %, 57 % and 70 % had pupated and this increased to 86%, 79 %, 80 %, 74 %, 65 %, 74 % and 70 % by day 10. Significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated on day 6 when injected with 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* compared to the non-injected group and insects injected with 8 µg ds*Kan*. On day 7, significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* compared to the non-injected group and those injected with 8 µg ds*Kan*. On day 8, significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 8 µg ds*ace-1* and 8 µg ds*para* compared to the non-injected group. By day 9, significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 8 µg ds*ace-1*, 4 µg ds*ace-1* + 4 µg *nAChR* and 8 µg ds*para* compared to the non-injected group. On day 10, significantly less ($P < 0.05$; ANOVA, Tukey post-hoc) insects pupated when injected with 4 µg ds*ace-1* + 4 µg *nAChR* and 8 µg ds*NADPHcytP450r* compared to the non-injected group. Thus, injection of all *S. littoralis* specific dsRNA into 3rd instar larvae caused a significant delay in pupation at various time points.

Mean pupal weights of 3rd instar non-injected insects and those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* were 324, 300, 297, 322, 310, 289 and 279, respectively (Figure 3.52). Only the pupal weights for insects injected with 8 µg ds*para* and 8 µg ds*NADPHcytP450r* specific dsRNA were significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than both the non-injected group and those injected with 8 µg ds*Kan*. In terms of adult emergence, for non-injected insects or those injected with 8 µg ds*Kan*, 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg *nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r*, 92 %, 86 %, 57 %, 65 %, 65 %, 39 % and 88 % of the insects that pupated emerged as adults (Figure 3.53). Significantly fewer ($P < 0.05$; ANOVA, Tukey post-hoc) adults emerged when larvae were injected with 8 µg ds*ace-1* and 8 µg ds*para* compared to non-injected insects and emergence of those injected with 8 µg ds*para* was significantly lower ($P < 0.05$; ANOVA, Tukey post-hoc) than when insects were injected with 8 µg ds*Kan*.

The time taken for 3rd instar larvae to emerge as adults after injection of *S. littoralis* specific dsRNA into the haemolymph was recorded. On day 19 (post-injection), 38 %, 53 %, 41 %, 41 %, 62 %, 28 % and 75 % of non-injected insects and those injected with 8 µg kanamycin, 8 µg *ace-1*, 8 µg *nAChR*, 4 µg *ace-1* + 4 µg *nAChR*, 8 µg *para*, and 8 µg *NADPHcytP450r* had emerged, respectively (Figure 3.54), this increased to 62 %, 63 %, 48 %, 62 %, 62 %, 28 % and 75 % by day 22 and 94 %, 84 %, 56 %, 70 %, 62 %, 36 % and 90 % by day 25. On day 22, there was no significant difference ($P > 0.05$; ANOVA, Tukey post-hoc) in emergence between non-injected insects and those injected with any dsRNA. However, by day 25, significantly less insects injected with 8 µg *para* specific dsRNA emerged as adults compared to non-injected insects.

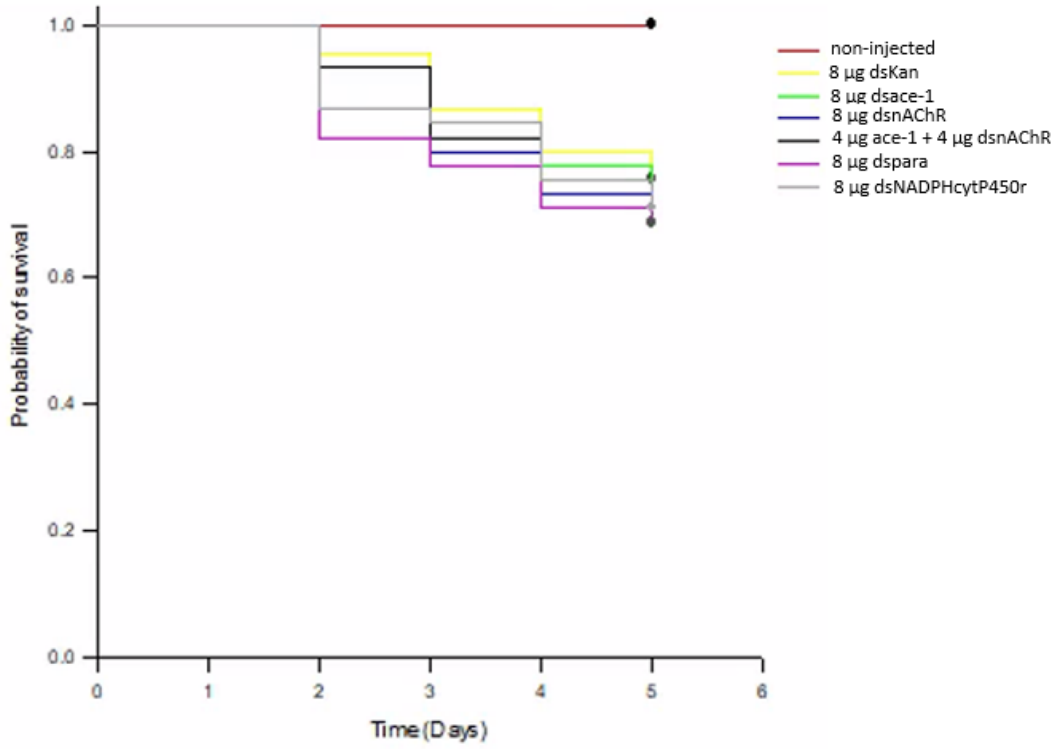

Figure 3.48 *S. littoralis* survival after 3rd instar larvae were injected with *S. littoralis* specific dsRNA. Controls were non-injected insects and insects injected with 8 µg ds*Kan* (n=45). All insects were transferred to artificial diet post-injection.

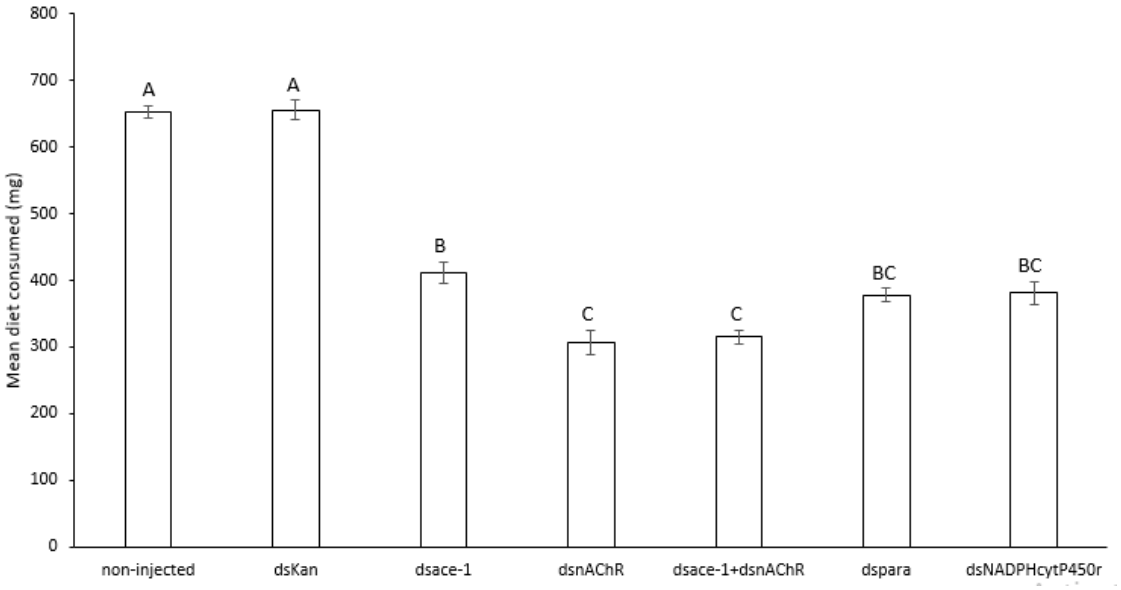

Figure 3.49 Weight of artificial diet consumed by *S. littoralis* larvae between 3 and 5 days post-injection with *S. littoralis* specific dsRNA at the 3rd instar stage. Insects injected with ds*ace-1*+ds*nAChR* (in combination) received 4 µg of both whereas all other insects received 8 µg of gene specific dsRNA. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively were removed.

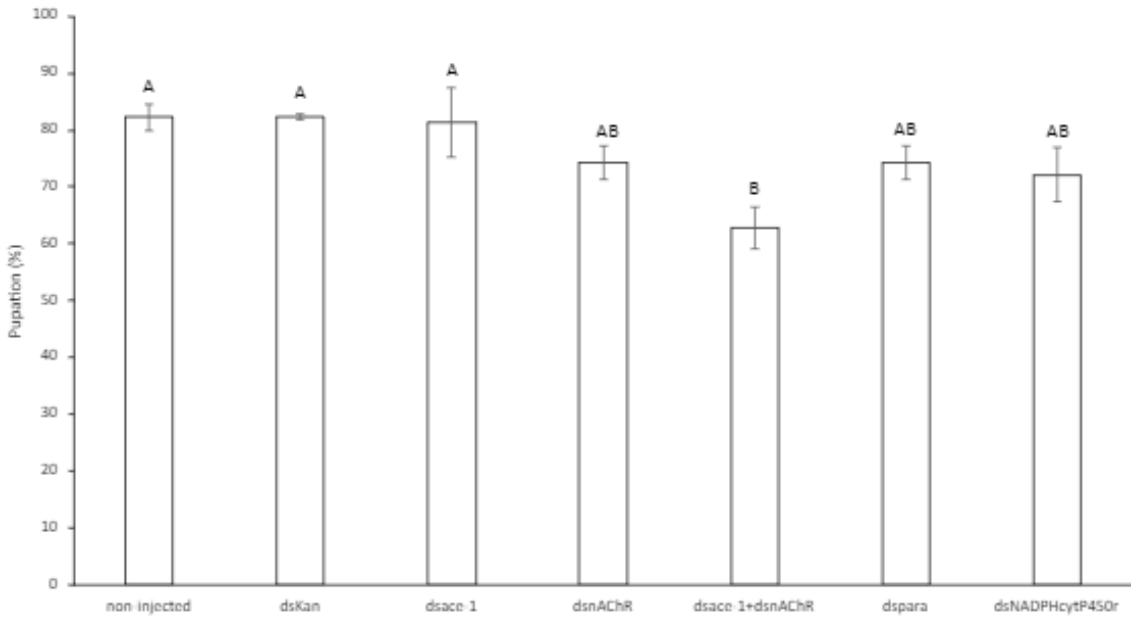

Figure 3.50 *S. littoralis* pupation after 3rd instar larvae were injected once with *S. littoralis* specific dsRNA. Insects injected with ds*ace-1*+ds*nAChR* (in combination) received 4 µg of both whereas all other insects received 8 µg of gene specific dsRNA. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

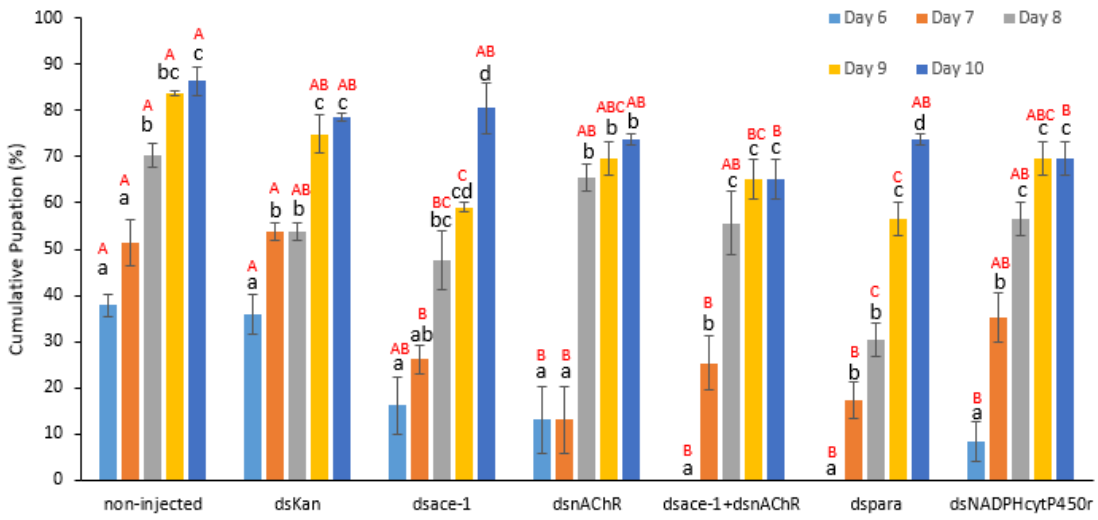

Figure 3.51 Time taken for *S. littoralis* larvae to pupate after dsRNA injection into the haemolymph. 3rd instar larvae were injected with 8 µg *S. littoralis* specific dsRNA or 4 µg ds*ace-1* + 4 µg µg *nAChR* and the time taken to pupate was recorded daily. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates and are given as percentages of surviving insects, error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences (lower case black letters denote differences in pupation across each day and capital red letters denote differences in pupation between each group on a specific day), light blue, orange, grey, yellow and dark blue bars represent cumulative pupation after 6, 7, 8, 9 and 20 days, respectively(P = 0.05 ANOVA, Post-hoc Tukey test).

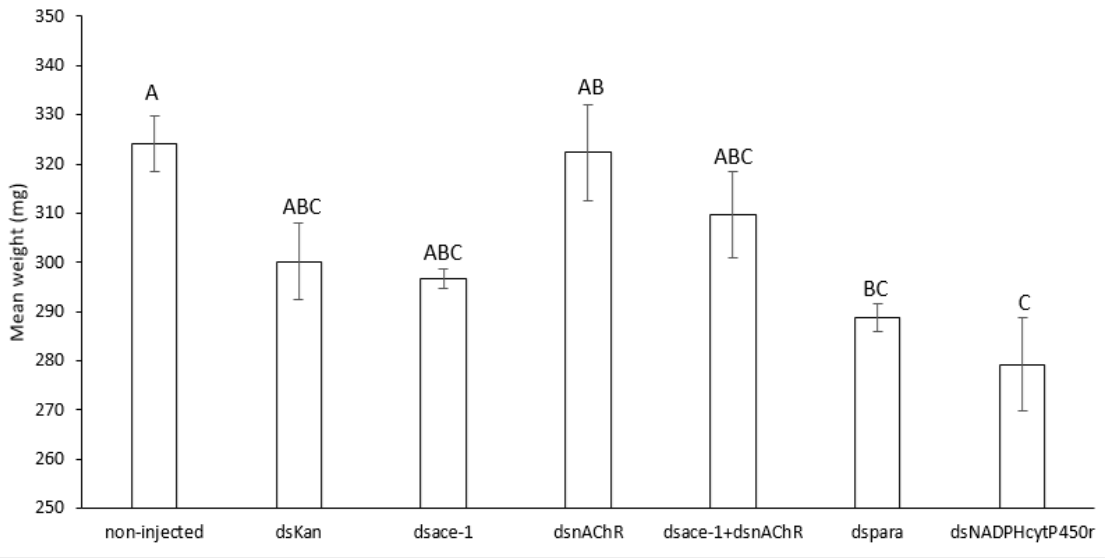

Figure 3.52 Weight of *S. littoralis* pupae after 3rd instar larvae were injected with 8 µg *S. littoralis* specific dsRNA or 4 µg ds*ace-1* + 4 µg ds*nAChR*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Error bars denote standard error of the mean of 3 groups (n=15) and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively were removed.

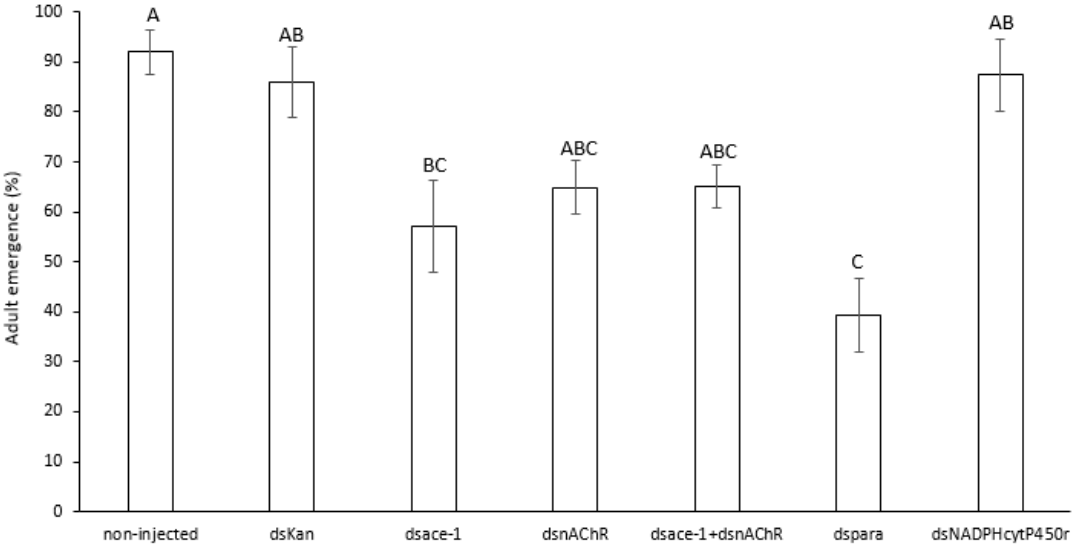

Figure 3.53 *S. littoralis* adult emergence after 3rd instar larvae were injected with 8 µg of gene specific dsRNA or 4 µg ds*ace-1* + 4 µg ds*nAChR*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of pupating insects, error bars denote standard error of the mean and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively were removed.

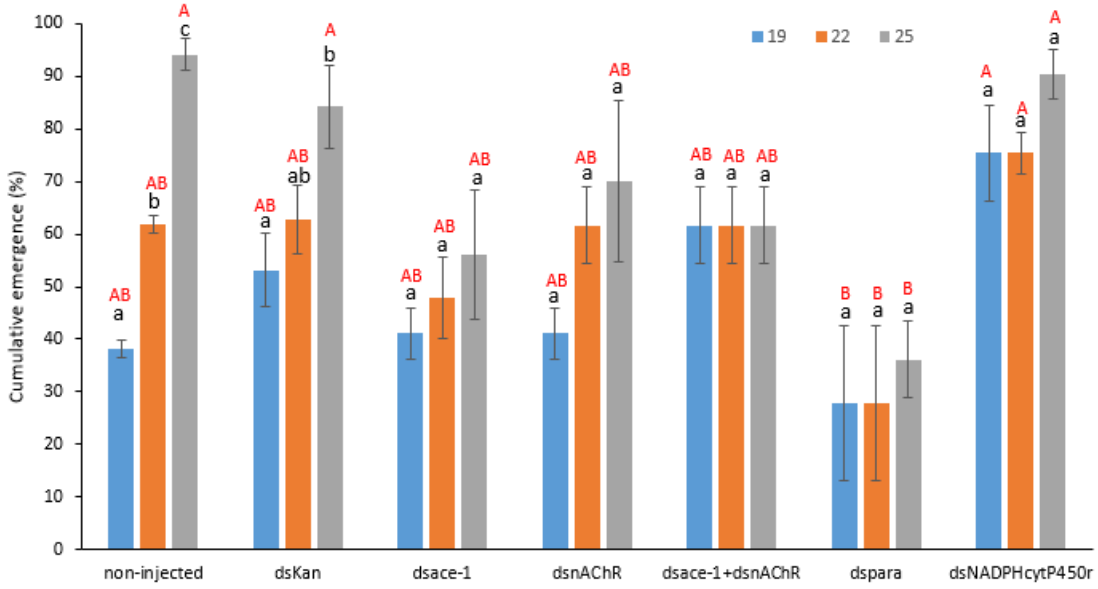

Figure 3.54 Time taken for *S. littoralis* larvae to emerge as adults after 3rd instar larvae were injected with 8 µg *S. littoralis* specific or 4 µg ds*ace-1* + 4 µg ds*nAChR*. Controls were non-injected insects and insects injected with 8 µg ds*Kan*. Values are the average of 3 biological replicates (n=15) and are given as percentages of pupating insects, error bars denote standard error of the mean of 3 groups and different letters denote significant differences in emergence (lower case black letters denote differences in pupation across each day and capital red letters denote differences in pupation between each group on a specific day), blue, orange and grey bars represent emergence after 19, 22 and 25 days, respectively (P = 0.05, ANOVA, Tukey post-hoc).

3.3.13 Soaking S. littoralis eggs in gene specific dsRNA

To determine the effects of soaking eggs in *S. littoralis* specific dsRNA, egg masses (~ 50 per biological replicate) were soaked in 200 µl dsRNA diluted in PBS (100 ng/µl) for 2 h then transferred to artificial diet. Eggs exposed to ds*ace-1* + ds*nAChR* were soaked in 100 µl each of ds*ace-1* + ds*nAChR* (100 ng/µl). When untreated or soaked in ds*Kan*, 200 µl PBS (pH 7) or ds*ace-1*, ds*nAChR*, ds*ace-1* + ds*nAChR*, ds*para* or ds*NADPHcytP450r*, 86 %, 75 %, 82 %, 40 %, 15 %, 30 %, 52 %, and 33 % of eggs hatched, respectively (Figure 3.55). Significantly fewer ($P < 0.05$; ANOVA, Tukey post-hoc) eggs hatched after soaking in ds*ace-1*, ds*nAChR*, ds*ace-1* + ds*nAChR* and ds*NADPHcytP450r* compared to when untreated or soaked in ds*Kan* or PBS and significantly fewer ($P < 0.05$; ANOVA, Tukey post-hoc) eggs hatched after soaking in ds*para* compared to when untreated or soaked in PBS but not when soaked in ds*Kan*. These results demonstrate this method of delivery to be effective in triggering an RNAi effect, at least for the investigated targets.

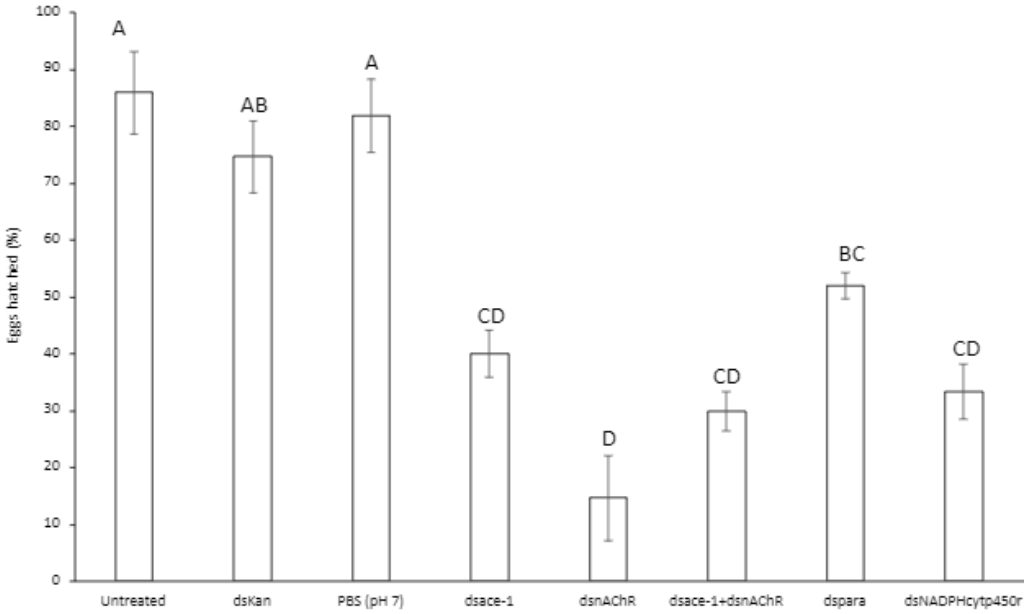

Figure 3.55 Mean *S. littoralis* egg hatching after soaking in 200 µl of 100 ng/µl *S. littoralis* specific dsRNA (diluted in PBS) for 2 h. The group exposed to ds*ace-1* + ds*nAChR* received 100 µl 100ng/µl of both. Controls were untreated eggs, (non-soaked), eggs soaked in PBS (pH 7), or eggs soaked in 200 µl of 100 ng/µl ds*Kan*. Error bars denote standard error of the mean of 3 replicates (n=50) and different letters denote significant differences.

3.3.14 Increasing pesticide susceptibility through dsNADPHycytp450r pre-exposure

To determine the effect of ds*NADPHcytP450r* pre-exposure on the susceptibility of *S. littoralis* to pesticides, larvae were reared on artificial diet containing 2 µg or 4 µg ds*NADPHcytP450r* for 9 days from the neonate stage. Subsequently, 0.5 µl of increasing concentrations of deltamethrin (2.5, 5, 10, 20, 40, 80 mg/ml), corresponding to 1.25, 5, 10, 20 and 40 µg/larva were applied topically to the head of 4th instar larvae and mortality was determined 24 h post exposure. Controls were insects reared on artificial diet ± 4 µg ds*Kan* prior to deltamethrin exposure and insects with 0.5 µl pure hexane applied topically to the head. No mortality was recorded 24 h after pure hexane application and the LC_{50} value for insects reared on artificial diet alone and then exposed to deltamethrin was similar to when reared on artificial diet containing 4 µg ds*Kan* then exposed to deltamethrin. Mortality was plotted against deltamethrin concentration and the equation of an exponential line of best fit was used to determine LC_{50}. For insects previously reared on artificial diet containing ds*Kan* prior to deltamethrin exposure (control group), LC_{50} was 11.8 mg/ml or 5.9 µg/larva (Figure 3.56a) compared to 11.56 mg/ml or 5.78 µg/larva (Figure 5.56b) and 9.33 mg/ml or ~ 4.7 µg/larva (Figure 5.57c) for the experimental groups previously reared on artificial diet containing 2 µg ds*NADPHcytP450r* specific dsRNA or 4 µg

ds*NADPHcytP450r*. respectively. Neverthless, there was no significant difference in morality observed between the groups (P > 0.05, ANOVA).

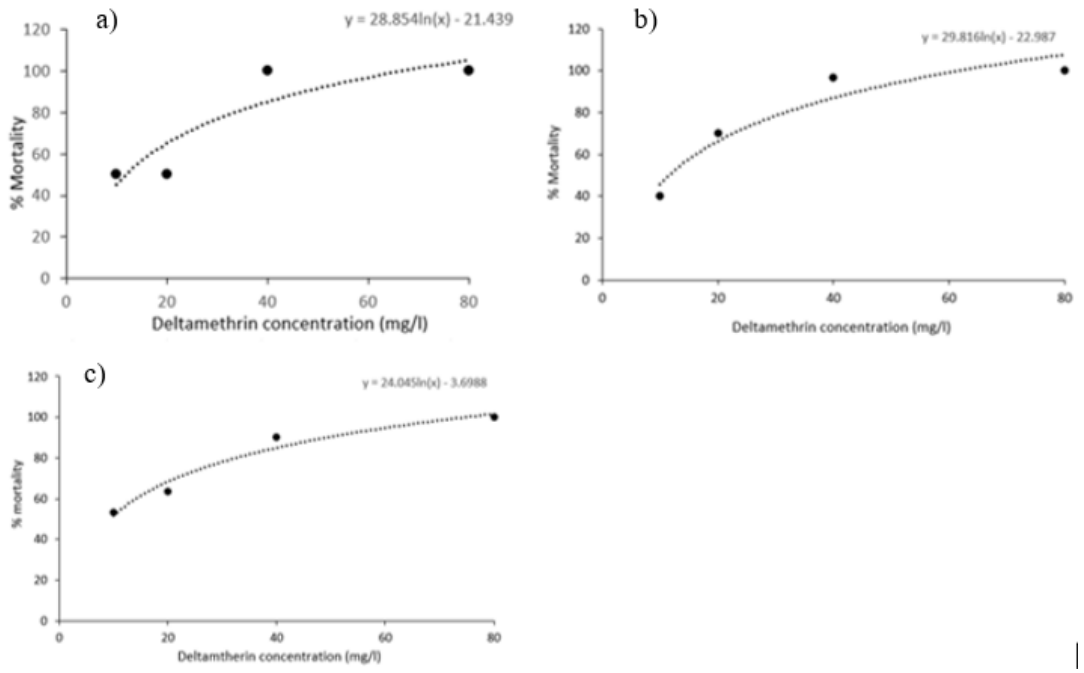

Figure 3.56 *S. littoralis* mortality 24 h after 0.5 µl of 2.5, 5, 10, 20, 40, 80 mg/ml deltamethrin (diluted in pure hexane) was applied topically to the head of 4th instar larvae. Controls were insects reared on an artificial diet ± 4 µg ds*Kan* and insects with 0.5 µl pure hexane applied topically to the head. An exponential line of best fit was applied to each data set. As no mortality was observed at concentrations 0, 2.5 or 5 mg/ml, these values were not included. a) Mortality of larvae previously reared on artificial diet containing 4 µg ds*Kan* b) Mortality of larvae previously reared continuously on artificial diet containing 2 µg ds*NADPHcytP450r* c) Mortality of larvae previously reared continuously on artificial diet containing 4 µg ds*NADPHcytP450r*.

The rationale of the subsequent study was to investigate the effect of ds*NADPHcytP450r* pre-exposure on the susceptibility of *S. littoralis* to chlorpyrifos. To facilitate this, 0.5 µl of increasing concentrations of chlorpyrifos; 0.67, 1.375, 2.75, 5.5, 11, 22, 44, 88, 176 and 352 mg/l were applied topically to the head of 4th instar larvae and mortality was determined 24 h post exposure. Controls were insects reared on artificial diet prior to chlorpyrifos exposure and 4th instar insects with 0.5 µl pure hexane applied topically to the head. These concentrations were chosen based on Bagni *et al* (2020) who reported an LC_{50} of ~ 44 mg/l 72 h post-application. No mortality was recorded for insects exposed to Chlorpyrifos at any concentration, so pupation and pupal weight were investigated to determine exposure effects. For those reared on artificial diet, exposed to 0.5 µl hexane or to 0.5 µl 0.67, 1.375, 2.75, 5.5, 11, 22, 44, 88, 176 and 352 mg/l chlorpyrifos, 87 %, 90 %, 87 %, 90 %, 87 %, 80 %,

87 %, 90 %, 80 %, 83 %, 80 % and 77 % insects pupated (Figure 3.57) and there was no significant difference (P > 0.05; ANOVA, Tukey post-hoc) between the instance of pupation from insects in any group. For pupal weights, for those reared on artificial diet, exposed to 0.5 µl hexane or to 0.5 µl 0.687, 1.375, 2.75, 5.5, 11, 22, 44, 88, 176 and 352 mg/l chlorpyrifos, mean weight was 229, 223, 223, 222, 227, 222, 224, 225, 223, 224, 229 and 232 mg, respectively (Figure 3.58). The results show that there was no significant difference (P > 0.05; ANOVA, Tukey post-hoc) between weights of pupae from any group. Thus, effect of ds*NADPHcytP450r* pre-exposure on the susceptibility of *S. littoralis* to chlorpyrifos could not be determined.

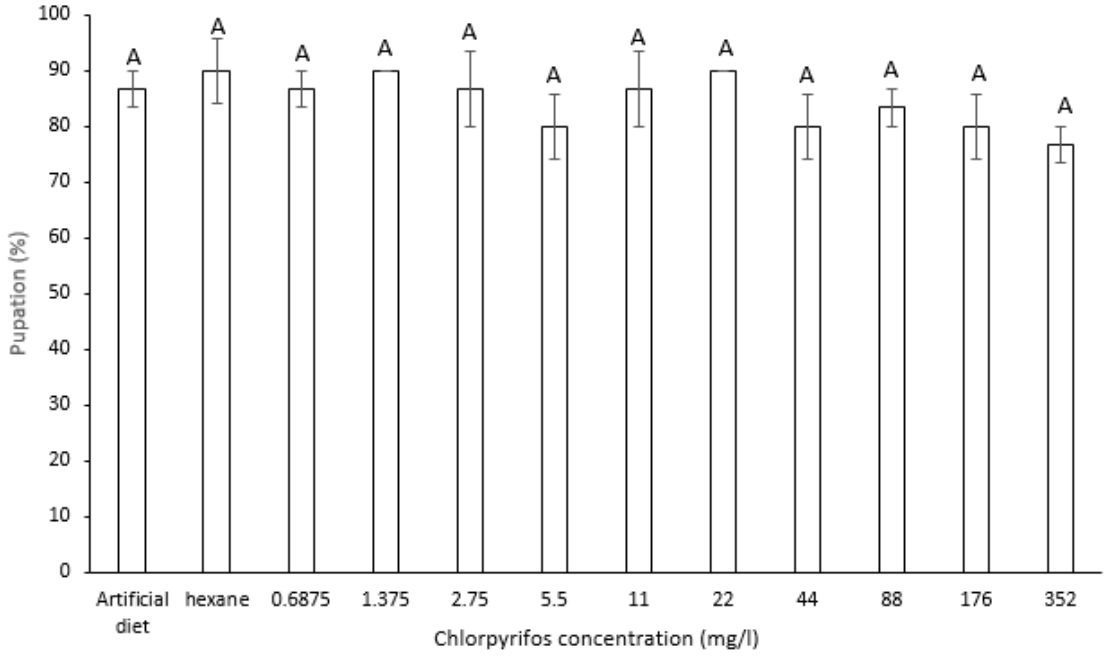

Figure 3.57 *S. littoralis* pupation after increasing concentrations of chlorpyrifos (diluted in pure hexane) was applied topically to the head. Controls were insects reared on an artificial diet and insects with 0.5 µl pure hexane applied topically to the head. Values are the average of 3 biological replicates (n=10), error bars denote standard error of the mean and different letters denote significant differences.

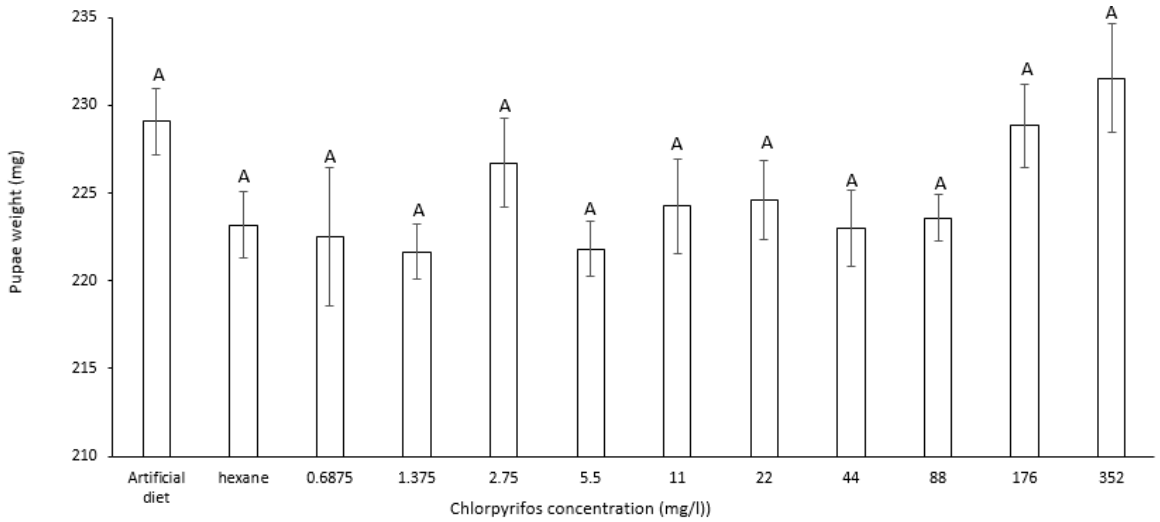

Figure 3.58 Weight of *S. littoralis* pupae after increasing concentrations of chlorpyrifos (diluted in pure hexane) was applied topically to the head. Controls were insects reared on an artificial diet and insects with 0.5 µl pure hexane applied topically to the head. Values are the average of 3 biological replicates (n=10), error bars denote standard error of the mean and different letters denote significant differences. Outliers either 1.5*IQR below or above Q1 and Q3, respectively, were removed.

3.3.15 Determining the homology between the designed ace-1 dsRNA sequence and the S. littoralis ace-2 sequence

To determine the homology of ds*ace-1* with *S. littoralis ace-2* and thus the possibility of ds*ace-1* unintentionally targeting *ace-2*, both sequences were aligned (Figure 3.59). This identified 56 % sequence similarity, with the longest stretch of exactly matching nucleotides being 15 bp.

Figure 3.59 Alignment of the *S. littoralis ace-1* dsRNA nucleotide sequence used in the present study and a section of *S. littoralis ace-2* available on NCBI. Alignment created with Clustal Omega, * denotes a match, space = not a match.

3.3.16 Determining the similarity between dsKan and the S. littoralis genome

As the *S. littoralis* genome has now been published (Wu *et al*, 2022) an NCBI BLAST search concluded that there was no significant similarity between ds*Kan* and the genome of *S. littoralis* (results not shown). Additionally, after *in silico* cleavage of ds*Kan* into its component siRNAs, a viroBlast search concluded that there were no matches between any length kanamycin specific siRNA with ds*ace-1*, ds*para*, ds*nAChR* or ds*NADPHcytP450r* (Table 3.1).

Table 3.1 Similarity between siRNAs specific to the bacterial kanamycin gene and *S. littoralis* specific genes used in the present study. A 470 bp dsRNA specific to the bacterial kanamycin gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each ds*Kan* n-mer and each gene specific dsRNA.

		Number of exact n-mer matches											
Species	Gene	16	17	18	19	20	21	22	23	24	25	26	27
Spodoptera littoralis	*ace-1*	0	0	0	0	0	0	0	0	0	0	0	0
Spodoptera littoralis	*para*	0	0	0	0	0	0	0	0	0	0	0	0
Spodoptera littoralis	*NaChR*	0	0	0	0	0	0	0	0	0	0	0	0
Spodoptera littoralis	*NADPHcytp450r*	0	0	0	0	0	0	0	0	0	0	0	0

3.4 Discussion

3.4.1 Baseline expression

To assess baseline gene expression prior to RNAi and to determine the most appropriate life stage to target in subsequent experiments, the expression of the *ace-1*, *ace-2*, *para*, *nAChR* and *NADPHcytP450r* genes was investigated at larval instars 1-6 and the pupae and adult stages. Prior to this, a single gene product was amplified via PCR which corresponded to the expected size for each specific gene. Subsequently, primer efficiency was assessed to ensure that each primer pair, designed to amplify each gene, was optimal for subsequent expression experiments and efficiencies ranged from 99.8 % to 108.98 %. Considering the acceptable efficiency range is 90 – 110 %, to account for PCR inhibitors such as ethanol contamination from previous RNA extraction and pipetting inaccuracies (Thermofisher, 2023), all primer pairs were acceptable for use in subsequent experiments.

In the present study, both *ace-1* and *ace-2* were expressed most highly in larval instar 1, with *ace-1* expression significantly higher in this instar compared to any other life stage but there being no significant difference in *ace-2* expression across any life stage. The expression of both *ace* genes has previously been assessed across the developmental stages of many insects. Contrasting with the present study, Zhao *et al* (2019) reported that both *ace-1* and *ace-2* were most highly expressed in the final larval instar (5) of the lepidopteran *S. exigua* and Salim *et al* (2017) also reported the highest *ace-1* expression in the lepidopteran *S. litura* in the final larval instar (6) but *ace-2* was most highly expressed in the egg stage and male pupae. Similarly, Wang *et al* (2016) reported *ace-1* and *ace-2* expression was highest in the last larval instar (5) of the lepidopteran *Cnaphalocrocis medinalis* (rice leafroller) and lowest in 1st instar larvae. Similarly, Jiang *et al* (2018) reported that *ace-1* and *ace-2* expression was highest in the final larval stage (5) and female adults of the lepidopteran *Pieris rapae* (Cabbage white butterfly), although they did not investigate expression in the 1st larval instar. However, Gao *et al* (2023) reported that *ace-1* and *ace-2* expression was significantly higher in the 3rd larval instar stage of the lepidopteran *S. frugiperda* than any other developmental stage.

As in the present study, Huchard *et al* (2006) reported the highest level of *ace-1* expression in the dipteran *Culex pipiens* (Common house mosquito) in larval instar 1 with expression being ~ 50 %, 45 %, 55 % and 90 % higher compared to larval instars 2, 3, 4 and adults,

respectively. Expression of *ace-2* was also highest in larval instar 1 with a ~ 80 %, ~ 70 %, ~ 80 % and ~ 95 % increase compared to larval instars 2, 3, 4 and adults, respectively. Furthermore, Li *et al* (2012) reported that *ace-1* expression was significantly higher in the hemipteran Brown Plathopper, *Nilaparvata lugens*, in the 1st instar larval stage than any other stage and that *ace-2* expression was also significantly higher in the 1st larval instar stage compared to other larval stages. Chen *et al* (2009), also reported significantly higher expression of *ace-1* in larval instar 1 of the lepidopteran *B. mori*, with expression ~ 3-5 -fold higher than any other larval instar but *ace-2* expression was significantly higher in male adults compared to any other developmental stage.

Thus, *ace-1* and *ace-2* expression differs across developmental stages both between and within insect orders and phylogenetic relatedness does not infer similarities in expression patterns. For example, although the *ace-1* gene of *S. frugiperda* shares greater sequence similarity to that of *S. exigua* (Gao *et al*, 2023) compared to *S. litura*, the expression patterns of *S. exigua* and *S. litura ace-1* genes are more similar to each other, with the highest *ace-1* expression being in the final instar stage of these insects (Zhao *et al* 2019; Salim *et al*, 2017). Furthermore, Chen *et al* (2009) reported that *B. mori ace-1* and *ace-2* expression changes dynamically across developmental days. For example, *ace-1* expression was highest on day 3 of larval instar 3, followed by a rapid reduction on day 4 and an increase to that of almost day 3 by day 5. However, *ace-2* expression increased from day 1 to day 3 but rapidly decreased between days 3-5 of larval instar 3. Furthermore, Zhao *et al* (2019) reported significantly higher *ace-1* and *ace-2* expression in 3-day old female pupae compared to 1 and 7-day old female pupae and any day of development for male pupae. Salim *et al* (2017) reported significantly higher (~ 8-fold) expression of *ace-1* in 12 day old male pupae compared to both 12-day old female pupae and 6 day old male and female pupae but *ace-2* expression was significantly higher in 1 day old male pupae than both 1 day old female pupae and 6 and 12 day old male pupae (~ 2.5 fold, ~ 9-fold and ~ 9-fold, respectively). Therefore, it is possible that *ace-1* and *ace-2* expression may change dynamically by day and also between sexes in *S. littoralis*, which may impact the relative expression values reported in the present study.

As pest insects are generally exposed to high concentrations of chemical insecticides during their most destructive stages (Gao *et al*, 2023) and as chemical stress can induce the

overexpression of *ace* genes via alternative splicing or post-translational modifications (Lee *et al*, 2015), it was perhaps expected that the late larval stages or the most destructive stage of *S. littoralis* (CABI, 2023a) would have displayed the highest acetylcholinesterase activity. However, the use of a laboratory strain in the present study, not previously exposed to insecticides, may provide a reason for the unexpected expression pattern. Thus, it would be interesting to include a field strain for comparison.

Generally, when insects possess both ace genes, *ace-1* is considered the major catalytic enzyme in acetylcholine hydrolyzation due to higher expression levels than *ace-2*, with Kim and Lee (2013) reporting that, of 100 species across 18 different orders, 67 exhibited higher *ace-1* activity. Also, high *ace-1* expression in central nervous system (CNS) tissues of *Pieris rapae* (Jiang *et al*, 2018), *Plutella xylostella* (*Diamondback moth*) (Baek *et al*, 2005), *Helicoverpa assulta* (Oriental tobacco budworm) (Lee *et al*, 2006), *S. Frugiperda* (Gao *et al*, 2023) and *Cimex lectularius* (Common bed bug) (Seong *et al*, 2011) suggests it likely plays a more significant role in neurotransmission than *ace-2* in these species. Additionally, point mutations in the *ace-1* gene confer resistance to organophosphates in many insect species such as *B. tabaci* (Alon *et al*, 2008) and *Chilo suppressalis* (Jiang *et al*, 2009) and neonicotinoids e.g. *Aedes aegypti* (Samal *et al*, 2021), suggesting the more important role of *ace-1* in neurotransmission compared to *ace-2*.

Contrastingly, *ace-2* is the predominantly expressed gene in 33 of 100 species (Kim and Lee, 2013) such as *A. mellifera* where the *ace-2* gene exhibits ~ 2,500 fold higher catalytic activity than *ace-1*, with high expression in CNS tissues (Kim *et al*, 2012). Also, resistance to pirimicarb in *M. persicae* is conferred by a single amino acid substitution in the *ace-2* gene (Nabeshima *et al*, 2003) and no point mutations were reported in the *ace-1* gene of resistant strains of this species. Furthermore, the tissue distribution pattern, kinetic properties and catalytic efficiency of *ace-2* are significantly higher than those of *ace-1* in *B. mori* (Cao *et al*, 2012; Chen *et al*, 2009), possibly because the domestication of this insect has meant it has not been subjected to widespread pesticide use (Chen, *et al*, 2009) and therefore the *ace-1* upregulation upon insecticide exposure seen in both mammals (Evron *et al*, 2007) and insects (Lee *et al*, 2015) may not occur. Thus, as the *ace* gene predominantly responsible for neurotransmission in *S. littoralis* is yet to be determined, future studies could investigate expression patterns across different tissues as well as the response in expression upon

insecticide exposure allowing more in-depth analysis into the expression patterns of *ace-1* and *ace-2* in this insect.

In the present study, the expression of *para* was significantly higher in larval instar 1 compared to all other investigated life stages. This contrasts with Tariq *et al* (2019) who reported an increase in expression of the VGSC gene (*para*) in *M. persicae* from larval instars 1-4 through to the adult stage, although expression between larval instars 1 and 2 did not differ significantly nor between larval instars 3, 4 and adults. Abd El Halim *et al* (2016) reported the highest expression of the voltage-gated sodium channel (VGSC) gene in the late pupa of *T. castaneum*, although expression was not significantly different compared to any larval stage, early pupa or adults. Furthermore, Zuo *et al* (2016) reported that expression of the *Rhopalosiphum padi* (Bird cherry-oat aphid) VGSC gene was significantly higher in instars 3 and 4 compared to instar 1 but not compared to instar 2 and adults. However, the VGSC in this insect is unique; with two subunits encoded by two genes (Zuo *et al*, 2016) and expression of the second gene was significantly lower in instar 3 compared to instar 4 and adults but expression in instars 1, 2, 4 and adults was not significantly different from one another. Therefore, as with *ace-1* and *ace-2*, it appears that the expression profile of *para* differs between insect species. Consequently, it is also possible that expression of this gene changes throughout the development of a specific life stage.

In the present study, *nAChR* expression was highest in adults and lowest in the 1st instar stage but there was no significant difference in expression across any of the investigated developmental stages. Previous research into other insects investigated the expression of each subunit of the *nAChR* gene separately. Due to the lack of available *S. littoralis* sequences at the beginning of this project, only expression of the α9 subunit was investigated. Regarding larval expression, Martin and Garczynski (2014) reported the highest expression of the α9 subunit in the final instar (5) of the lepidopteran *Cydia pomonella*, the lowest in 4th and higher expression in male adults than female, although significance was not reported. Expression patterns of the other 7 α and 2 *β subunits* investigated differed considerably to that of the α9 subunit. Xu *et al* (2016), reported that expression of the α9 subunit in the lepidopteran *C. suppressalis* was significantly higher in the egg stage than any other life stage (larval instars 1-5, pupae and adults) and was also significantly lower across all larval instars compared to pupae and adults, although neither

were separated by sex. Expression of all other α subunits was significantly higher in the 1st instar larval stage. Shan *et al* (2020) did not investigate expression of the α9 subunit of the dipteran *Bradysia odoriphaga* (Chive midge) but did report differential expression of 5 α and 2 *β* subunits across the developmental stages, with the expression of 5 subunits significantly higher in the egg stage than any other stage. Furthermore, the expression of all subunits was higher in instar 1 than any other larval stage and expression across instars 2, 3 and 4 did not differ significantly to one another. Also, expression of all subunits was significantly higher in male adults than female and expression of 5 subunits was significantly higher in male pupae than female pupae. Again, expression profiles were different across each subunit. Yin *et al* (2023) did not investigate α subunit 9 nAChR expression in *B. tabaci*, but reported that expression of *β* subunit 1 was significantly higher in the egg stage than any other. Furthermore, expression in larval instar 3 was significantly higher than any other larval stage and expression in male and female adult pupae was significantly lower than and other life stage, which differs with that of *β* subunit 1 in *C. suppressalis* where expression was significantly higher in larval instar 1 than any other life stage and was significantly higher in adults compared to larval instars 2-5 (Xu *et al*, 2016). Therefore, the expression patterns of *nAChR* subunits differ between insect species. Future studies to determine expression of all *S. littoralis* subunits both across developmental stages and tissues, will provide greater knowledge regarding the subunit most responsible for cholinergic functionality in this species.

In the present study, *NADPHcytP450r* expression was highest in the adult stage and lowest in the pupal stage and expression in the 4th and 5th instar and pupae was significantly lower than in larval instars 1, 2, 3, 6 and the adult stage where expression did not differ significantly. Similarly, Jing *et al* (2018) reported expression of *NADPHcytP450r* was highest in the adult stage of *Aphis citridus* and was significantly higher than larval instars 3 and 4 but not instars 1 and 2. Furthermore, He *et al* (2020a) reported that expression of *NADPHcytP450r* was significantly higher in male and female adults of *B. tabaci* compared to the egg stage, larval instars 1, 2, 3 and 4 and expression in male adults was significantly higher than female adults. Shi *et al* (2021) reported that, for two transcripts of *NADPHcytP450r*, expression in *S. litura* was lowest in larval instars 1 and 2 and the pupae stage and significantly higher in larval instars 3, 4, 5 and 6 for transcript 1 and larval instars 3, 4, 5 for transcript 2, although they did not determine expression in the adult stage.

Contrastingly, Wang *et al* (2016) reported that *NADPHcytP450r* expression in *R. padi* adults was significantly lower than expression in larval instars 1, 2 and 3 but not 4, with expression in larval instar 2 significantly higher than any other stage. Similarly, Yuan *et al* (2020) reported that *NADPHcytP450r* expression in *Diaphorina citri* (the Asian citrus psyllid) was significantly lower in 15-day old adults than all larval instars. Qiao *et al* (2021) reported that *NADPHcytP450r* expression was lowest in the 4th instar and adult stages and expression in the egg stage was significantly higher than any other life stage. Similarly, Moural *et al* (2020) reported that *NADPHcytP450r* expression in *L. decemlineata* 1 day old eggs was significantly higher (> 3-12-fold) than any other life stage and expression in 5 day old eggs, larval instars 1, 3 ,4, pupae and male and female adults was not significantly different to one another but expression in larval instar 2 was significantly higher than every other stage except 1 day old eggs. Therefore, *NADPHcytP450r* expression profiles differ depending on insect species and expression also differs between developmental days of a specific life stage (Huang *et al*, 2015; Moural *et al*, 2020). Given this finding, not just for *NADPHcytP450r*, but for all other genes investigated, determining expression levels daily during *S. littoralis* development, as well as determining expression separately based on sex, may provide greater insights.

3.4.2 Delivering dsRNA by feeding

As mentioned previously, eliciting an RNAi response via feeding is desirable compared to direct injection which is not feasible in the field. Therefore, the effects of feeding a single dose of 8 µg ds*ace-1*, ds*para,* ds*nAChR* and 4 µg ds*ace-1* + 4 µg ds*nAChR* to 4th instar *S. littoralis* larvae were determined via percolation through an artificial diet. This instar was chosen as it is one of the most damaging *S. littoralis* stages (CABI, 2023a). As mentioned previously, the *ace-1*, *para* and *nAChR* genes are targets of commercial insecticides routinely used in *S. littoralis* control, therefore a significant impact on survival was expected. However, upon exposure to dsRNA specific to any gene, there was no significant impact on survival, pupation, adult emergence or gene expression. This contrasts with Malik *et al* (2016) who reported a 98% reduction in *ace-1* expression and 90 % reduction in *B. tabaci* survival after feeding on transgenic tobacco plants expressing *ace-1* specific dsRNA. Furthermore, Abd El Halim *et al* (2016) reported a 1.75-fold reduction in *para* expression, 51 % increase in mortality and 51 % reduction in adult emergence upon feeding 6th instar *T. castaneum* on flour disks containing 150 ng *ace-1* specific dsRNA/mg diet.

Difficulty in achieving RNAi through oral delivery of dsRNA to lepidopteran species has been previously reported (Christiaens *et al*, 2018a; Terenius *et al*, 2011; Guan *et al*, 2018a; Sivakumar *et al*, 2007). As mentioned in section 1.9.1, many factors affect the susceptibility of an insect to RNAi, particularly in lepidopteran insects, such as the presence of dsRNA degrading nucleases in the haemolymph, midgut (Peng *et al*, 2018) and saliva (Guan *et al*, 2018a) and the extremely high alkaline pH (>9.0) of the midgut which causes chemical hydrolysis of dsRNA (Christiaens *et al*, 2018a). Therefore, the absence of gene knockdown reported in the present study upon oral feeding of dsRNA perhaps suggests nuclease degradation is inhibiting the RNAi response. Furthermore, the lower nuclease activity in the midgut and haemolymph of the coleopteran *P. americana* in comparison to the lepidopteran *S. litura* (Wang *et al*, 2016) suggests a reason for the success reported by Abd El Halim *et al* (2016).

It is also possible that dsRNA may have degraded prior to ingestion, as stability can differ depending on the diet dsRNA is incorporated into, with persistence of ~ 14 days on flour disks used to feed *T. castaneum* but only ~ 48 h in *A. pisum* diet (Cao *et al*, 2018), therefore it may have been useful to investigate dsRNA stability in the diet used in the present study. However, as the artificial diet was provided to larvae immediately after dsRNA percolation, degradation is unlikely. Furthermore, as high dsRNA concentrations are necessary to elicit an RNAi response in lepidopteran insects and as the concentration of dsRNA used in the present study (8 µg) is regarded as low-intermediate in comparison to other feeding studies (Terenius *et al*, 2011) it is also possible that the dsRNA concentration used in the present study was not high enough and therefore it may have been useful to include a wide range of concentrations. Additionally, as post-RNAi gene knockdown in Lepidoptera is often transient, with transcript levels able to rebound within 48 h (Zhang *et al*, 2015), it may have been useful to monitor gene expression across a range of time points. This is further suggested by Meng *et al*, (2015) who reported the highest reduction in gene expression 60 h post injection of *ace-1* specific dsRNA in the pond wolf spider, *Pardosa pseudoannulata* and Gao *et al* (2023) who reported ~ 20 %, 70 %, 25 % and 25 % reduction in gene expression at 12, 24, 36 and 48 h post-injection of *ace-1* specific dsRNA into 3rd instar *S. frugiperda* larvae.

Although nuclease degradation of dsRNA has not yet been confirmed in *S. littoralis*, gavage feeding was used to deliver dsRNA directly into the larval gut in an attempt to evade the possibility of nucleases present in the saliva and haemolymph. In this experiment, 5th instar larvae were used as they represent one of the most destructive stages of *S. littoralis* (CABI, 2023a) but also because earlier instars proved technically too difficult to gavage. Resultantly, the expression of *ace-1*, *para*, *nAChR* and *NADPHcytP450r* remained unchanged, which again suggests the possibility of dsRNA degradation and that investigating expression across multiple time points may have been useful. Although larval survival was not recorded for the gavage study, unsuccessful pupation and emergence may be regarded as mortality. Thus, as neither feeding dsRNA via an artificial diet or gavaging elicited a significant detrimental effect, this may suggest that, although nucleases present in the saliva and haemolymph of lepidopteran insects have the ability to degrade nucleases (Guan *et al*, 2018a; Wang *et al*, 2016), perhaps the main nuclease activity in *S. littoralis* is present in the midgut, although this would require further investigation. However, the significant reduction in pupation reported in the present study upon gavage feeding 4 µg ds*ace-1* + 4 µg ds*nAChR*, may highlight the efficacy of delivering dsRNA targeting multiple genes in this species, although this would require further investigation.

As mentioned, although it may have been useful to include higher dsRNA concentrations in the present study, the idea that gavage feeding, which evades salivary and haemolymph nucleases, did not elicit the intended mortality perhaps suggests that concentrations would need to either be considerably higher or delivered in a more stable form if it was to be used as a control strategy in the field. However, the use of such high dsRNA concentrations would likely not be indicative of realistic levels of exposure in field conditions (Ivashantu *et al*, 2015). Furthermore, the concentration used in the present study was higher than in Griebler *et al* (2008) who showed that feeding 1 µg dsRNA specific to the allostatin gene via droplet feeding to 5th instar *S. frugiperda* led to an 80 % transcript reduction and Rodríguez-Cabrera *et al* (2010) who reported 90 % reduction in serine-proteinase gene (SfT6) expression through feeding 3 µg dsRNA to 4th instar *S. frugiperda* larvae. Although this may suggest that *S. littoralis* is more recalcitrant to RNAi than *S. frugiperda*, Rodríguez-Cabrera *et al* (2010) concluded that success in this instance may been due to the use of freshly moulted larvae and also due to starvation 24 h prior to experiments which greatly reduced dsRNA-degrading activity in the midgut. This is perhaps suggested further by the improved knockdown (82 %

compared to controls) of the melanin synthesis gene, *yellow*, in *Bactrocera tryoni* (Queensland fruit fly) after previous knockdown of the two gut nucleases *dsRNase1* and *dsRNase2* (Tayler *et al*, 2019).

To determine the effect of continuously feeding dsRNA targeted to the *ace-1*, *nAChR*, *para* and *NADPHcytP450r* genes and also to determine the effects of feeding dsRNA to early *S. littoralis* instars, neonate larvae were fed continuously for 9 days with artificial diet containing 1 µg ds*ace-1*, 2 µg ds*ace-1*, 1 µg ds*nAChR*, 2 µg ds*nAChR*, 1 µg ds*para*, 2 µg ds*para*, 2 µg ds*NADPHcytP450r*, 4 µg ds*NADPHcytP450r*, 1 µg ds*ace-1* + 1 µg ds*nAChR* or 2 µg ds*ace-1* +2 µg ds*nAChR*. The resultant significant reduction in larval weight is perhaps surprising considering that feeding dsRNA to 4th instar larvae did not elicit a response in the present study. However, Sharif *et al* (2022) reported a significant decrease in larval weight upon feeding 20 µg dsRNA specific to the *ace-1* gene via artificial diet to *H. armigera* neonate larvae along with 60 %, 40 % and 30 % mortality for larval instars 1, 2 and 3, respectively, but no significant effect was reported when larval instars 4 and 5 were provided with the same treatment. Furthermore, Sharif *et al* (2022) reported that dsRNA remained stable in the midgut juice of larval instars 1-3 for 1 h but was completely degraded in the midgut juice of larval instars 4 and 5 within the same time period, which may be the reason for the greater success reported when feeding neonate larvae in the present study, however this would require further investigation. Furthermore, the use of a 10-fold concentration (20 µg) by Sharif *et al* (2022) may suggest why artificial diet feeding of *ace-1* specific dsRNA led to a significant reduction in survival in their study in contrast to the present study.

Furthermore, Kumar *et al* (2009) reported that continuously feeding *H. armigera* with *ace-1* specific siRNA via an artificial diet from the neonate to the pre-pupation stage led to significant growth reduction of 20%, 40 % and 81 % for larvae exposed to 25 Nm, 37.5 and 50 nM, respectively). Similar to the present study, they reported low levels of larval mortality, with those reared on artificial diet containing 25, 37.5 and 50 nM siRNA suffering ~ 15 %, 16 % and 20 % mortality, respectively). They also reported that withdrawing siRNA from the diet after feeding from neonate to the second instar led to no significant difference in the weight of insects reared on 25 nM siRNA compared to controls and, although the

weight of those reared on 37.5 and 50 nM of siRNA was still significantly lower than those reared on artificial diet, weight increased by ~ 20 % and ~ 80 % compared to when fed continuously from neonate through to the time of weighing. Thus, the removal of dsRNA from the diet after 9 days in the present study may provide a reason as to why pupal malformation was reported by Kumar *et al* (2009) upon continuous feeding of *ace-1* specific dsRNA from the neonate stage through to pupation but there was no significant difference in pupal weight upon *ace-1* specific dsRNA feeding in the present study. Furthermore, Sharif *et al* (2022) reported significant transcript level reduction of the *ace-1* gene, with ~ 50 %, 80 %, 75 %, 75 % and 90 % reductions at 2, 4, 6, 8 and 10 days continuous feeding from neonate of *H. armigera*. Again, the significant reduction in expression reported by Sharif *et al* (2022) compared to the lack of reduction in the present study may be the result of the 10-fold higher dsRNA concentration used in their study.

3.4.3 Delivering dsRNA by direct haemolymph injection

In an attempt to evade the salivary (Guan *et al*, 2018a) and midgut (Peng *et al*, 2018) nucleases present in many lepidopteran insects, *S. littoralis* specific dsRNA was injected directly into the haemolymph of 4th instar larvae. To determine if a dose-response effect was present, increasing concentrations of ds*ace-1*, ds*nAChR* and ds*para* were injected. Although no significant difference in the survival of 4th instar larvae was reported, regardless of gene target or dsRNA concentration, survival was reduced by 16 %, 18 % and 18 % upon injection of 8 µg ds*ace-1*, ds*para* and ds*nAChR*, respectively, compared to 2 %, 4 %, 4 %, respectively, upon artificial diet delivery (section 3.4.8). Whilst this may suggest that delivery via haemolymph injection is more effective at eliciting an RNAi response than artificial diet delivery in *S. littoralis*, as reported previously in various lepidopteran insects (Zhang *et al*, 2022, Cooper *et al*, 2021, Terenius *et al*, 2011), the > 10 % reduction in survival upon ds*Kan* injection and the lack of significant difference between survival when injected with ds*Kan* and any *S. littoralis* specific dsRNA perhaps suggests that mortality may be the result of injection trauma. This is perhaps further confirmed by the lack of similarity between the designed ds*Kan* and its component n-mers with the *S. littoralis* genome and the dsRNA sequences used in the present study (Section 3.4.16).

In the present study, the expression of the *ace-1*, *para* and *nAChR* genes was not significantly reduced upon gene specific dsRNA injection. As mentioned previously, it is possible that the concentration of dsRNA used in the present study is insufficient to elicit

an RNAi response, although lower dsRNA concentrations are generally required when injecting dsRNA compared to delivery via an artificial diet (Cooper *et al*, 2021). However, Khalil *et al* (2023) reported that injecting an 8-fold lower concentration (1 µg) of dsRNA specific to an aquarporin gene (AQP3) into *S. littoralis* caused a significant reduction in gene expression, although this only became significantly lower 72 h post injection. These findings reiterate the need to investigate gene expression across multiple time points in future studies. Further to expression, it may also have been useful to investigate AChE activity across various time points as gene knockdown is not synonymous with reduced AChE activity and a 64 % reduction in AChE activity did not significantly affect *B. germanica* (Revuelta *et al*, 2009) or *L. migratoria* (Zhou and Xia *et al*, 2009) survival.

The lack of *ace-1* downregulation in the present study makes it difficult to decipher whether *ace-2* has a compensatory effect for *ace-1* expression reduction, as hypothesised in the present study and reported in *B. mori* (Cao *et al*, 2012). Therefore, investigating expression across time points may have allowed further investigation into the role played by *ace-2* in this insect. Although 56 % sequence similarity was identified between the designed *ace-1* dsRNA and the *ace-2* gene (section 3.4.15), the longest stretch of exactly matching nucleotides was 15 bp which may suggest that accidental *ace-2* knockdown is unlikely, as 21-25 bp is generally considered the minimum for triggering the RNAi machinery (Elbashir *et al*, 2001 and Tijsterman and Plasterk, 2004). However, as Santos *et al* (2019) reported that the length of siRNA produced by DICER is species specific, it is also possible that the homology required, between designed siRNA and the corresponding gene in a given NTO, to trigger successful RNAi may also differ between species (Arpaia *et al*, 2020). Thus, as the minimum number of matches required to trigger RNAi in *S. littoralis* is currently unknown and only a partial *ace-2* sequence was available, it is possible that the *ace-1* specific dsRNA used in the present study could also target the *ace-2* gene which would make expression analysis difficult.

Overall, the lack of mortality upon injecting ds*ace-1*, ds*para* and ds*nAChR* may again be due to nucleases in the haemolymph of lepidopteran insects. Although direct injection into the haemolymph generally yields greater success regarding RNAi experiments compared to feeding via an artificial diet (Chatterjee *et al*, 2021; Terenius *et al*, 2011), Garbutt *et al* (2013) reported that dsRNA was partially and completely degraded after 1 h and 3 h

incubation, respectively, in *Manduca sexta* (Tobacco hornworm) haemolymph, although this is yet to be confirmed in *S. littoralis*. However, the idea that the injection of 8 µg of ds*ace-1* and ds*para* led to significantly reduced pupation in the present study but feeding via an artificial diet did not, may suggest the efficacy of haemolymph injection for eliciting an RNAi response, whilst also highlighting the potential of targeting these genes as a means of *S. littoralis* control. This significant reduction in pupation was also reported by Salim *et al* (2021) upon injection of 5 µg *ace-1* specific dsRNA into 6th instar *Spodoptera litura*.

To investigate whether injecting larvae at differing instar stages influences RNAi efficacy in *S. littoralis*, 3rd instar larvae were injected with 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg ds*nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r* which led to a 24 %, 27 %, 31 %, 29 %, 31 % reduction in survival compared to non-injected insects. Although this is higher than the 16 %, 18 % and 18 % reduction in survival upon injection of ds*ace-1*, ds*para* and ds*nAChR* into 4th instar larvae, the 29 % survival reduction exhibited by insects injected with ds*Kan*, which was not significantly different to survival of those injected with *S. littoralis* specific dsRNA, suggests that mortality may be the result of injection trauma rather than a direct result of the applied dsRNA. Furthermore, the greater mortality suffered by 3rd instar larvae compared to 4th instar larvae is likely due to their smaller size.

Nevertheless, when injected with 8 µg ds*ace-1*, 8 µg ds*nAChR*, 4 µg ds*ace-1* + 4 µg ds*nAChR*, 8 µg ds*para* and 8 µg ds*NADPHcytP450r*, surviving insects consumed significantly less artificial diet than insects that were reared on artificial diet or injected with 8 µg ds*Kan*. This contrasts with the lack of significant difference in diet consumption when 4th instar larvae were injected with 8 µg ds*ace-1* in the present study, suggesting that injecting younger larvae may increase RNAi efficacy. This could be the result of decreased nuclease activity in the haemolymph of earlier instars, akin to the lower midgut nuclease activity reported by Sharif *et al* (2022) in early *H. armigera* larval instars, although this would require further investigation.

When 3rd instar insects were injected with 8 µg ds*ace-1*, ds*nAChR* or ds*para*, pupation decreased by 1 %, 8 % and 8 %, respectively, and there was no significant difference to controls. However, when 4th instar insects were injected with 8 µg ds*ace-1*, ds*nAChR* or

ds*para*, pupation reduced by 24 %, 21 % and 31 %, respectively, and was significantly lower than controls for those injected with ds*ace-1* and ds*para* but not ds*nAChR*. This may suggest that administering dsRNA specific to these genes at a later stage has a greater effect on pupation. Contrastingly, adult emergence decreased by 35 % and 53 % for those injected with 8 µg ds*ace-1* and ds*para* at the 3rd larval instar stage but decreased by only 29 % and 28 % when injected with 8 µg ds*ace-1* and ds*para* at the 4th instar stage suggesting that injecting at an earlier stage may have a greater effect on adult emergence.

3.4.4 Delivering dsRNA via egg soaking

Due to the technical difficulty of injection (Xu *et al*, 2022) or electroporation (Ando and Fujiwara, 2013), *S. littoralis* eggs were soaked in 200 µl PBS buffer containing 100 ng/µl ds*ace-1*, ds*nAChR*, ds*para* or ds*NADPHcytP450r* or 100 µl each of 100 ng/µl ds*ace-1*+ds*nAChR*. Significantly fewer eggs hatched after soaking in ds*ace-1*, ds*nAChR*, dsace–1 + ds*nAChR* and ds*NADPHcytP450r* compared to when untreated or soaked in ds*Kan* or PBS buffer. As mentioned previously, the non-cholinergic roles of the *ace-1*, *para* and *nAChR* genes in insects have been documented, along with the role of cytochrome P450s in growth and development. For example, *ace-1* is critical for embryonic development in *R. padi* and *Sitobion avenae* (English grain aphid) (Xiao *et al*, 2014). Therefore, it is possible that these genes also influence embryonic development and larval hatching in *S. littoralis*. Furthermore, it is possible that the success reported in the present study is the result of the use of freshly laid eggs which may have fewer nucleases than more developed eggs or later developmental stages (Xu *et al*, 2022) akin to the lower nuclease activity in the midgut of earlier *H. armigera* larval instars reported by Sharif *et al* (2022). However, Yamaguchi *et al* (2011) reported that injecting *B. mori* embryos with siRNA specific to the *tyrosine hydroxylase* gene, important for larval pigmentation, resulted in larval discoloration but the phenotype returned to that of non-injected embryos once larvae reached the 1st larval instar stage. Thus, it would have been interesting to monitor the growth and development of surviving *S. littoralis* larvae to confirm whether the effect of dsRNA exposure at the egg stage is transient.

Due to time constraints, it was not possible to determine gene expression post-dsRNA delivery at this stage and without this information it is difficult to confirm whether reduced

egg hatching rates were the direct result of gene knockdown. Furthermore, it would have been useful to confirm the presence of dsRNA within the egg by fluorescently labelling dsRNA and subsequently imaging via fluorescence microscopy, as described by Chao *et al* (2022). Additionally, although Chao *et al* (2022) confirmed that dsRNA can penetrate the eggshell of *S. frugiperda*, they used star polycation nanocarriers to enhance uptake. Thus, because the ability for naked dsRNA to penetrate the *S. littoralis* eggshell has not yet been confirmed, it is not possible to verify that the reduced larval hatching rates are the direct result of dsRNA exposure which is perhaps more important considering that PBS buffer was used as a novel method for dsRNA delivery. Nevertheless, the idea that there was no significant difference between the number of larvae hatching when soaked in PBS buffer compared to unsoaked eggs, suggests that PBS buffer does not affect larval hatching and the phenotype observed in this study was due to RNAi.

3.4.5 Increasing pesticide susceptibility through dsNADPHycytp450r pre-exposure

NADPHcytP450r is required for electron transfer from NADPH to cytochrome P450 and therefore for *P450* functionality (Zhu *et al*, 2012). As mentioned in section 1.7.2, no singular P450 is responsible for insecticide detoxification both within and between species. Consequently, the aim of the present study was to determine the effect of ds*NADPHcytP450r* exposure on the susceptibility of *S. littoralis* to a range of pesticides. Unfortunately, the LC_{50} for chlorpyrifos against *S. littoralis* could not be determined in the present study as even the highest concentration did not induce mortality. The concentrations used in the present study were based on Bagni *et al* (2020) who reported an LC_{50} of ~ 44 mg/l 72 h post-application in 4th instar *S. littoralis* (corresponding to 0.022 µg/larvae). However, Dewer *et al* (2015) reported an LC_{50} of 0.31 µg/larva and Ismail *et al* (2021) reported LC_{50} values of 1.71 mg/l, 18.44 mg/l and 30.15 mg/l for a lab strain and two field strains, respectively, of *S. littoralis* 4th instar larvae. Therefore, the variability between various populations of *S. littoralis* suggests that the concentration used in the present study may not have been sufficiently high to induce mortality.

The effect of ds*NADPHcytP450r* exposure on the susceptibility of *S. littoralis* to deltamethrin was subsequently determined. An LC_{50} of 11.8 mg/ml was reported in the present study which lies within the recommended exposure range for field treatment (8 to 50 mg/l) (Malbert-Colas *et al*, 2020). Although this is higher than the LC_{50} (7.6 ml/ml)

reported by Lalouette *et al* (2016), it is possible that deltamethrin susceptibility also varies between *S. littoralis* population, as is the case for chlorpyrifos. Feeding 2 µg or 4 µg ds*NADPHcytP450r* to neonate larvae for 9 days led to a 2 % and 20 % reduction in the concentration of deltamethrin necessary to induce 50 % insect mortality which suggests that *P450s* may play a role in *S. littoralis* insecticide detoxification as is the case in many insects (section 1.7.2). However, this would require further investigation.

The publication of the *S. littoralis* genome (Wu *et al*, 2022) would enable the expression of all *cytP450* genes to be monitored upon insecticide exposure (ie via RNA-seq) and the upregulation of particular cytP450 enzymes would highlight those responsible for the detoxification of a wide range of insecticides. This would allow direct targeting of specific *P450s* through RNAi which may increase insecticide susceptibility, thus possibly reducing the concentrations necessary for field application. However, as other enzymes such as esterases (ESTs) or glutathione S-transferases (GSTs) can detoxify insecticides (Schluep and Buckner, 2021), it is possible that compensatory effects could occur.

Increased susceptibility to insecticides has previously been reported as the result of ds*NADPHcytP450r* exposure. For example, injection of ds*NADPHcytP450r* led to a 35 % increase in mortality when *S. litura* were exposed to the previously determined LC_{50} for Phoxim (Ji *et al*, 2019), feeding ds*NADPHcytP450r* to *Laodelphax striatellus* (Small brown planthopper) led to a 30 % increase in mortality upon buprofezin exposure (Zhang *et al*, 2016) and injection of ds*NADPHcytP450r* into *A. pisum* increased mortality by 30 %, 32 % and 44 % upon exposure to chlorpyrifos, imidacloprid and cypermethrin, respectively. Therefore, the results of the present study agree with previous research and suggest that lower concentrations of a widely used synthetic pyrethroid (Malbert-Colas *et al*, 2020) may be necessary to induce *S. littoralis* mortality as a result of ds*NADPHcytP450r* exposure, thus reducing the harm to the environment and non-target organisms generally reported with insecticide use (Nicolopoulou-Stamati *et al*, 2016). However, the small sample size of 10 insects/treatment in the present study means that further work is necessary to confirm these results. It would also be interesting to investigate the effect of *NADPHcytP450r* specific dsRNA exposure on susceptibility to a range of other insecticides.

3.5 Conclusion

The hypothesis of the present study was that exposure to *ace-1*, *nAChR* and *para* specific dsRNA would induce mortality in *S. littoralis*. Although survival was not significantly impacted upon administration of dsRNA targeted to any gene via any delivery method, the growth retardation upon continuous feeding of neonate larvae, the disruption to pupation and adult emergence upon 3rd instar larvae injection and the significant reduction in larval emergence through egg soaking highlight the non-cholinergic roles of these genes whilst suggesting the efficacy of targeting these genes via RNAi. The *NADPHcytP450r* gene was included in the present study in the effort of increasing the susceptibility of *S. littoralis* to a range of insecticides. The reduced LC_{50} for deltamethrin upon *NADPHcytP450r* exposure suggests the possibility for reduced concentrations of this insecticide to control this insect. However, the lack of gene knockdown and mortality reported in this chapter also suggest that the strong nuclease activity present in the saliva, midgut and haemolymph of many Lepidoptera may also be present in *S. littoralis* therefore this will be investigated in the subsequent chapter, along with possible methods to improve RNAi efficiency in this insect.

Chapter 4. Increasing RNAi efficacy in *Spodoptera littoralis* through (i) improved stability via a nanoparticle-based delivery system and (ii) immune system priming via pre-injection of non-species-specific dsRNA

4.1 Abstract

Difficulty in achieving RNAi in Lepidoptera is often attributed to high nuclease activity in the haemolymph and midgut juice which can degrade dsRNA before it has the ability to trigger the RNAi mechanism. At the time of writing, dsRNA degradation within the highly polyphagous lepidopteran *Spodoptera littoralis* had not been documented. *Ex vivo* studies revealed that 1 µg dsRNA was degraded within 30 min in both pure haemolymph and midgut juice which suggests a reason for the general lack of RNAi response upon dsRNA ingestion and injection reported in chapter 3. To optimise efficiency of RNAi in the field, it is imperative to increase dsRNA stability under the harsh conditions it faces once administered to an insect. The efficacy of formulating dsRNA with nanoparticles as a means of increasing dsRNA stability and uptake in the presence of nucleases has been previously documented in other insects. The natural occurrence and availability of chitosan, along with its biodegradability, lack of toxicity, low cost and simple preparation make it an excellent candidate nanocarrier. Thus, dsRNA specific to the *S. littoralis ace-1*, *para*, *nAChR* and *NADPHcytP450r* genes was formulated with chitosan to create dsRNA-CS-TPP complexes. Complexation with CS-TPP increased dsRNA stability in pure *S. littoralis* haemolymph but not pure midgut juice suggesting a reason as to why delivery of dsRNA-CS-TPP via artificial diet to 4th instar or neonate larvae did not significantly reduce ($P > 0.05$) survival, pupation or adult emergence. Further to the use of nanocarriers, RNAi efficiency can be increased via pre-exposure to non-species-specific dsRNA which primes the immune system via the upregulation of genes within the core RNAi machinery. Thus, dsRNA specific to a bacterial kanamycin gene (ds*Kan*) was injected into 5th instar *S. littoralis* larvae prior to species specific dsRNA. The resultant lack of significant gene knockdown compared to controls further suggests the high degradative capacity of *S. littoralis* nucleases. Nevertheless, the significant decrease in *NADPHcytP450r* and *nAChR* expression upon ingestion of ds*NADPHcytP450r* by 4th instar larvae and injection of ds*ace-1*+ds*nAChR* into 5th instar larvae, respectively, suggests that eliciting an RNAi response is possible in this insect. Although, the variable response and lack of effect on phenotype or survival suggest that further research into improving the stability and delivery of dsRNA in this species is imperative to enable the feasibility of a novel biopesticide targeting *S. littoralis*.

4.2 Introduction

RNA interference (RNAi) is a natural immune defence mechanism that can be triggered artificially through the introduction of a specifically designed long dsRNA molecule into an organism of choice. The consequent post transcriptional down-regulation of gene expression (Hammond *et al*, 2001) can lead to significant phenotypic changes and insect mortality (Sharma *et al*, 2021), depending on gene choice. Thus, dsRNA-based biopesticides or genetically engineered plants expressing dsRNA offer highly specific pest control methods without the environmental concerns surrounding most chemical insecticides. As mentioned previously, dsRNA can be delivered via various methods but achieving RNAi via oral feeding is preferable as it indicates how an insect will respond to dsRNA in the field, where direct injection is not feasible. However, insects differ in their susceptibility to RNAi due to many factors (section 1.9.1).

As dsRNA must persist long enough to permit cellular uptake (Bolognesi *et al*, 2012), nuclease degradation in the saliva, haemolymph or midgut of an insect is generally considered a major factor influencing RNAi susceptibility (Chao *et al*, 2022). For example, difficulty in achieving a successful RNAi response in *A. pisum* may be attributed to dsRNA degradation by nucleases in the haemolymph which can completely degrade dsRNA within 3 h (Christiaens *et al*, 2014). Similarly, difficulty in achieving RNAi via dsRNA feeding in the lepidopterans *S. frugiperda* and *H. zea compared to the* high sensitivity of the coleopteran *D. virgifera virgifera* (Baum *et al*, 2007) may be attributed to increased dsRNA stability in the coleopteran midgut (Ivashuta *et al*, 2015). Furthermore, dsRNA remains intact in the haemolymph of the blattodean insect *B. germanica*, which is highly amenable to RNAi through both oral induction and injection (Huang and Lee, 2011; Guo *et al*, 2010), for more than 24 h (Garbutt *et al*, 2013) but is completely degraded after 1 h in the haemolymph of the lepidopteran *Manduca sexta* in which RNAi success is variable (Garbutt *et al*, 2013), although some success has been reported through direct haemolymph injection (Zhuang *et al*, 2008; Zhuang *et al*, 2007; Eleftherianos *et al*, 2006). Although, Whyard *et al* (2009) and Burke *et al* (2019) reported success through feeding dsRNA to *M. sexta*, the use of neonate and second instar larvae, respectively, may have increased success due to lower nuclease activity in earlier instars, such as in *H. armigera* (Sharif *et al*, 2022). Furthermore, dsRNA is most stable at ~ pH 4.0 – 5.0 (Romeis and Widmer, 2020), thus is generally more stable in the weakly acidic guts of most coleopteran and hemipteran insects (Wynant *et al*, 2014b)

compared to orthopteran, dipteran hymenopteran and lepidopteran guts which are generally alkaline (Cooper *et al*, 2020; Christiaens *et al*, 2018a).

To optimise the efficacy of RNAi in the field, it is imperative to increase dsRNA stability under the harsh conditions it faces once administered to an insect. As described in sections 1.11.3-5, nanoparticles can be used to enhance dsRNA stability. Nanoparticles generally range between 1 to 100 nm (Khan *et al*, 2019) but can be anywhere up to 500 nm in size (Kunte *et al*, 2019). They can be molecules such as peptides, sugars, lipids and cationic polymers (Blanco *et al*, 2015) which have the unique ability to promote the movement of siRNA and dsRNA across cell membranes and protect them from enzymatic degradation which has allowed their use as drug carriers for human therapy (Khan *et al*, 2019; Ahmadzada *et al*, 2018). These unique properties highlight the use of nanoparticles as efficient carriers of nucleic acids in insects, especially those recalcitrant to orally induced RNAi (Kunte *et al*, 2019). For example, Castellanos *et al* (2018) reported a significant increase in mortality when dsRNA targeting the *V-ATPaseA* gene was liposome encapsulated and fed to *Euschistus heros* (Brown stink bug), compared to naked dsRNA. Furthermore, Zhang *et al* (2022a) reported that simultaneous topical application of dsRNA targeted to the vestigial (*vg*) and Ultrabithorax (*Ubx*) genes, when complexed in a star polycation nanocarrier, led to ~ 50 % silencing of both genes and 63 % increase in wing aberration rate in the hemipteran *M. persicae* which is generally recalcitrant to orally induced RNAi (Ghodke *et al*, 2019). Additionally, Wei *et al* (2021) reported that complexation of the nanocarrier-mediate transdermal (*NPF1*) gene with a star polycation nanocarrier reduced expression by 91.53% which led to a significant decrease in diet consumption, weight gain, body size and fruit drill hole rate in the lepidopteran Oriental fruit moth, *Grapholita molesta*.

The natural occurrence and availability of chitosan, the second most abundant biopolymer after cellulose (Zhang *et al*, 2010) along with its biodegradability, lack of toxicity, low cost and simple preparation via chitin deacetylation (Dass and Choong, 2008) make it an excellent candidate nanocarrier. Zhang *et al* (2010), were the first to report successful RNAi in insects via the use of a chitosan-based nanocarrier when they showed that targeting the chitin synthase gene in *A. gambiae* reduced expression by 63 %, chitin content by 44 % and increased susceptibility to Diflubenzuron by 27 %. Subsequently, many successful RNAi

attempts via chitosan delivery were reported in *Aedes aegypti* with Das *et al* (2015) reporting 62 % silencing of the SNF7 gene and 47 % mortality, Zhang *et al* (2015) reporting 32 % silencing of the *Sema1a* gene and 44 % larval antennal lobe defects and Chen *et al* (2019) reporting 80 % gene silencing of the *DOPAl synthase* gene.

Improved RNAi efficacy via chitosan delivery has also been reported in lepidopteran insects. For example, Wang *et al* (2019) reported that feeding dsRNA specific to the *Glyceraldehyde-3-phosphate dehydrogenase* gene complexed with chitosan in *C. suppressalis* reduced gene expression by 55 % which also reduced mortality by 55 %. Furthermore, Wang *et al* (2023) reported that feeding dsRNA specific to the Krüppel-homologue *kr-h1* gene complexed with chitosan led to significant gene silencing (57.9%), pupal weight reduction (29.29%) and adult emergence rate (34.74%) in the lepidopteran *T. absoluta*. Additionally, Kolge *et al* (2023) reported that loading dsRNA specific to the *H. armigera chitinase* and *lipase* genes onto chitosan nanoparticles offered improved protection from nucleases across pHs 5, 7, 9 and 11, whilst also improving its uptake into the gut. Additionally, cross-linkers such as sodium tripolyphosphate (TPP) can enhance siRNA and dsRNA protection (Raja *et al* (2015); Dhandapani *et al*, (2019) and uptake into cells (Katas *et al*, 2006) due to its small size, anionic charge, low toxicity and ability to quickly form ionic interactions with positively charged amino groups of chitosan (Ko *et al*, 2002).

Further to the use of nanocarriers, pre-exposure to non-species-specific dsRNA can prime the immune system and enhance RNAi efficiency. For example, Fan *et al* (2022a) reported that pre-injection of dsRNA specific to the e*GFP* gene led to upregulation of two core RNAi genes (*OfDicer2* and *OfAgo2*) in *Ostrinia furnicalis*. Consequently, the expression of two experimental genes, *OfEF1α and OfCTP8,* was reduced by 46.9% and 44.1% in the haemolymph and midgut, respectively for *OfEF1α*, and 91.9% and 80.0% in the haemolymph and integument, respectively for *OfCTP8*.

The present study first investigated the stability of dsRNA in the haemolymph and midgut juice of *S. littoralis*. Subsequently, the efficacy of chitosan-TPP (CS-TPP) nanoparticles as nanocarriers to deliver dsRNA targeted to the *S. littoralis* acetylcholinesterase 1 (ds*ace-1*), voltage gated sodium channel (ds*para*), nicotinic acetylcholine receptor (ds*nAChR*) and NADPH cytochrome P450 reductase (*dsNADPHcytP450r*) genes was investigated as a means of improving dsRNA delivery and uptake in this species. Finally, the pre-injection of dsRNA

specific to the bacterial kanamycin gene (ds*Kan*) was used as a means of priming the immune system, prior to delivering dsRNA specific to *S. littoralis* genes.

4.3 Results

4.3.1 dsRNA stability in S. littoralis haemolymph and midgut juice

The stability of dsRNA in *S. littoralis* haemolymph and midgut juice was investigated via agarose gel electrophoresis and band intensity was estimated using ImageJ. The expected 485 bp band corresponding to *S. littoralis dsace-1* was not present after incubation for 30 min in pure haemolymph (Figure 4.1) but appeared when haemolymph was diluted and band intensity increased with increasing dilutions, with a 1/100 dilution leading to a ~ 12 % increase in band intensity compared to a 1/50 dilution. The expected 485 bp band corresponding to *dsace-1* was not present after dsRNA incubation for 30 min in pure nor 1/50 or 1/100 dilution of midgut juice nor after incubation in RNAse. Additionally, no bands were present when pure or diluted haemolymph or midgut juice (alone) were electrophoresed but was present when *ace-1* specific dsRNA was incubated in PBS pHs 6.8, 8.8 and DEPC treated water.

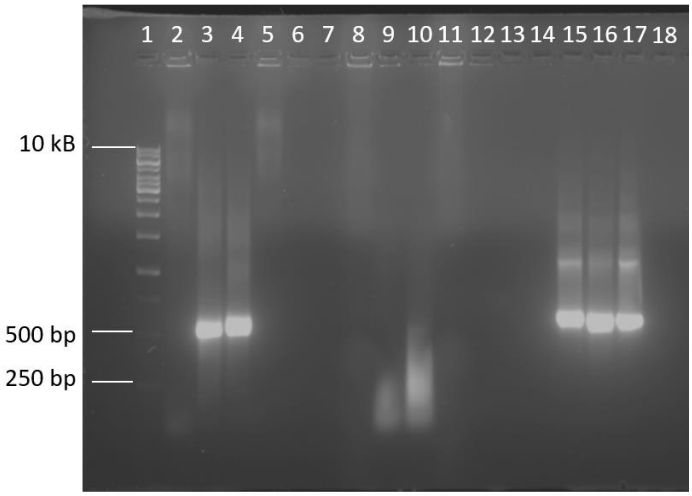

Figure 4.1 Stability of dsRNA in *S. littoralis* haemolymph and midgut juice. 1 µg of *S. littoralis ace-1* specific dsRNA was incubated at 37**°** for 30 min in the pure and diluted haemolymph and midgut juice of 5th instar *S. littoralis* (diluted in PBS pH 6.8 and 8.8, respectively) prior to 1.5% (w/v) agarose gel electrophoresis (120 v, 50 min) with EtBr staining. Lane 1: Generuler 1Kb ladder, lanes 2, 3 and 4: 1 µg *ace-1* dsRNA incubated in pure haemolymph, 1/50 haemolymph, 1/100 haemolymph, respectively, lanes 5, 6 and 7: pure haemolymph (alone), 1/50 haemolymph, 1/100 haemolymph , respectively, lanes 8, 9 and 10: dsRNA incubated in pure midgut juice, 1/50 midgut juice, 1/100 midgut juice, respectively, lanes 11, 12 and 13: pure midgut juice (alone), 1/50 midgut juice, 1/100 midgut juice, respectively, 14: empty, 15: Control 1 – PBS pH 6.8 + 1 µg ds*ace-1*, 16: Control 2 – PBS pH 8.8 + 1 µg ds*ace-1*, 17: Control 3 - DEPC water + 1 µg ds*ace-1*, 18: Control 4 – RNAseA + 1 µg ds*ace-1*.

4.3.2 Confirming the formation of dsRNA-chitosan-TPP complexes

The formation of dsRNA-CS-TPP complexes was confirmed by agarose gel electrophoresis. Naked ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and ds*Kan* were resolved to their indicative molecular masses of 484, 499, 486, 473 and 470 bp, respectively (Figure 4.2). Formulated dsRNA-CS-TPP complexes did not leave the wells and the bands relating to nucleic acids remained at the top of the agarose gel (Figure 4.2).

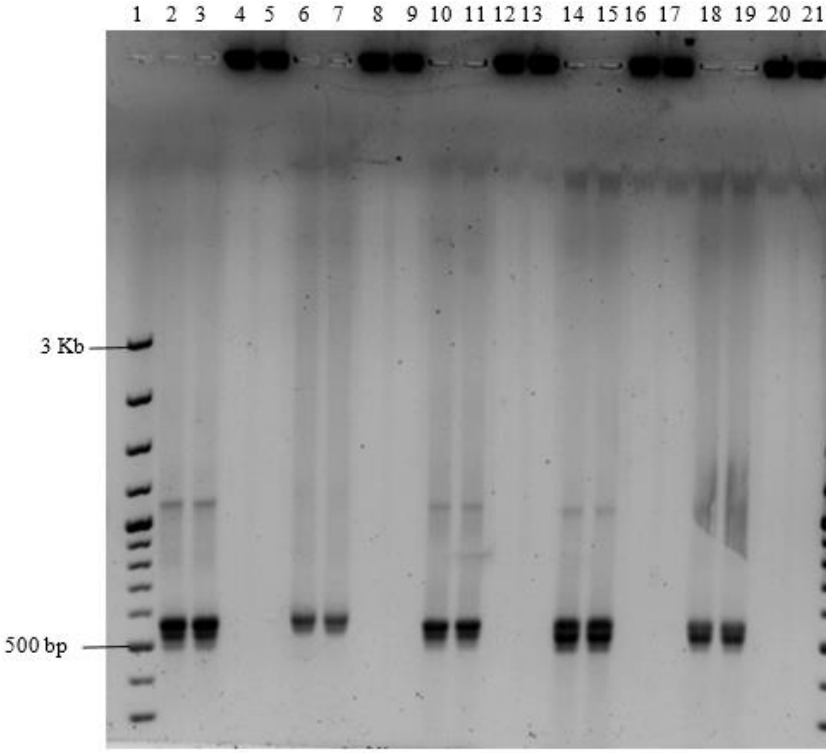

Figure 4.2 *S. littoralis* gene specific dsRNA complexed with chitosan tripolyphosphate (CS-TPP) nanoparticles. Generated nanoparticles were incubated in PBS pH 6.8 and 8.8 at RT for 30 min prior to 1.5% (w/v) agarose gel electrophoresis (120 v, 50 min) with EtBr staining. Lane 1: Generuler 100 bp+ ladder, 2 and 3: ds*ace-1* incubated in PBS 6.8 and 8.8, lanes 4 and 5: ds*ace-1*-CS-TPP in PBS pH 6.8 and 8.8, lanes 6 and 7: ds*para* in PBS 6.8 and 8.8, lanes 8 and 9: ds*para*-CS-TPP in PBS pH 6.8 and 8.8, lanes 10 and 11: ds*nAChR* in PBS 6.8 and 8.8, lanes 12 and 13: ds*nAChR*-CS-TPP in PBS pH 6.8 and 8.8, lanes 14 and 15: ds*NADPHcytP450r* in PBS 6.8 and 8.8, lanes 16 and 17: *NADPHcytP450r*-CS-TPP in PBS pH 6.8 and 8.8, lanes 18 and 19: ds*Kan* in PBS 6.8 and 8.8, lanes 20 and 21: ds*Kan*-CS-TPP in PBS pH 6.8 and 8.8.

4.3.3 Increasing dsRNA stability in S. littoralis haemolymph and midgut juice via formulation with chitosan-TPP nanoparticles

The stability of dsRNA when complexed with CS-TPP was investigated in *S. littoralis* haemolymph and midgut juice via agarose gel electrophoresis and band intensity was estimated using ImageJ. The expected 485 bp band corresponding to ds*ace-1* was not present after naked dsRNA was incubated for 30 min in pure haemolymph or 1/20 dilution but appeared when haemolymph was diluted to 1/50 and 1/100 and band intensity increased with increasing dilutions (Figure 4.1). When ds*ace1*-CS-TPP was incubated in pure or diluted haemolymph, the 485 bp dsRNA band was not present but fluorescence from intact dsRNA was detected in each corresponding well (lanes 3, 5, 7 and 9). When incubated in 1/100 haemolymph dilution, the band corresponding to the ds*ace-1*-CS-TPP complex was ~ 10 %, 41 % and 47 % brighter than when diluted in 1/50, 1/20 and pure haemolymph, respectively. The expected 485 bp band corresponding to ds*ace-1* was not present when naked ds*ace-1* was incubated for 30 min in pure or diluted midgut juice but, upon formulation with CS-TPP, fluorescence was present in the wells corresponding to incubation in 1/20, 1/50 and 1/100 midgut juice dilutions. When chitosan (alone) was incubated in pure haemolymph, only a high molecular weight band (> 3 Kb) was present (similar to electrophoresis of pure haemolymph) and no bands were present when chitosan was incubated in pure midgut juice or when midgut juice (alone) was electrophoresed. When dsRNA-CS-TPP was incubated in PBS pHs 6.8 and 8.8, fluorescence remained in the wells.

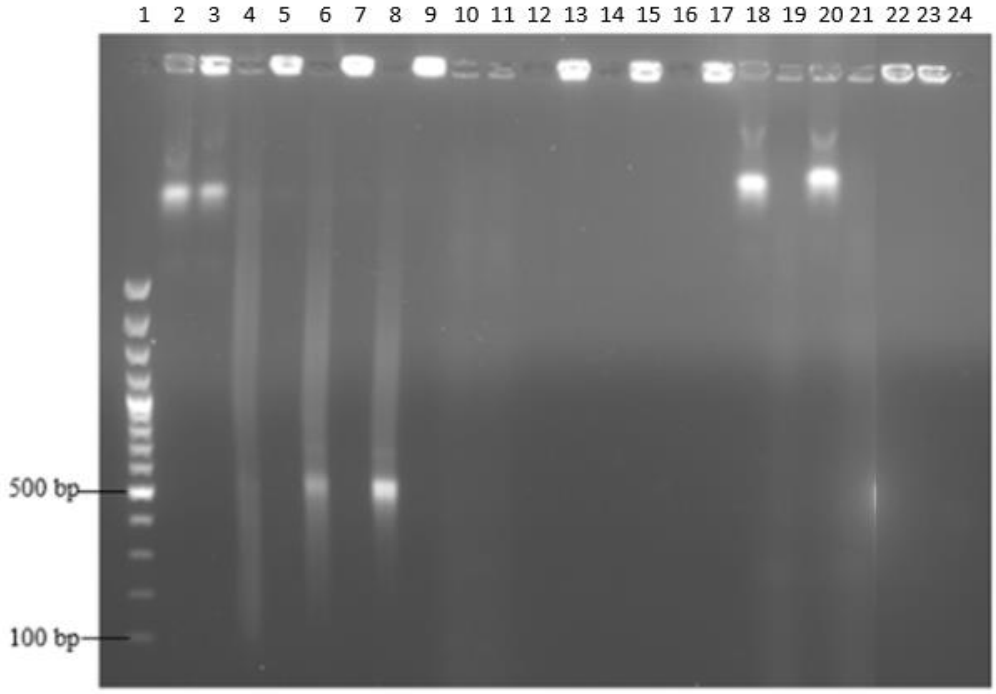

Figure 4.3 Stability of dsRNA and dsRNA-CS-TPP in *S. littoralis* haemolymph and midgut juice. 2 µl (0.1 µg/µl) of naked *S. littoralis ace-1* specific dsRNA (ds*ace-1*) and complexed with chitosan (ds*ace*-1-CS-TPP containing 0.1 µg dsRNA/µl) were incubated at 37**°** for 30 min in extracted *S. littoralis* pure and various dilutions of haemolymph and midgut juice (diluted in PBS pH 6.8 and 8.8, respectively) prior to 1.5% (w/v) agarose gel electrophoresis (120 v, 50 min) with EtBr staining. Lane 1: Generuler 100 bp+ ladder, lanes 2 and 3: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP incubated in pure haemolymph, lanes 4 and 5: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/20 haemolymph, lanes 6 and 7: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/50 haemolymph, lanes 8 and 9: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/100 haemolymph, lanes 10 and 11: 2 µl ds*ace-1* and ds*ace-1*-CS-TPP in pure midgut juice, lanes 12 and 13: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/20 midgut juice, lanes 14 and 15: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/50 midgut juice, lanes 16 and 17: 2 µl ds*ace-1* and ds*ace*-1-CS-TPP in 1/100 midgut juice, lanes 18 and 19: pure haemolymph and midgut juice + 2 µl DEPC water, lanes 20 and 21: chitosan incubated in pure haemolymph and midgut juice, lanes 22 and 23: 2 µl ds*ace*-1-CS-TPP in PBS pH 6.8 and 8.8, lane 24: RNAseA + 2 µl ds*ace-1*.

4.3.4 Delivery of naked dsRNA and dsRNA-CS-TPP complexes via artificial diet to 4th instar larvae through a single feeding event

To determine the effect of feeding gene specific naked dsRNA and dsRNA-CS-TPP complexes on survival, pupation, adult emergence and gene expression, fourth instar larvae were reared on artificial diet with 8 µg ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* or ds*ace-1*-CS-TPP, ds*para*-CS-TPP, ds*nAChR*-CS-TPP, ds*NADPHcytP450r*-CS-TPP percolated throughout. Controls were insects reared on artificial diet ± 8 µg dsRNA specific to the kanamycin resistance gene (ds*Kan*), ds*Kan* complexed with chitosan-TPP (ds*Kan*-CS-TPP) and chitosan

(alone). When insects were reared on artificial diet alone or with *dsKan* or *dsKsan-CS-TPP* incorporated 4 %, 4 % and 2 % mortality was recorded by day 5 (Figure 4.4). This is comparable with 2-7% mortality for insects reared on artificial diet containing any *S.littoralis* specific dsRNA whether naked or complexed with chitosan and 4 % when reared on artificial diet with chitosan alone. Kaplan-Meier log-rank survival analysis identified no significant difference in survival (P = 0.994) between insects reared on any diet. Regarding pupation, when reared on artificial diet alone or containing *dsKan* or *dsKan-CS-TPP*, 91 %, 88 % and 84 % of insects pupated compared to 81 – 88 % when insects were reared on artificial diet containing any *S. littoralis* specific dsRNA or 81 % when insects were reared on artificial diet and chitosan (alone) (Figure 4.5) and there was no significant difference (P > 0.05; ANOVA, post-hoc Tukey) in pupation between insects in any group. For those reared on artificial diet or artificial diet containing ds*Kan* ords*Kan*-CS-TPP, 87, 81 and 84 % of those that pupated emerged as adults, which is comparable to 84-92 % for those reared on artificial diet containing dsRNA specific to any *S. littoralis* gene (Figure 4.6) and there was no significant difference (P > 0.05; ANOVA, post-hoc Tukey) between the instance of adult emergence for any group.

Gene expression was investigated via RT-qPCR after 4th instar larvae ingested artificial diet containing ds*ace-1*, ds*nAChR*, ds*para* or ds*NADPHcytP450r* or ds*ace-1*-CS-TPP, ds*nAChR*-CS-TPP, ds*para*-1-CS-TPP, ds*NADPHcytP450r*-1-CS-TPP with expression compared to insects fed with artificial diet containing ds*Kan*-CS-TPP. As described in section 3.4.7, only expression below 0.5- and above 2.0 times that of the control sample will be classed as a biologically significant change in gene expression. The expression of *ace-1* was 1.62 and 1.36 when insects ingested naked ds*ace-1* or ds*ace-1*-CS-TPP, respectively, thus was not significantly different than when insects ingested ds*Kan*-CS-TPP (Figure 4.7a). When insects ingested ds*nAChR*, the expression of *nAChR* was not significantly different to when ingesting ds*Kan*-CS-TPP (0.66) but significantly decreased to 0.26 upon ds*nAChR*-CS-TPP ingestion (Figure 4.7b). The expression of *para* was 0.92 and 1.09 when insects ingested ds*para* or ds*para*-1-CS-TPP, respectively, thus was not significantly different than when insects ingested ds*Kan*-CS-TPP (Figure 4.7c). The expression of *NADPHcytP450r* was significantly decreased to 0.07 and 0.06 when insects ingested ds*NADPHcytP450r* or ds*NADPHcytP450r*-1-CS-TPP, respectively, compared to when they ingested ds*Kan*-CS-TPP (Figure 4.7d).

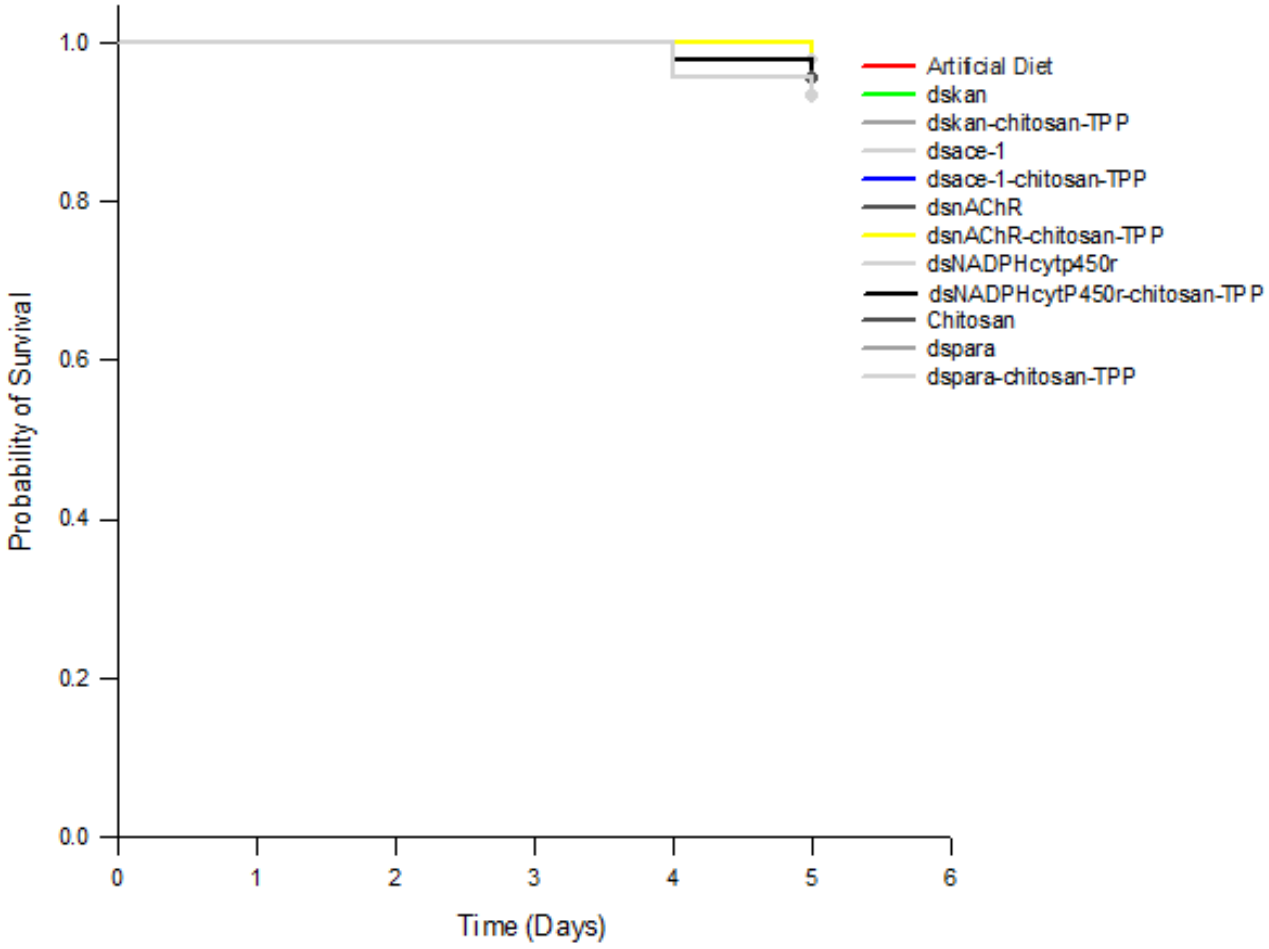

Figure 4.4 Survival of *S. littoralis* larvae after 4th instar insects ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan were used as controls (n=45). Insects were fed once with artificial diet containing dsRNA or dsRNA-CS-TPP then were transferred to artificial diet for the remainder of the trial.

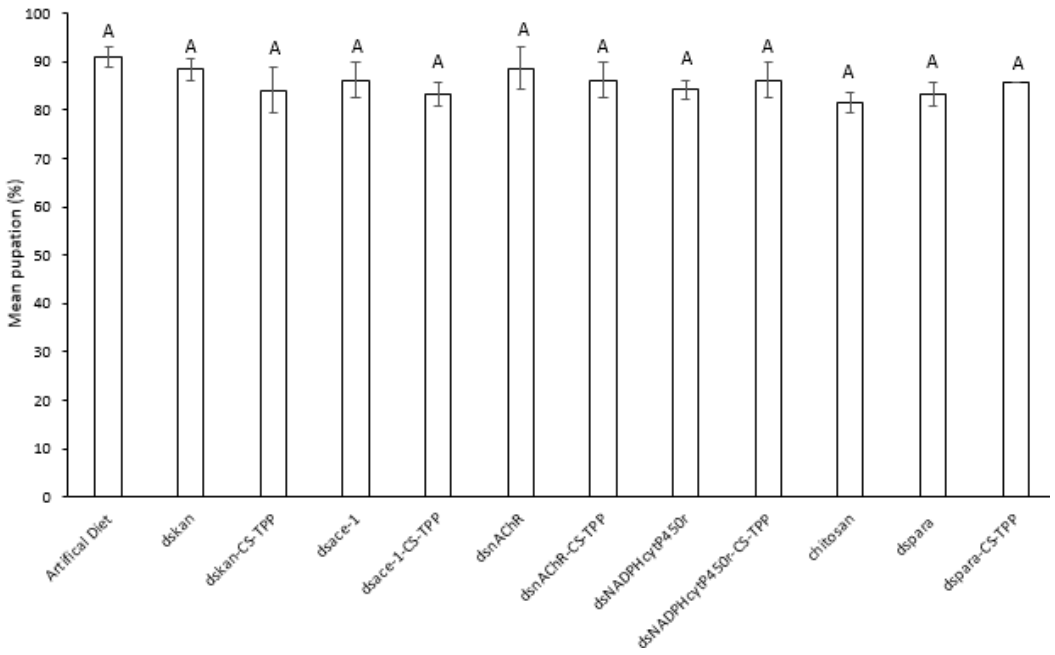

Figure 4.5 *S. littoralis* pupation after 4th instar larvae ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Controls were insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

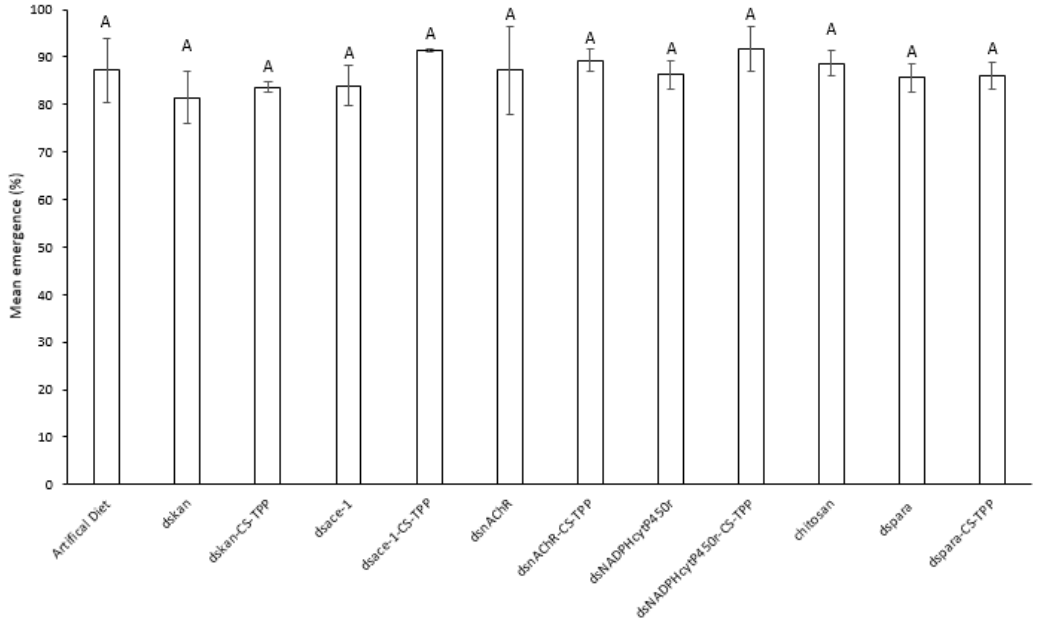

Figure 4.6 *S. littoralis* adult emergence after 4th instar larvae ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Controls were insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

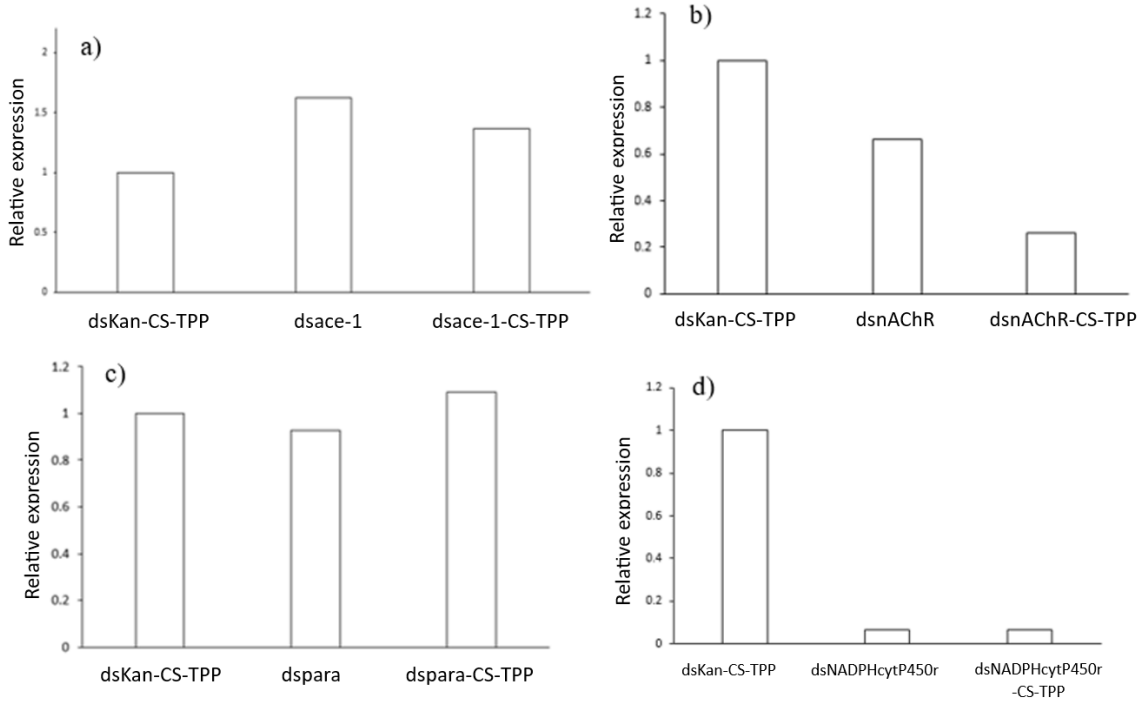

Figure 4.7 *S. littoralis* gene expression after 4th instar larvae ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. a) *ace-1* expression, b) *nAChR* expression, c) *para* expression d) *NADPHcytP450r* expression. Expression was determined after 48 h via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to expression of insects reared on artificial diet containing 8 µg ds*Kan*-CS-TPP (shown as Y=1).

4.3.5 Delivery of naked dsRNA and dsRNA-chitosan complexes via artificial diet to neonate larvae through a single feeding event

To determine the effect of feeding gene specific naked dsRNA and dsRNA-CS-TPP complexes on survival, pupation, adult emergence and gene expression, neonate larvae were reared on artificial diet with 8 µg ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* or ds*ace-1*-CS-TPP, ds*para*-CS-TPP, ds*nAChR*-CS-TPP, ds*NADPHcytP450r*-CS-TPP percolated throughout. Controls were insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan. When insects were reared on artificial diet alone or containing dsKan or dsKan-CS-TPP 11 %, 13 % and 13 % mortality was recorded by day 5, respectively (Figure 4.7). This is com*para*ble with 13 – 18 %% mortality for insects reared on artificial diet containing any *S. littoralis* specific dsRNA whether naked or complexed with chitosan. Kaplan-Meier log-rank survival analysis identified no significant difference in survival (P = 0.994) between insects reared on any diet. Regarding pupation, 88, 85 and 88 % of insects reared on artificial diet alone or containing *dsKa*n or *dsKan-CS-TPP* pupated, respectively, compared to and 81-87 %, when reared on

artificial diet containing any *S. littoralis* specific dsRNA whether naked or complexed with chitosan (Figure 4.8) and there was no significant difference ($P > 0.05$; ANOVA, post-hoc Tukey) in pupation between insects in any group. For those reared on artificial diet or artificial diet containing *dsKan* or *dsKan*-CS-TPP, 83, 82 and 80 % of insects that pupated emerged as adults, respectively, compared to 73 – 87 % for insects who were reared on artificial diet containing dsRNA specific to *S. littoralis* whether naked or complexed with chitosan (Figure 4.9) and there was no significant difference ($P > 0.05$; ANOVA, post-hoc Tukey) between the instance of adult emergence for any group.

Gene expression was investigated via RT-qPCR after neonate larvae ingested artificial diet containing ds*ace-1*, ds*nAChR*, ds*para* or ds*NADPHcytP450r* or ds*ace-1*-CS-TPP, ds*nAChR*-CS-TPP, ds*para*-1-CS-TPP, ds*NADPHcytP450r*-1-CS-TPP with expression compared to insects fed with artificial diet containing ds*Kan*-CS-TPP. The expression of *ace-1* was 1.30 and 0.98 when insects ingested ds*ace-1* or ds*ace-1*-CS-TPP, respectively, thus was not significantly different than when insects ingested ds*Kan*-CS-TPP (Figure 4.10a). The expression of *nAChR* was 1.23 and 1.46 when insects ingested ds*nAChR* or ds*nAChR*-CS-TPP, respectively, thus was not significantly different than when insects ingested ds*Kan*-CS-TPP (Figure 4.10b). The expression of *NADPHcytP450r* was 0.85 and 0.96 when insects ingested naked ds*NADPHcytP450r* or ds*NADPHcytP450r*-1-CS-TPP, respectively, thus was not significantly different than when insects ingested ds*Kan*-CS-TPP (Figure 4.10c).

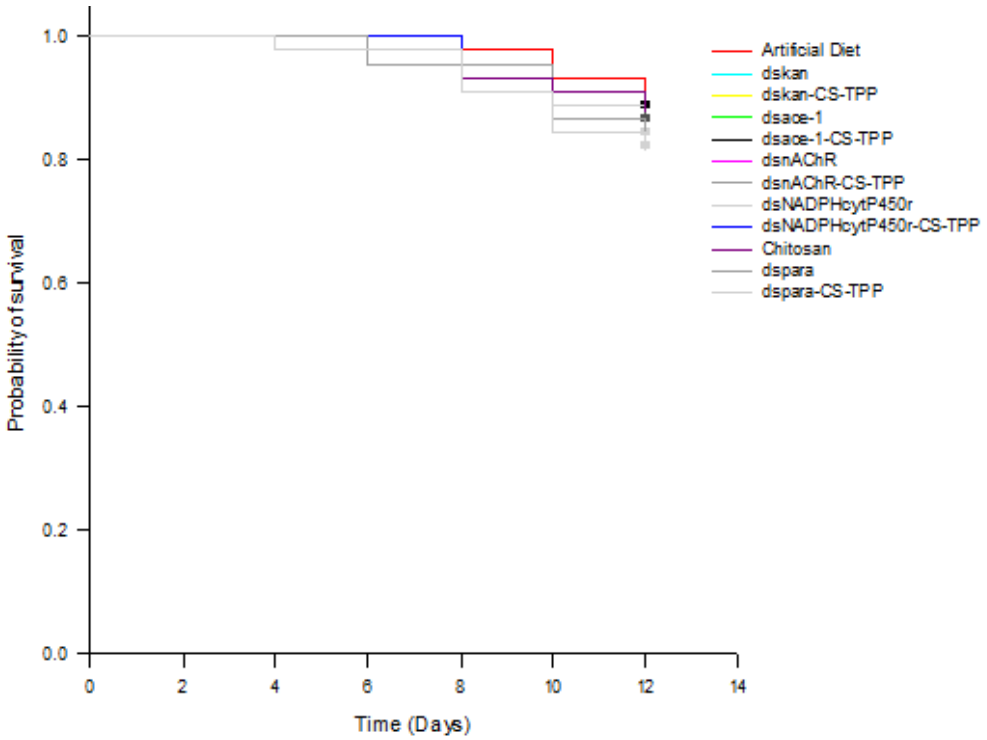

Figure 4.8 Survival of *S. littoralis* after neonates ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan were used as controls (n=45). Insects were fed once with artificial diet containing dsRNA or dsRNA-CS-TPP then were transferred to artificial diet for the remainder of the trial.

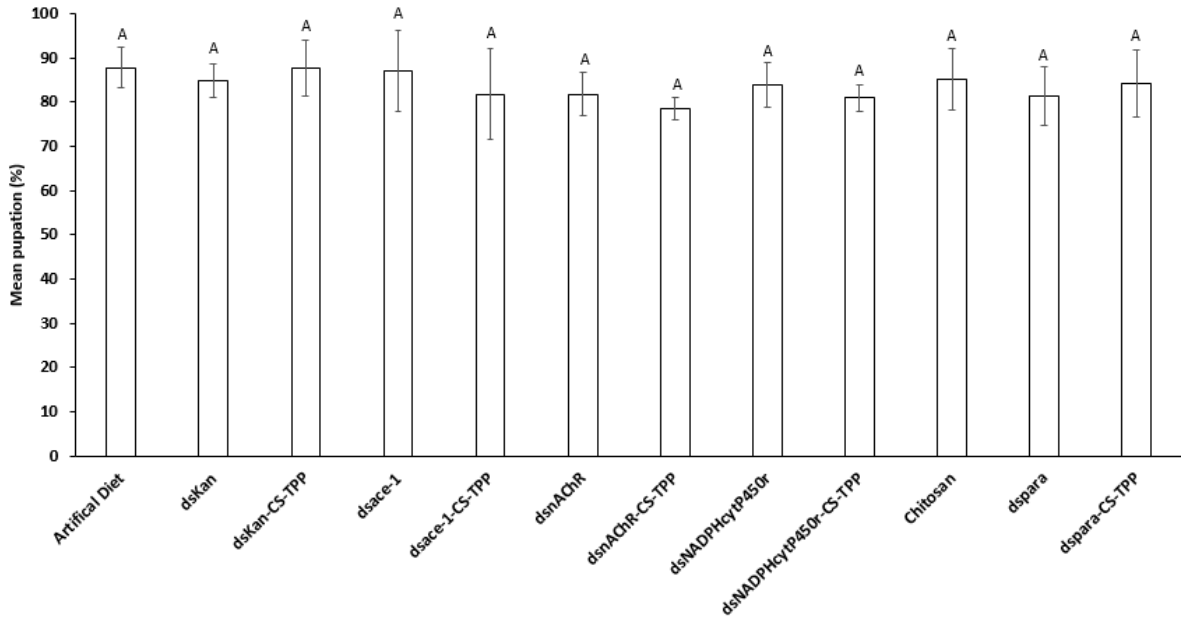

Figure 4.9 *S. littoralis* pupation after neonates ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan were used as controls. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

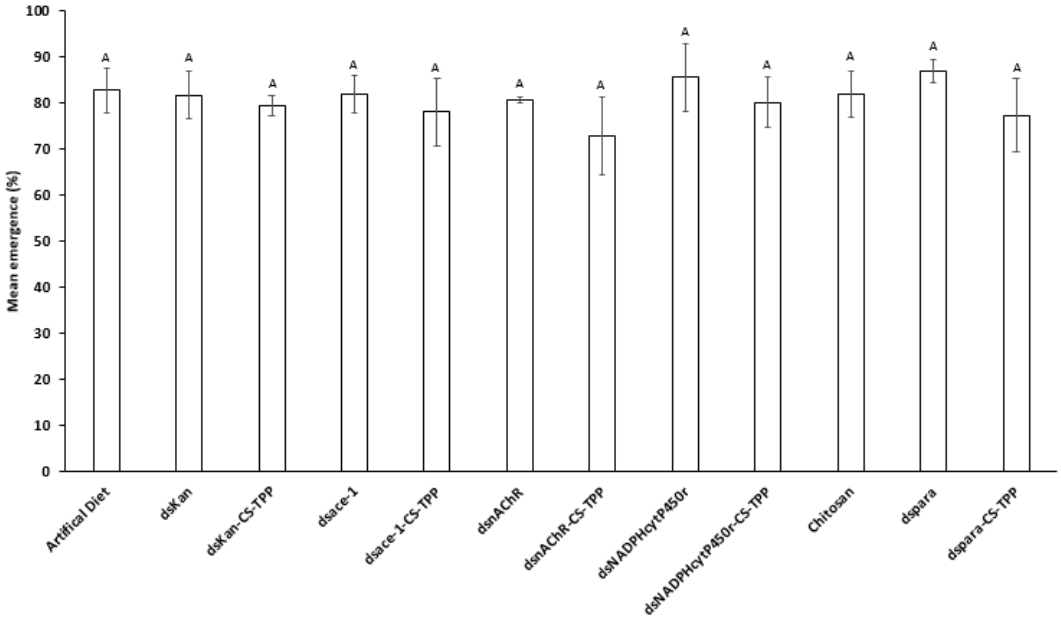

Figure 4.10 *S. littoralis* adult emergence after neonates ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR*, *para* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. Insects reared on artificial diet ± 8 µg ds*Kan*, ds*Kan*-CS-TPP and chitosan were used as controls. Insects were fed once with artificial diet containing dsRNA then were transferred to artificial diet for the remainder of the trial. Values are the average of 3 biological replicates (n=15) and are given as percentages of surviving insects, error bars denote standard error of the mean and different letters denote significant differences.

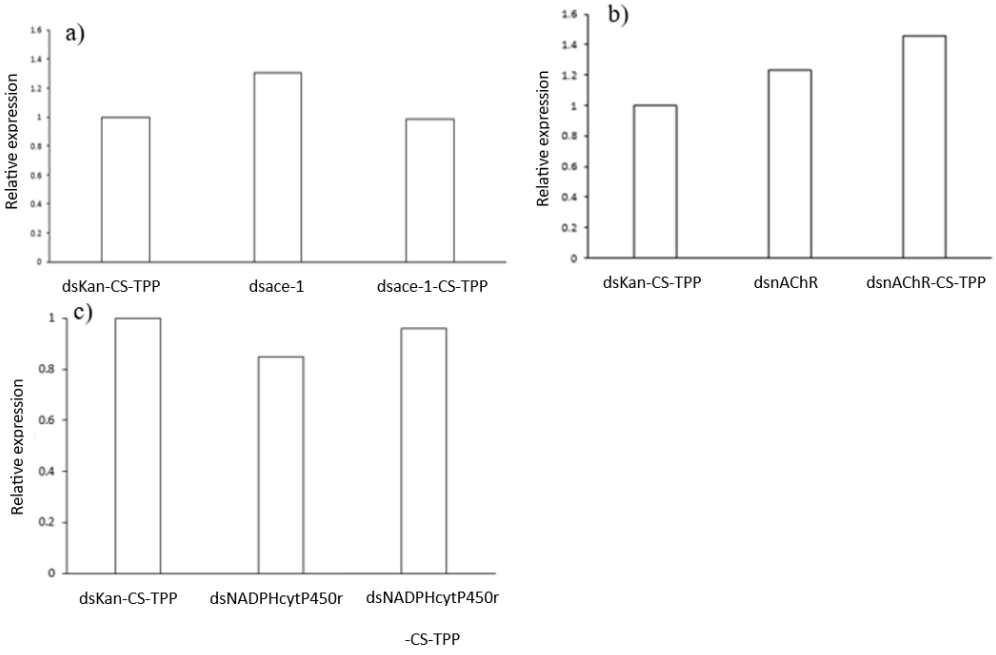

Figure 4.11 *S. littoralis* gene expression after neonate larvae ingested artificial diet with 8 µg dsRNA specific to the *ace-1*, *nAChR* or *NADPHcytP450r* genes or each gene specific dsRNA complexed with chitosan-TPP percolated throughout. a) *ace-1* expression, b) *nAChR* expression, c) *NADPHcytP450r* expression. Expression was determined after 48 h via RT-qPCR and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to expression of insects reared on artificial diet containing 8 µg ds*Kan*-CS-TPP (shown as Y=1).

4.3.6 Immune system priming through dsKan pre-injection

As previous studies have reported the upregulation of core RNAi pathway genes upon injection of non-species-specific dsRNA and a resultant increase in gene knockdown (Ye *et al*, 2018; Zhang *et al*, 2022), the effect of pre-injecting 5 µg ds*Kan* into *S. littoralis* 5th instar larvae prior to 10 µg species specific dsRNA was investigated, following the protocol detailed in Fan *et al* (2022a). Expression values are relative to insects injected with DEPC water followed by 10 µg ds*Kan*. Expression of *ace-1* was 0.78 and 0.94 when injected with water followed by ds*ace-1* or ds*ace-1*+ds*nAChR*, respectively, and 1.15, 0.93 and 1.21 when insects were injected with ds*Kan* followed by ds*Kan*, ds*ace-1* and ds*ace-1*+ds*nAChR*,respectively. Thus, pre-injection with kanamycin specific dsRNA did not significantly reduce the expression of *ace-1*. Expression of *nAChR* was 0.83 and 0.17 when injected with water followed by ds*ace-1* or ds*ace-1*+ds*nAChR*, respectively, and 0.78, 0.76 and 0.23 when insects were injected with ds*Kan* followed by ds*Kan*, ds*ace-1* and ds*ace-1*+ds*nAChR*, respectively. Thus, *nAChR* expression was significantly lower when ds*ace-1* and ds*nAChR* were injected in combination regardless of whether insects were pre-injected with DEPC water or ds*Kan*. Expression of *para* was 1.07 and 1.03 and 1.35 when injected with water followed by ds*para* or ds*Kan* followed by ds*Kan* or ds*para*, respectively. Thus, pre-injection with kanamycin specific dsRNA did not significantly reduce the expression of *para*.

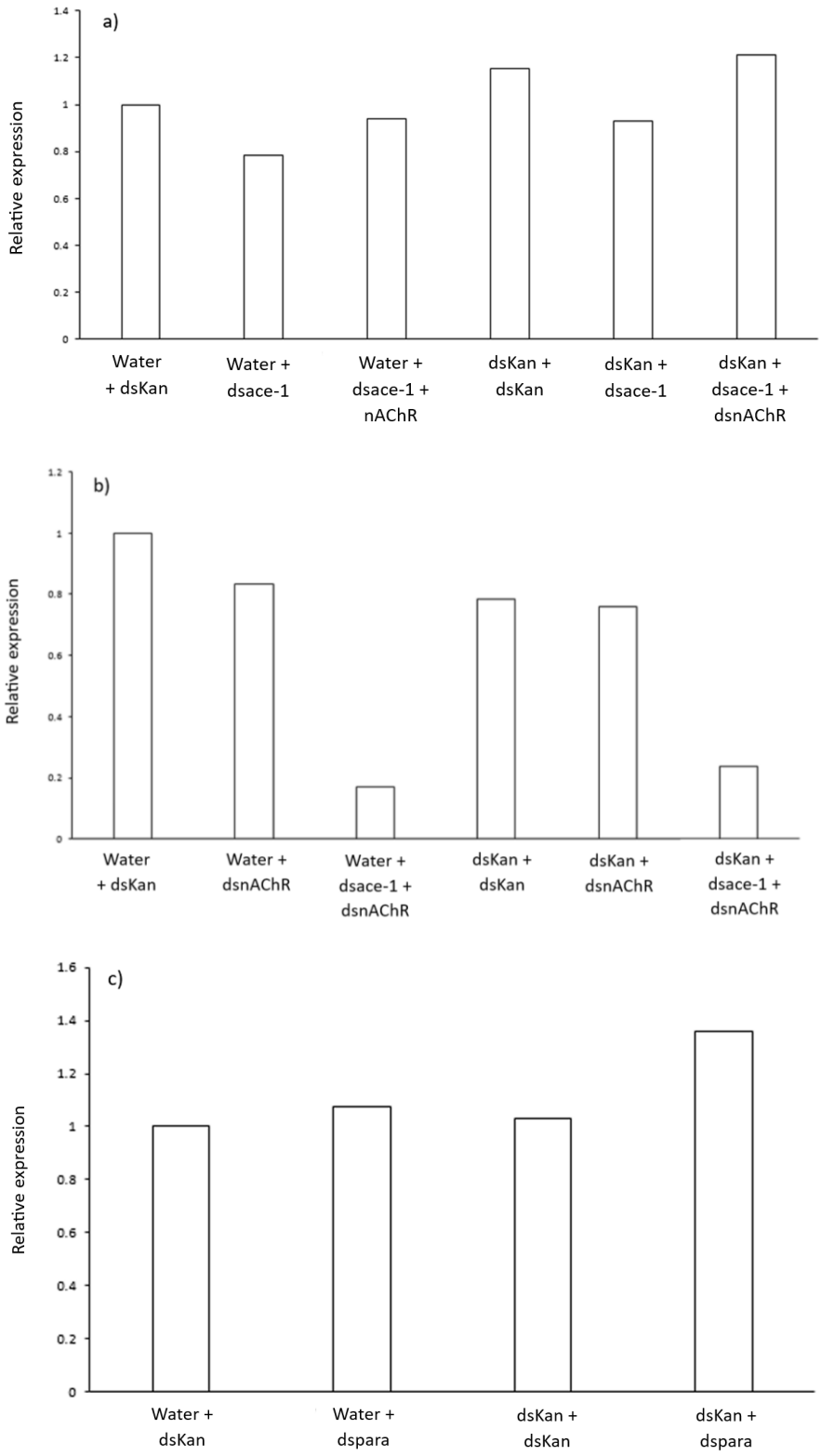

Figure 4.12 Mean fold change in *S. littoralis* gene expression after 5th instar larvae were injected with DEPC water or 5 µg ds*Kan* followed by 10 µg either ds*Kan*, ds*ace-1*, ds*nAChR* or ds*para* 2 h later. a) *ace-1* expression, b) *nAChR* expression, c) *para* expression. Gene expression was investigated via qPCR 24 h after the second injection and data was analysed using the $2^{-\Delta\Delta Ct}$ method with *RPL13A* as the endogenous reference gene and all values are relative to expression of insects injected with DEPC water followed by 10 µg ds*Kan* 2 h later (shown as Y=1).

4.4 Discussion

RNAi technology offers a novel means of pest control via the incorporation of dsRNA specific to an essential gene of a target pest into novel biopesticides. However, the efficacy of these compounds against lepidopteran insects is likely low as they are generally considered recalcitrant to RNAi (Terenius *et al*, 2011) due to poor intracellular transport of dsRNA (Shukla *et al*, 2016) and reduced dsRNA stability in the saliva, haemolymph and midgut due to the presence of nucleases and the alkaline pH of the lepidopteran midgut (Guan *et al*, 2018; Garbutt *et al* 2013, Fan *et al*, 2022b, Ioannidis *et al*, 2022). The stability of dsRNA in *S. littoralis* haemolymph and midgut juice was initially investigated as this was unknown at the time of writing. Subsequently, chitosan nanoparticles were used to enhance RNAi efficacy through increased dsRNA stability in these harsh environments. Furthermore, as Fan *et al* (2022a) reported increased upregulation of the RNAi machinery and increased RNAi efficacy through the pre-injection of non-species-specific dsRNA into *O. furnicalis*, this hypothesis was tested by pre-injecting dsRNA specific to the bacterial kanamycin resistance gene into *S. littoralis* prior to delivery of experimental dsRNA in an attempt to increase RNAi efficacy in this insect.

4.4.1 dsRNA is rapidly degraded in S. littoralis haemolymph and midgut juice

In the present study, 1 µg dsRNA was completely degraded in the pure haemolymph and midgut juice of 5th instar *S. littoralis* within 30 min yet remained stable after incubation in PBS at both pHs 6.8 and 8.8 (corresponding to the pH of the haemolymph and midgut juice, respectively). Thus, suggesting that nuclease degradation rather than pH is the major contributing factor in dsRNA instability in *S. littoralis*. Considering enzymes extracted from the whole bodies of *Locusta migratoria*, *P. americana* and *Zophobas atratus* display their highest nuclease activity at pH 9, *S. litura* at pH 11 (Peng *et al*, 2018) and *T. castaneum* at pH 8 (Peng *et al*, 2020) dsRNA degradation in a range of insects is alkaline-activated. Thus, the results of the present study suggest that, although the high pH of *S. littoralis* midgut juice may enhance the activity of midgut nucleases and thus contribute to dsRNA degradation in this environment, it is perhaps not sufficient to degrade dsRNA alone.

Degradation of dsRNA in the lepidopteran haemolymph is well documented. For example, Singh *et al* (2017) reported that 300 ng dsRNA specific to the eGFP gene was degraded within 1 h in the haemolymph of the lepidopterans *S. frugiperda*, *Heliothis virescens*, Virginia Tiger

moth (*Spilosoma virginica*), *Manduca sexta*, *Cydia pomonella*, Small purplish gray moth (*Iridopsis humaria*), Cabbage looper (*Trichoplusia ni*), Orange sulphur butterfly (*Colias eurytheme*) and saltmarsh caterpillar (*Estigmene acrea*) and a lack of band corresponding to siRNA confirmed dsRNA was not processed efficiently as a result. Furthermore, 200 ng *eGFP* specific dsRNA was degraded within 1 h in 5th instar *M. sexta* haemolymph (Garbutt *et al*, 2013). However, considering earlier time points were not assessed by Singh *et al* (2017), Garbutt *et al* (2013) nor in the present study, it is possible that dsRNA degraded more rapidly. For example, Fan *et al* (2022b) reported degradation of 1 *µg* of eGFP specific dsRNA in < 1 min in the haemolymph of 5th instar *O. furnicalis*. However, dsRNA specific to the *SeCHY2* gene remained intact for 1 h in 5th instar *Spodoptera exigua* haemolymph although this may have been due to the use of a higher dsRNA concentration (2 µg) (Vatanparast and Kim, 2017).

In the present study, dsRNA was degraded in 5th instar *S. littoralis* midgut juice within 30 min and diluting 1/100 did not increase stability. This contrasts with haemolymph degradation, whereby diluting 1/50 led to a prominent band representing dsRNA, suggesting that the midgut juice of *S. littoralis* has higher nuclease degrading capabilities compared to haemolymph. As mentioned previously, this may be the result of increased enzymatic activity under high pH conditions rather than the presence of more individual nucleases or higher concentrations of a particular nuclease. Comparatively higher midgut nuclease activity with respect to haemolymph degradation has been documented previously. For example, Liu *et al* (2016) reported that 2 µg dsRNA specific to the GFP gene was degraded completely within 20 minutes in *B. mori* midgut juice but remained intact for 3 h in haemolymph. Furthermore, dsRNA specific to the *SeCHY2* gene remained intact in 5th instar *S. exigua* haemolymph for 1 h but degraded within 40 min in 5th instar midgut juice (Vatanparast and Kim, 2017) and gut nuclease degradation was > 200-fold higher in comparison to haemolymph degradation in 6th instar *S. litura* Peng *et al* (2018). Additionally, this phenomenon is not restricted to Lepidoptera, with the gut nuclease degradation activity ~ 300,000 –fold, > 5,000 and > 250 –fold higher than haemolymph degradation in *Locusta migratoria*, *P. americana* and the superworm (*Zophobas atratus*), respectively (Peng *et al*, 2018). However, these results contrast with *O. furnicalis*, where dsRNA specific to the *eGFP* gene degraded in < 1 min in haemolymph extract but remained intact for ~ 5 min in midgut juice (Fan *et al*, 2022b).

Additionally, the nuclease degrading potential of the lepidopteran midgut juice can differ between life stages. Sharif *et al* (2022) reported stronger degradation in the midgut juice of later *H. armigera* instars compared to earlier, with 5 µg dsRNA specific to the *AchE* gene being degraded within 1 h in the midgut juice of larval instars 4 and 5 but remaining intact in larval instars 1, 2 and 3. This was reiterated by Vatanparast and Kim (2017) whereby *SeCHY2* specific dsRNA remained intact for 48 h, 12 h and 2 h in the midgut juice of *S. exigua* larval instars 1, 2 and 3, respectively, compared to 1 h and 40 min in larval instars 4 and 5, respectively. Additionally, Peng *et al* (2020) reported stronger nuclease activity in later *S. litura* larval instars, with significantly higher nuclease activity after 30 min in larval instars 3, 4, 5 and 6 compared to instars 1 and 2. However, contrasting with Vatanparast and Kim (2017) and Sharif *et al* (2022), dsRNA degrading activity did not increase with instar progression and was significantly higher in 3rd instar larvae compared to 4th, 5th and 6th, however this may be due to the use of crude enzyme extracts from whole insects (Peng *et al*, 2020).

Considering initiation of the RNAi process requires dsRNA to remain intact long enough to encounter DICER, the complete degradation of dsRNA in the midgut juice and haemolymph of *S. littoralis* reported in the present study suggests a possible reason for the general lack of effect on survival, pupation, emergence and gene expression upon feeding via artificial diet, gavage or 4th instar haemolymph injection reported in sections 3.4.8, 3.4.10 and 3.4.11. This correlation between dsRNA degradation and low RNAi efficiency has been reported previously (Singh *et al*, 2017; Cao *et al*, 2018; Prentice *et al*, 2017). For example, injecting dsRNA specific to the *chitinase* gene into the blattodean *P. americana,* in which haemolymph degradation activity is low (9%), reduced gene expression by 76% but in *Spodoptera litura*, which exhibits high haemolymph nuclease activity (95 %) gene expression was reduced by only 20% (Wang *et al*, 2016). Furthermore, the widely reported lower nuclease activity in the midgut juice of younger lepidopteran instars suggests a reason as to why the continuous feeding of dsRNA specific to the *S. littoralis ace-1*, *para*, *nAChR* and *NADPHcytP450r* genes to neonate larvae significantly reduced larval weight (section 3.49) and the greater nuclease activity in older instars may suggest why this did not translate into a significant effect on survival, pupation, pupae weight or gene expression. Additionally, the significant reduction in the consumption of artificial diet after 3rd instar larvae were injected with 8 *µg dsace-1* (section 3.4.12) compared to the lack of effect when 4th instar larvae were injected (3.4.11)

may suggest lower nuclease degrading activity in the haemolymph of younger larval instars. However, as differing dsRNA-degrading activity has not yet been reported across *S. littoralis* larval instars, this would require further investigation.

As insect saliva exhibits nuclease activity (Calvo and Ribeiro (2006); Caljon *et al* (2012); Lomate *et al* (2016)) further studies could investigate dsRNA stability in *S. littoralis* salivary secretions. However, with Guan *et al* (2018) reporting that ~ 50 % of dsRNA remained stable in the presence of the *O. furnicalis* salivary nuclease *REase* for 24 h but Fan *et al* (2022b) reporting complete degradation within < 1 min and ~ 5 min in *O. furnicalis* haemolymph and midgut juice, respectively, this may suggest that salivary nucleases may have comparatively lower degradation capabilities in *S. littoralis*. However, it is still possible that degradation by salivary nucleases may have been a contributing factor in the low RNAi response (reported in chapter 3) as a result of feeding dsRNA via an artificial diet. Furthermore, the significant knockdown of *NADPHcytP450r* upon ingestion of ds*NADPHcytP450r* by 4th instar larvae may infer differential stability of dsRNA molecules due to structural differences. For example, differences in GC content and thus the number of hydrogen bonds (Chan *et al*, 2009).

4.4.2 Chitosan nanocarriers Improve dsRNA stability in S. littoralis haemolymph and midgut juice

With the intention of protecting dsRNA from the harsh environments found in *S. littoralis* haemolymph and midgut juice, ds*ace-1*, ds*para*, ds*nAChR* and ds*NADPHcytP450r* were formulated with chitosan as a non-viral delivery vector. Although viral vectors can be used in gene therapy to efficiently transfect cells, (Lundstrom, 2020; Bulcha *et al* (2021)), non-viral vectors are generally easier to synthesise, generate a lower immune response, have an unrestricted plasmid size (Leong *et al* (1998); Kim *et al* (2005)) and offer enhanced safety (Kolliopoulou *et al* (2017). Non-viral chitosan-based carrier systems can be used to deliver dsRNA, siRNA, plasmid DNA, proteins and peptides (Dhandapani *et al*, 2019). The advantageous properties of chitosan such as low toxicity and immunogenicity, biodegradability and high positive charge which enables electrostatic interaction between negatively charged nucleic acids, thus ability to protect dsRNA from enzymatic degradation (Park *et al*, 2006) led to the choice of chitosan as the nanocarrier in the present study and TPP was used in an attempt to enhance dsRNA protection (Raja *et al* (2015); Dhandapani *et al*, (2019)) and uptake into cells (Katas *et al*, 2006).

In the present study, naked dsRNA was degraded completely in pure and 1/20 dilution of haemolymph. The complexation of dsRNA with CS-TPP improved its stability in each haemolymph dilution (1/20, 1/50 and 1/100) and importantly, also in pure haemolymph. This is demonstrated by fluorescence in the wells corresponding to dsRNA-CS-TPP which suggests efficient dsRNA take up by the nanoparticle and the inability of chitosan to move through the agarose gel due to its large size (Wang *et al*, 2023). This is supported by the lack of fluorescence in the wells when chitosan alone was electrophoresed. These results may suggest that direct injection of dsRNA-CS-TPP into the haemolymph of *S. littoralis* may increase RNAi efficacy. However, the increase in dsRNA stability when dsRNA-CS-TPP molecules were incubated in increasing haemolymph dilutions, suggests that protection in pure haemolymph is not complete. Nevertheless, this would require further investigation.

In the present study, naked dsRNA was completely degraded in pure midgut juice and 1/20, 1/50 and 1/100 dilutions after 30 min. Formulation with CS-TPP did not increase dsRNA stability in pure midgut juice, suggesting that nuclease activity in *S. littoralis* pure midgut juice is still too high to enable protection. This is supported by the increase in dsRNA stability offered by CS-TPP formulation with the lowest (1/20) midgut juice dilution. This contrasts with Dhandapani *et al* (2019) who reported that formulation with CS-TPP protected dsRNA from nuclease degradation in the pure midgut juice of *A. aegypti* for > 24 h, whereas naked dsRNA degraded within 1 h (Singh *et al*, 2017). However, as 1 µg dsRNA was degraded within 30 min in pure *S. littoralis* midgut juice in the present study and Singh *et al* (2017) reported degradation of 300 ng of dsRNA within 1 h in *A. aegypti* and did not investigate degradation at time points below 1 hr, it is possible that the nuclease degradation capacity of the *S. littoralis* midgut is greater than that *of A. aegypti*. This would support the idea that nuclease degradation in *S. littoralis* midgut juice is so great that it overcomes protection provided by formulating the specific dsRNA, used in the present study, with CS-TPP. Thus, as dsRNA in the field would encounter midgut juice in its pure form, it is unlikely that dsRNA formulation with CS-TPP would enable the possibility of a realistic RNAi based biopesticide for the control of *S. littoralis*.

The improvement of dsRNA stability when dsRNA-CS-TPP was incubated in even the lowest midgut juice dilution suggests that a concurrent approach whereby a gene encoding a gut nuclease is targeted alongside dsRNA-CS-TPP could improve RNAi efficacy in this species. For

example, Spit *et al* (2017) reported that the simultaneous knockdown of two nucleases in the gut of the coleopteran *Leptinotarsa decemlineata*, *Ld_dsRNase1* and *Ld_dsRNase2,* led to significantly higher mortality (~ 32 % increase) compared to controls upon ingesting 50 ng dsRNA specific to the *Ld_lethtgt gene,* which may suggest the efficacy of targeting similar genes in *S. littoralis.* However, when this experiment was repeated with *Schistocerca gregaria,* an insect which displays higher native gut nuclease activity and lower RNAi susceptibility than *L. decemlineata* (Wynant *et al*, 2014b), no improvement was reported (Spit *et al*, 2017). Thus, due to the high nuclease activity and low RNAi susceptibility exhibited by *S. littoralis,* this approach may be difficult. Furthermore, as pH can differ across lepidopteran gut compartments, with the midgut generally pH 7-12 (Chen *et al*, 2017) compared to relatively weak alkalinity of the foregut and hindgut (Zhang *et al*, 2022b), it is possible that nuclease activity may also differ across gut compartments. If this were the case and nuclease activity was higher in the foregut and hindgut of *S. littoralis* compared to the midgut, improving dsRNA stability in the midgut may not affect overall RNAi efficacy because dsRNA would encounter every gut compartment before reaching its site of action. However, this would require further research.

4.4.3 Does formulation of dsRNA with CS-TPP improve the efficacy of orally induced RNAi?

In the present study, feeding dsRNA-CS-TPP to both 4th instar and neonate *S. littoralis* larvae had no significant effect on mortality, pupation or adult emergence. This contrasts to Kolge *et al* (2023) who reported that feeding *H. armigera* dsRNA specific to the *lipase* and *chitinase* genes complexed with CS-TPP nanoparticles led to 2- and 2.7 – fold gene downregulation, respectively, suppressed enzyme activity (2- and 5.3 – fold respectively), reduced pupation and impaired adult emergence whilst also causing 80 and 100 % mortality, respectively. However, feeding naked dsRNA specific to both genes caused no significant effect on pupation and emergence. Although the authors also reported complete degradation of naked dsRNA in *H. armigera* pure midgut juice, complexation with CS-TPP offered strong dsRNA protection. Furthermore, Gurusamy *et al* (2020) reported that 2 µg dsGFP was fully degraded within 1 h in pure *S. frugiperda* midgut juice but complexation with chitosan greatly improved its stability. Furthermore, complexation of chitosan with the inihibitor of apoptosis (*iap*) gene led to increased mortality and decreased gene expression, pupal weight and egg hatching rates in *S. frugiperda* compared to naked ds*iap*. Thus, the ability for CS-TPP formulation to protect dsRNA in *H. armigera* and *S. frugiperda* may infer

lower nuclease activity in these species which may suggest that the nuclease activity in pure *S. littoralis* midgut juice is still strong enough to degrade dsRNA even when complexed with CS-TPP.

The significant knockdown of *nAChR* expression upon ds*nAChR*-CS-TPP ingestion by 4th instar larvae compared to the lack of effect from naked ds*nAChR* ingestion in the present study suggests that some protection is offered by CS-TPP nanoparticles. As stability was only investigated after 30 min incubation in midgut juice, it is possible that dsRNA remained intact for a shorter time, which may have been long enough to elicit a response. As the quantity of dsRNA nor the length of time dsRNA needs to persist as a fully intact molecule to enable an RNAi response in *S. littoralis* is unknown, it is possible that some ds*nAChR* was able to enter the gut epithelia and interact with the DICER enzyme, triggering the RNAi response. Furthermore, as the degradation of only 1 µg of dsRNA was investigated in the present study, yet larvae ingested 8 µg, it is possible that the higher concentration may persist for long enough to allow efficient RNAi. This may also provide a reason for the significant reduction in *NADPHcytP450r* expression upon ingestion of 8 µg naked ds*NADPHcytP450r* and ds*NADPHcytP450r*-CS-TPP compared to controls. Additionally, the lack of significant knockdown of one gene (ie *ace-1*) compared to the significant knockdown of *nAChR* or *NADPHcytP450r* is not surprising as variable RNAi response between genes within a specific insect has been reported many times previously (Baum *et al*, 2007; Das *et al*, 2015; Sandal *et al*, 2023 etc).

The lack of phenotypic response observed despite gene knockdown is likely attributed to the transient nature of RNAi in Lepidoptera, whereby transcript levels can rebound (Zhang *et al*, 2015). As the length of time needed for gene knockdown to persist in order to elicit a response on *S. littoralis* phenotype is unknown, it is possible that the effect did not persist for a sufficient time and investigating knockdown across various time points may be useful. Also, as expression was only reduced and not eradicated completely, it is possible that complete knockdown may be necessary to elicit a phenotypic response in this species. As mentioned previously, comparatively lower nuclease activity in the haemolymph has been reported in younger lepidopteran instars. Thus, the lack of significant reduction in *nAChR* (or any other) gene expression upon ingestion of dsRNA-CS-TPP by neonate compared to 4th instar larvae is perhaps surprising but as nuclease activity has not been investigated across *S.*

littoralis instars, this would require further investigation. Additionally, it would be useful to investigate the efficacy of CS-TPP nanoparticles in enhancing dsRNA stability in the midgut juice and haemolymph of younger larval instars.

The lack of effect on survival, pupation or emergence upon ingestion of chitosan (alone) in the present study suggests a lack of insecticidal effect in this species at the concentration used. This is important as insecticidal activity of nanoparticles has been reported in many insect species (Moorthi *et al*, 2015). For example, sublethal concentrations of silver and zinc oxide nanoparticles (10 mg/ml) both significantly reduced *S. littoralis* larval and pupal weight and zinc oxide nanoparticles significantly increased the length of the larval period to 18.4 days compared to 16.8 for control insects (Ibrahim and Ali, 2018). This could infer that dsRNA complexation with certain nanoparticles may be problematic due to unintended effects on non-target insects, humans and the environment (Ibrahim and Ali, 2018). Thus, the concentration of CS-TPP used in the present study is safe against *S. littoralis* whilst enhancing, to some degree, the stability of dsRNA in the haemolymph and midgut juice but further research regarding the safety of CS-TPP against other NTOs is necessary before dsRNA-CS-TPP could be applied in the field.

Many nanoparticles can enhance RNAi efficacy in Lepidopteran insects. For example, Christiaens *et al* (2018a) reported that although naked dsRNA specific to the *GFP* gene was degraded in *S. exigua* midgut juice within 2 h, complexation with guanidine-containing polymers protected dsRNA for > 30 h. Additionally, targeting the *chitin synthase B* gene in *S. exigua* led to 53 % mortality when dsRNA was complexed with guanylated polymers compared to only 16 % with naked dsRNA. Furthermore, Sandal *et al* (2023) reported that dsRNA specific to the *GFP* gene was degraded within 5 h in the Lepidopteran *Earias vittella* and feeding naked dsRNA specific to six species-specific genes had no significant effect on mortality, phenotype or gene expression. However, dsRNA complexation with three different nanoparticles; chitosan, carbon quantum dots (CQD) and Lipofectamine led to significant gene knockdown. For example, dsCadherin–Chitosan and dsCadherin–CQD significantly reduced gene expression by 78.6% and 93.6% compared to naked dsCadherin and dsAminopeptidase–Chitosan, dsAminopeptidase–CQD, and dsAminopeptidase–lipofectamine reduced gene expression by 83.9%, 83.8%, and 81.3% respectively, compared to naked dsRNA. Wang *et al* (2020) also compared the efficacy of chitosan, CDQ and

lipofectamine nanoparticles in their ability to protect dsRNA from degradation in the pure midgut juice of *Chilo suppressalis*. Although naked dsGFP degraded by 83 % within 10 min in pure midgut juice and was barely detectable after 60 min, 75%, 71%, and 70% dsRNA remained after 60 min when complexed with chitosan, CQD, and lipofectamine, respectively. Furthermore, feeding naked dsRNA specific to the *glyceraldehyde-3-phosphate dehydrogenase gene* (*G3PDH)* to *C. suppressalis* did not trigger significant mRNA reduction and there was no significant effect on phenotype or mortality. Contrastingly, complexation of ds*G3PDh* with Chitosan, CQD and lipofectamine significantly reduced expression by 57 %, 43 % and 69 %, respectively, and 55%, 70%, and 32% of insects died after ingesting dsG3PDH complexed with chitosan, CQD, and lipofectamine (respectively).

In a similar study, Das *et al* (2015) compared the efficacy of chitosan, CQDs and silica nanoparticles (ASNPs) in delivering dsRNA specific to the *SNF7* and steroid receptor coactivator (*SRC*) genes to *A. aegypti*. Feeding CQD complexed with *dsSNF7* and *dsSRC* reduced gene expression by 40 % and 71 %, respectively, complexation with chitosan reduced gene expression by 62 % and 10 % but feeding with dsRNA-ASNP complexes made no significant difference to gene expression. Furthermore, although nanoparticles themselves exhibited no toxicity towards *A. aegypti*, complexation of dsSNF7 and dsSRC with chitosan caused 47 % and 27 % mortality, respectively, and 53 % and 75 % when complexed with CQD, respectively, but complexation with ASNP was ineffective in causing mortality. The authors concluded that the variable pH gradients across the midgut, in which the anterior is neutral, between the oesophagus and the junction of the ceca is moderately alkaline and behind the cecal junction is strongly alkaline (Dadd *et al*, 1975), is likely a major factor in the variable efficacy of the three nanoparticles. The ASNP-dsRNA complex was unstable both at buffer pH 4 and 10 and dsRNA began disintegrating from ASNP in < 48 h. dsRNA also disintegrated from chitosan at pH 7 and pH10 after 72 h, however, CQDs retained 100% dsRNA after 72 h incubation in all tested pH conditions. Considering pH can vary across lepidopteran gut compartments (Chen *et al*, 2017; Zhang *et al*, 2022b), this may provide a reason as to the lack of significant effect on phenotype and mortality of *S. littoralis* through dsRNA-CS-TPP administration in the present study, whilst perhaps highlighting the efficacy of CDQ nanoparticles in this species.

Perhaps another reason for the overall general lack of RNAi response through the use of dsRNA-CS-TPP reported in the present study may be due to accumulation of dsRNA in the

endosomes (Gilleron *et al*, 2013). Lepidopteran insect cells may endocytose dsRNA by clathrin-mediated endocytosis or SID-1 receptors (Shukla *et al*, 2016). For example, the inefficient processing of dsRNA to siRNA in *H. virescens* was possibly due to accumulation of dsRNA in the endosomal compartments due to less efficient endosomal escape (Shukla *et al*, 2016). If dsRNA-nanoparticle complexes cannot escape the endosome they are degraded in lysosomes (Di Guglielmo *et al*, 2003) thus cannot reach their site of action in the cytoplasm. Considering the endosomal escaping abilities of chitosan nanoparticles are limited in comparison to some other nanoparticles (Ragelle *et al*, 2013) only small amounts of dsRNA can be released into the cytoplasm (Wang *et al*, 2020) which may not be enough to either trigger the RNAi response or induce a strong enough response to impact gene expression, phenotypic changes or mortality. As the buffering capacity of CQD nanoparticles can cause osmotic swelling leading to endosomal rupture (Akinc *et al*, 2005), complexes are more likely to be released into the cytoplasm. Therefore, future work could consider the efficacy of CQD nanoparticles in inducing RNAi in *S. littoralis*. However, although CQDs are generally considered non-toxic to humans and mammals, cytotoxicity may occur depending on concentration or surface modifications (Havrdova *et al*, 2016). Considering naked CQDs cannot conjugate with dsRNA without surface functionalization (Das *et al*, 2015), extensive biosafety analyses would need to be carried out to determine safety of these nanoparticles before real-world exposure. This is further emphasised by the ability for CQDs to inhibit bacteria and fungi (Kostov *et al*, 2022) which may infer possible effects on non-target beneficial organisms.

Although CQDs efficiently protected dsRNA and enabled an RNAi response in *E. vittella*, *C. suppressalis* and *A. aegypti*, their efficacy may be limited in *S. littoralis* due to comparatively higher nuclease activity considering naked dsRNA was degraded within 30 min in *S. littoralis* pure midgut juice and CS-TPP did not improve stability. Contrastingly, naked dsRNA remained stable in *E. vittella*, *C. suppressalis* and *A. aegypti* midgut juice for > 3 h, ~ 60 min and > 30 min, respectively (Sandal *et al*, 2023; Wang *et al*, 2020, Giesbrecht *et al*, 2020) and chitosan formulation enabled dsRNA to persist for > 60 min and > 24 h in *C. suppressalis* and *A. aegypti*, respectively (Wang *et al*, 2020; Dhandapani *et al*, 2019). Nevertheless, this would be a valuable area for future investigations.

4.4.4 Immune system priming through pre-injection of non-species-specific dsRNA

As a further strategy to improve RNAi efficacy in *S. littoralis*, dsRNA specific to a bacterial kanamycin resistance gene was injected into 5th instar larvae prior to a secondary injection with *S. littoralis* specific dsRNA. As well as gene regulation, RNAi plays an important role in viral defence (Obbard *et al*, 2009) and exposure to a virus can increase the activity of components of the RNAi machinery via gene upregulation (Xie *et al*, 2017; Marques *et al*, 2013). Pre-exposure to dsRNA non-specific to an organism can also trigger up-regulation of core genes in the RNAi pathway in certain insect species, thus priming the immune response. For example, Guo *et al* (2015) reported that pre-ingestion of *dseGFP* led to significantly increased expression of the *Dcr2a*, *Dcr2b*, *Ago2a* and *dAgo2b* genes in 1st, 2nd, 3rd and 4th instar *L. decemlineata*. Resultantly, subsequent ingestion of dsRNA specific to the *dSAHase L. decemlineata* gene significantly decreased survival of 4th instar larvae compared to those not pre-exposed to ds*eGFP*. Furthermore, Garbutt and Reynolds (2012) reported significant upregulation of the *dicer-2* and *ago2* genes in *Manduca sexta* upon injection with ds*eGFP*, which could be prolonged via multiple doses, although other core RNAi pathway genes were not upregulated. Similarly, Ye *et al* (2019) reported that pre-injection of 600 ng ds*eGFP* into *A. pisum* prior to injection with 600 ng dsRNA specific to the *hunchback* gene led to significant silencing (31 %) but injection of ds*hunchback* alone at this dose had no effect. Furthermore, injection with a larger dose (600 ng) of ds*eGFP* significantly reduced gene expression (81 %) compared to the lower dose.

In the present study, pre-injection of ds*Kan* did not significantly reduce the expression of *ace-1*, *nAChR* or *para*. The lack of effect in *S. littoralis* compared to *A. pisum* and *M. sexta* upon direct dsRNA injection is likely the result of the higher nuclease activity in *S. littoralis* haemolymph, whereby dsRNA is completely degraded within 30 min, but remains intact to some degree for ~ 1 h and 2 h in *A. pisum* and *M. sexta* haemolymph, respectively, (Christiaens *et al*, 2014; Garbutt *et al*, 2013). Furthermore, the improved RNAi success reported by Guo *et al* (2015) upon pre-ingestion of dsGFP in *L. decemlineata* is likely due to the low nuclease activity in the midgut juice of this insect whereby only slight degradation of dsRNA occurs after 1 h and dsRNA is not fully degraded until 6 h (Prentice *et al*, 2017). This is supported by the high amenability of *L. decemlineata* to RNAi (He *et al*, 2020c; Mei-qi *et al*, 2020). However, considering 1 µg dsGFP specific dsRNA is degraded in < 1 min in the haemolymph of *O. furnicalis* (Fan *et al*, 2022b) it is perhaps surprising that ds*GFP* pre-injection was successful in priming the immune system and improving RNAi efficacy in this

insect (Fan *et al*, 2022a), although this may be due to the degradation of only 1 µg dsRNA being investigated but 5 µg dsRNA being injected. Also, considering dsRNA stability differs depending on dsRNA length in *O. furnicalis* (Cooper *et al*, 2020) and considering the length of the ds*GFP* molecule used by Fan *et al* (2022a) was not mentioned, it is possible that the dsRNA used in this instance was more stable than previously reported by Fan *et al* (2022b). Overall, it is probable that the high nuclease activity present in the haemolymph of *S. littoralis* is causing degradation of ds*Kan* before it can lead to the upregulation of core RNAi machinery genes. Nevertheless, the significant knockdown of *nAChR* expression upon injection of ds*ace-1*+ds*nAChR* in combination suggests that *S. littoralis* is amenable to RNAi via direct haemolymph injection but RNAi response is highly variable depending on target gene. This perhaps suggests that dsRNA can persist for long enough to reach the RNAi machinery and reiterates the idea that assessing degradation at time points less than 30 min may be beneficial. It may also suggest that differential composition of each dsRNA molecule may affect i) their stability and ii) their ability to be processed via the RNAi machinery. Comparatively, gavage feeding 5th instar *S. littoralis* with 8 µg ds*ace-1*+*nAChR* had no significant effect on *nAChR* expression (section 3.4.10) which may be due to the evasion of salivary and midgut nucleases offered by haemolymph injection. This is perhaps supported by the stronger degradative ability of *S. littoralis* midgut compared to haemolymph reported in the present study.

4.5 Conclusion

The major aim of the present study was to assess the stability of dsRNA in *S. littoralis* haemolymph and midgut juice prior to investigating the efficacy of a CS-TPP nanoparticle and immune system priming (via pre-injection of non-species-specific dsRNA) in improving RNAi susceptibility. The results indicate both *S. littoralis* haemolymph and midgut juice possess a strong degradative capacity. However, increased stability of naked dsRNA upon haemolymph dilution and the ability of complexation with CS-TPP to increase the stability of dsRNA even in pure haemolymph compared to the lack of increased stability in *S. littoralis* midgut juice suggests that the midgut exhibits a comparatively stronger dsRNA degradation capacity. Additionally, as dsRNA remained stable when incubated in a high alkaline pH buffer, mimicking that of *S. littoralis* midgut but without the presence of nucleases, nuclease degradation is likely the major contributor to dsRNA instability in this species, although the alkaline pH of the midgut juice may increase nuclease activity.

Overall, nuclease degradation is likely a major reason for the general lack of RNAi response upon feeding via an artificial diet and gavage compared to some significant results upon direct dsRNA injection into the haemolymph (chapters 3 and 4). Additionally, considering dsRNA was degraded in 1/100 dilution of midgut juice but remained stable to some degree at 1/20 dilution upon formulation with CS-TPP, this may suggest the efficacy of a concurrent approach, whereby midgut nucleases are targeted alongside the use of dsRNA-CS-TPP. Overall, the successful knockdown of *nAChR* and *NADPHcytP450r* expression suggests that RNAi is possible in this species, although methods to enhance dsRNA stability within the harsh conditions of *S. littoralis* midgut juice are necessary to enable the possibility of RNAi-based biopesticides targeting this species.

Chapter 5. Determining the biosafety of four dsRNA molecules targeting *Spodoptera littoralis* against a range of non-target organisms

5.1 Abstract

Chemical insecticides are commonly used in agricultural systems to control pest insects. However, the threat they pose to non-target organisms including humans and beneficial pollinator insects has led to the ban of many of these compounds, meaning that alterative, environmentally friendly pest control strategies are necessary. RNA interference (RNAi) is a natural gene regulation mechanism which can be triggered artificially in a target organism via the introduction of dsRNA. Consequently, its incorporation into biopesticides offers the targeting of single genes in a specific organism which can lead to pest mortality. Thus, these novel compounds have the potential to overcome the safety concerns associated with commercial insecticides. Despite the potential high specificity, unintended effects on non-target organisms (NTOs) are possible, especially in species with high sequence homology to the designed dsRNA. Thus, rigorous testing of dsRNA-based biopesticides is essential prior to field implementation. The present study evaluated the biosafety of dsRNA targeted to the *ace-1*, *para*, *nAChR*, *NADPHcytP450r* and *ace-1+nAChR* genes in *S. littoralis*, firstly via a toxicology test against the important pollinator species *Bombus terrestris* and then an *in silico* bioinformatics approach against a range of NTOs to identify areas of high sequence similarity that could indicate possible adverse effects. Survival was not significantly reduced when *B. terrestris* was exposed to 50% sucrose solution containing dsRNA targeted to any *S. littoralis* specific gene and was significantly higher than when exposed to the pyrethroid insecticide, esfenvalerate. However, the degradation in pure *B. terrestris* midgut juice could reduce the impact of using this species as a representative for RNAi toxicity assays. Bioinformatics analyses identified homology between *S. littoralis* specific siRNAs and the corresponding gene in a range of, predominantly lepidopteran, NTOs. Although this could indicate the potential for adverse effects, many factors influence the risk that dsRNA poses to NTOs. Overall, this work provides evidence regarding the biosafety of dsRNA molecules targeting *S. littoralis* against a range of NTOs.

5.2 Introduction

Although chemical insecticides can double crop yield (Washuck *et al*, 2022) many harm the environment and non-target organisms (NTOs) including humans (Nicolopoulou-Stamati *et al*, 2016). In 2008, children represented ~45% of all reports to US poison control centres due to pesticide exposure (Roberts *et al*, 2012) and early-life exposure to organophosphates effects neurodevelopment and behaviour whilst increasing leukaemia and brain tumour risk (Zahm and Ward, 1998). Furthermore, gestational exposure can reduce birth weight (Longnecker *et al*, 2001) and cause birth defects (Addissie *et al*, 2020).

Chemical insecticides also threaten non-target beneficial organisms or those who benefit humans either directly or indirectly. For example, dung beetles, who provide vital ecosystem services by maintaining soil quality, promoting plant growth and aiding in seed dispersal (Manning *et al*, 2021), and stoneflies, who positively impact freshwater stream health (MDEP, 2023) and are used in biomonitoring (Zweig and Rabeni, 2001) are both highly sensitive to chemical insecticides (MLA, 2020, McVeigh, 2021). Additionally, the ladybird, *Hippodamia variegata*, an important natural predator of various aphid pest species, is sensitive to the neonicotinoid imidacloprid, with even sublethal doses significantly reducing hatching and emergence rates (Wumuerhan *et al*, 2020).

Pollinators enhance the seed, fruit and vegetable production of many of the world's leading food crops (Klein *et al*, 2007) thus are often considered one of the most important beneficial organisms (Rader *et al* 2015). Animal pollinators such as birds, bats and insects (Potts *et al*, 2016) account for yield increases of an estimated $235–577 billion annually (Potts *et al*, 2016). Insect pollination specifically is relied upon, to some degree, by 75% of food crops (Klein *et al*, 2007) and is responsible for almost 10% of the economic value of food consumed by humans (Gallai *et al*, 2009). The most important pollinator species belong to the Hymenoptera, with bees aiding 90% of leading crop types (Klein *et al*, 2007).

Bee populations are rapidly declining due to invasive species competition (Stout and Morales, 2009), climate change (Vanbergen, 2013), pathogens (Doublet *et al*, 2015) and parasites (Goulson *et al*, 2015) but chemical insecticides are a major threat (Camp and Lehmann, 2021). Neonicotinoids have been used extensively in more than 120 countries to protect crops such as maize, potato and rice (EFSA, 2012) thus are one of the most broadly

studied insecticides (Pisa *et al*, 2015). Their mode of action, to mimic the neurotransmitter acetylcholine (ACh) by binding to nicotinic acetylcholine receptors (*nAChR*s) on the synaptic membrane, (Tomizawa and Casida, 2005) is advantageous as it renders them less toxic to mammals and other vertebrates (Volvodić and Bažok, 2021) who have differences in *nAChR* binding sites and fewer *nAChR*s with a high affinity to neonicotinoids (Liu *et al*, 2010). Furthermore, their systemic nature extends their effects to all plant organs and tissues regardless of application method (Cabezas and Farinós, 2022) which allows their use as seed treatments, often regarded one of the safest and cheapest means of young seed/plant protection from both soil and above-ground pests (Volvodić and Bažok, 2021). However, this systemicity also enhances the risk posed by neonicotinoids to non-target invertebrates, by increasing the number of pathways by which they are exposed (Cabezas and Farinós, 2022).

Firstly, dust generated by drilling insecticide treated seeds can directly harm beneficial pollinators (Girolami *et al*, 2012) whilst leaching into surrounding soils and waterways can affect below ground NTOs (Antoine and Forest, 2020). Pelosi *et al* (2021) reported insecticide contamination in 180 soil samples and 92 % of earthworms in both seed treated and untreated habitats. Additionally, leached neonicotinoids can be absorbed by plant and tree roots (Bonmatin *et al*, 2015) then translocated to various tissues, (Aajoud *et al*, 2008) hence can contaminate pollen and nectar (Stoner and Eitzer, 2012) with Friedle *et al* (2021), reporting a mixture of up to 13 different insecticides in 90 % of pollen samples. Beneficial insects may also be exposed to insecticides via contaminated guttation droplets (Mörtl *et al*, 2017), foliar application (Gerecke *et al*, 2002) or through trophic levels, with Wumuerhan *et al* (2020) reporting a 44 % decrease in *H. variegata* predatory behaviour when exposed to imidacloprid directly, compared to a 56 % decrease when feeding on imidacloprid poisoned *A. gossypii*. High environmental persistence further exacerbates NTO exposure risk. Bonmatin *et al* (2005) reported that imidacloprid persisted in 97 % of soils two years post-sowing although degradation rate depends upon soil type, moisture content, temperature and pH (Bonmatin *et al*, 2015).

The honeybee, *A. mellifera,* is particularly sensitive to neonicotinoids likely due to its genome presenting only half of the genes encoding enzymes involved in the detoxification of xenobiotics, e.g., cytochrome P450s (*P450s*), carboxyl/cholinesterases (CCEs) and glutathione-s-transferases (GSTs) (Claudianos *et al*, 2006), compared to other insect species.

As, *A. mellifera* plays an important role in pollinating a range of crops and is highly tractable (Pisa *et al*, 2015), most insecticide toxicity studies have focussed solely on this insect (Barbosa *et al* 2015), which is also used as a representative for pesticide regulation decisions and risk assessments in both the USA and Europe (Camp and Lehmann, 2021). However, their suitability for such has recently been questioned, as many features of their life history differ considerably from other pollinators such as bumblebees (Gradish *et al*, 2019), leading to recent studies focussing on a wider range of invertebrates (Pisa *et al*, 2015).

The buff-tailed bumblebee, *Bombus terrestris*, is an important pollinator species. Its ability to survive and forage in various environmental conditions (Hart *et al*, 2021) and to utilise buzz pollination (Hart *et al*, 2021), whereby flowers are incidentally fertilised via bee vibrations which gather and remove pollen (Vallejo-MarÍn, 2018), enables it to pollinate crops which *A. mellifera* cannot, such as tomatoes and blueberries. Resultantly, although *B. terrestris* is native mainly to Europe, it has been used as a valuable pollinator of greenhouses throughout the world (Inoue *et al*, 2008), with 70,000 colonies used in Japanese greenhouses in 2004 alone (Kunitake and Goka, 2006). Consequently, various toxicity testing studies now include *B. terrestris* (Reid *et al*, 2020, Cabezas and Farinós, 2022) and although it is generally less sensitive to a range of insecticides compared to *A. mellifera* (Arena and Sgolastra, 2014), it is highly susceptible to the neonicotinoid imidacloprid and the pyrethroid deltamethrin (Reid *et al*, 2020).

Due to the threat posed by neonicotinoids, many have been banned for outdoor use in the EU (Pisa *et al*, 2015) and new, more environmentally friendly, pest control methods are required. RNA interference (RNAi) is a natural gene regulation mechanism which can be triggered artificially in a target organism via the introduction of dsRNA. Consequently, dsRNA-based biopesticides could overcome the safety concerns associated with commercial insecticides as they target a single gene within a chosen organism (Whyard *et al*, 2009) with the potential to reduce gene expression, induce phenotypic changes and even cause mortality (Vogel *et al*, 2019). However, unintended effects on NTOs are possible (Christiaens *et al*, 2020b), especially in those species with high sequence homology to the designed dsRNA (Fletcher *et al*, 2020). Thus, rigorous testing of dsRNA-based biopesticides is essential prior to field implementation.

Two main methods are available to determine, and aim to minimise, the chance of unintended gene silencing in NTOs. Toxicology tests assess the effects of dsRNA exposure on a model organism and an *in silico* bioinformatics approach can enable the identification, and thus avoidance, of similar sequences between dsRNA and the corresponding gene of an NTO, thus minimising the potential for off-target binding (Christiaens *et al*, 2018b). Here, the potential risks associated with dsRNA specific to the *ace-1* (ds*ace-1*), *para* (ds*para*), *nAChR* (ds*nAChR*), *NADPHcytP450r* (ds*NADPHcytP450r*) and *ace-1+nAChR* (ds*ace-1+nAChR*) genes targeting *Spodoptera littoralis* will be investigated. An *in vivo* toxicity assay will determine biosafety against the non-target model insect *B. terrestris* and bioinformatics analyses will assess sequence similarity between *S. littoralis* gene specific dsRNA and the corresponding gene in a plethora of NTOs, in an attempt to identify possible areas of concern. Although it was previously considered that 21-25 bp of the designed dsRNA must match the target mRNA to trigger the RNAi machinery (Elbashir *et al*, 2001 and Tijsterman and Plasterk, 2004), Santos *et al* (2019) reported that the length of siRNA produced by DICER is species specific, and predominantly 18-26 nt (Santos *et al*, 2019). Consequently, it is possible that the homology required, between designed siRNA and the corresponding gene in a given NTO, to trigger successful RNAi could also differ between species (Arpaia *et al*, 2020), thus the similarities between all possible component siRNAs and the corresponding gene in NTOs will be assessed.

5.3 Results

5.3.1 Effect of S. littoralis gene specific dsRNA on B. terrestris survival

To determine the effect of *S. littoralis* gene specific dsRNA on *B. terrestris* survival, adults were reared on 50% sucrose (diluted in DEPC water) containing 8 µg *dsace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* or dsace1+*nAChR* (4 µg of both). Controls were 50% sucrose ± esfenvalerate or 8 µg dsRNA specific to the microbial kanamycin resistance gene (ds*Kan*). *B. terrestris* reared on 50% sucrose containing 25 µg esfenvalerate suffered > 70% mortality by day 1 and 100% mortality by day 4 (Figure 5.1) compared to ~ 30% mortality after 6 days for bees reared on 50% sucrose and sucrose containing ds*Kan*. Furthermore, when reared on 50% sucrose with *S. littoralis* gene specific dsRNA incorporated, *B. terrestris* mortality was 33% for ds*ace-1*, 33% for ds*para*, 37% for ds*nAChR*, 37% for ds*NADPHcytP450r* and 41% for *ace-1*+ *nAChR* by day 6. Kaplan-Meier log-rank survival analysis identified that survival of *B. terrestris* adults reared on 50% sucrose containing esfenvalerate was reduced significantly ($P < 0.001$) compared to all other diets by day 1 and this remained the case throughout the trial ($P=0.000$; ANOVA, Tukey post-hoc). Survival was not significantly reduced when *B. terrestris* was reared on 50% sucrose containing any *S. littoralis* specific dsRNA or ds*Kan* compared to 50% sucrose alone by day 6 ($P=0.000$).

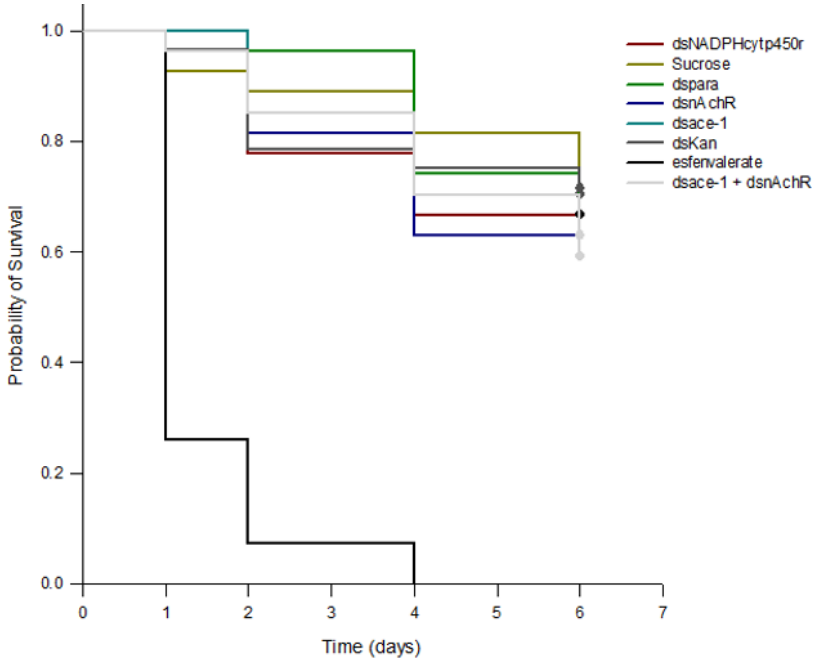

Figure 5.1 Survival of *Bombus terrestris* adults reared on 50% sucrose (diluted in DEPC water), containing 8 µg dsRNA specific to the *S. littoralis ace-1*, *para*, *nAChR*, *NADPHcytP450r* genes or 4 µg of *ace-1* + *nAChR* in combination. Controls were insects fed 50% sucrose ± 25 µg esfenvalerate pesticide or dsRNA specific to the bacterial kanamycin resistance gene (ds*Kan*) (27 adults/treatment). *B. terrestris* were provided with treated 50% sucrose solution for 2 consecutive days, then were transferred to 50% sucrose for the remainder of the trial.

5.3.2 dsRNA stability in B. terrestris midgut juice

The stability of dsRNA in *B. terrestris* midgut juice was investigated via agarose gel electrophoresis and band intensity was estimated using ImageJ. When 1 µg ds*ace-1* was incubated in PBS pH 8.8 and DEPC treated water, the expected 485 bp band corresponding to ds*ace-1* was present but was not when incubated in pure RNAse (Figure 5.2). No nucleic acids were present when pure midgut juice (alone) was electrophoresed. The expected 485 bp band corresponding to ds*ace-1* was not present after incubation for 30 min in pure midgut juice but appeared when midgut juice was diluted and band intensity increased with increasing dilutions. A 1/20 midgut juice dilution led to ~ 57% increase in band intensity compared to 1/10 dilution, a 1/50 dilution led to ~ 2% band intensity increase compared to 1/20 dilution and a 1/100 dilution led to ~ 9% band intensity increase compared to 1/50 dilution. The highest band intensity corresponded to dsRNA incubated in PBS pH 8.8 and was ~65 %, ~18 %, ~16% and ~7% brighter than bands corresponding to dsRNA incubated in 1/10, 1/20, 1/50 and 1/100 midgut juice dilutions, respectively).

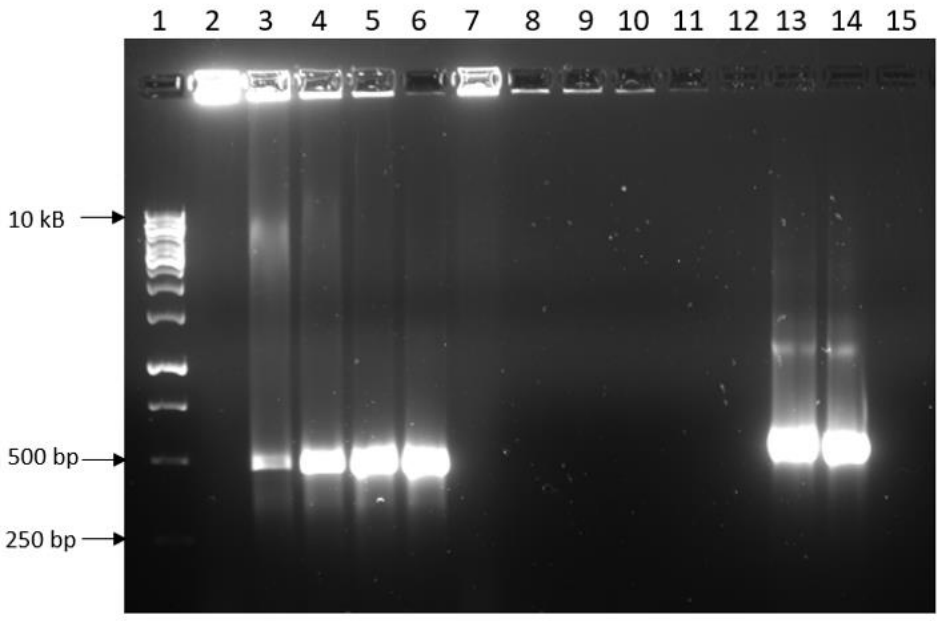

Figure 5.2 Stability of dsRNA in *B. terrestris* midgut juice. 1 µg of *S. littoralis ace-1* specific dsRNA (ds*ace-1*) was incubated at 37° for 30 min in extracted *B. terrestris* pure midgut juice and various dilutions (in PBS pH 8.8) prior to 1.5% (w/v) agarose gel electrophoresis (120 v, 50 min) with EtBr staining. Lane 1: Generuler 1Kb ladder, lanes 2, 3, 4, 5 and 6: 1 µg ds*ace-1* incubated in pure midgut juice and 1/10, 1/20, 1/50 and 1/100 midgut juice dilutions, respectively, lanes 7, 8, 9, 10 and 11: pure midgut juice (alone) and 1/10, 1/20, 1/50 and 1/100 midgut juice dilutions, respectively, 12: Empty, 13: Control 1 – PBS pH 8.8 + 1 µg ds*ace-1*, 14: Control 2 – DEPC water + 1 µg ds*ace-1*, 15: Control 3 – RNAse + 1 µg ds*ace-1*.

5.3.3 Bioinformatics analyses of dsRNA and siRNA sequences against B. terrestris

An NCBI BLAST search concluded that there were no significant similarities between the full-length sequences of ds*ace-1*, *dspara*, *dsnAChR*, *dsNADPHcytP450r* or ds*Kan* and the genome of *B. terrestris* (results not shown). Additionally, after *in silico* cleavage of each dsRNA molecule into their component siRNAs (16-27 mer), a viroBlast search concluded that there were no matches between any length ds*Kan* specific siRNA with *B. terrestris ace-1*, *para*, *nAChR* or *NADPHcytP450r* (Table 5.1) nor between *S. littoralis* ds*ace-1* and ds*nAChR* with the corresponding genes in *B. terrestris* (Table 5.2). However, there were six 16-mer, four 17-mer, two 18-mer and one 19-mer match(es) between the *S. littoralis* and *B. terrestris* specific *para* gene sequences. In addition, there was a single 16-mer match between the *S. littoralis* and *B. terrestris NADPHcytP450r* gene sequences.

Table 5.1 Similarity between siRNAs specific to ds*Kan* and specific genes in *Bombus terrestris*. The 470 bp ds*Kan* molecule was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the entire sequence length of specific *B. terrestris* genes.

		Number of exact n-mer matches											
Species	Gene	16	17	18	19	20	21	22	23	24	25	26	27
Bombus terrestris	*ace-1*	0	0	0	0	0	0	0	0	0	0	0	0
Bombus terrestris	*para*	0	0	0	0	0	0	0	0	0	0	0	0
Bombus terrestris	*NaChR*	0	0	0	0	0	0	0	0	0	0	0	0
Bombus terrestris	*NADPHcytp450r*	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.2 Similarity between siRNAs specific to *S. littoralis* ds*ace-1*, ds*para*, ds*nAChR* and ds*NADPHcytP450r* and corresponding *B. terrestris* genes. 485, 499, 486 and 473 bp dsRNAs specific to the *S. littoralis ace-1*, *para*, *nAChR* and *NADPHcytP450r* genes, respectively, were cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the entire sequence length of corresponding *B. terrestris* genes.

		Number of exact matches for each n-mer											
Species name	Gene	16	17	18	19	20	21	22	23	24	25	26	27
Bombus terrestris	*ace-1*	0	0	0	0	0	0	0	0	0	0	0	0
Bombus terrestris	*para*	6	4	2	1	0	0	0	0	0	0	0	0
Bombus terrestris	*NaChR*	0	0	0	0	0	0	0	0	0	0	0	0
Bombus terrestris	*NADPHcytp450r*	1	0	0	0	0	0	0	0	0	0	0	0

5.3.4 Bioinformatics analyses of dsRNA and siRNA sequences against various NTOs

An NCBI BLAST search concluded that there was no significant similarity between the full length ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and ds*Kan* sequences and the human or *A. mellifera* genomes (results not shown). Subsequently, a viroBlast search was used to ascertain similarities between all component siRNA sequences relevant to ds*ace-1*, ds*para*, ds*nAChR*, ds*NADPHcytP450r* and the corresponding gene in a range of NTOs.

In relation to *ace-1*, there were many exact matches between siRNA n-mers and the corresponding gene in several, predominantly lepidopteran, insects. The *S. litura ace-1* gene displayed the most similarity with > 400 16-mer matches, then *Spodoptera exigua* with > 150 16-mer matches (Table 5.3). Surprisingly, the *Sesamia inferens ace-1* gene displayed greater similarity to *S. littoralis* specific *ace-1* siRNA n-mers than the *H. armigera*, *Helicoverpa assulta* and *Chilo auricilius ace-1* gene, despite sharing a common ancestor less recently with *S. littoralis* (Figure 5.3). Furthermore, there were no matches between any *S. littoralis* specific *ace-1* siRNA n-mer and the *ace-1* sequences for four lepidopteran insects, *Melitaea cinxia*, *Plutella xylostella*, *Bombyx mandarina* and *Autographa californica*, despite *M. cinxia* and *P. xylostella* being more closely related to *S. littoralis* than *S. inferens*. In relation to non-lepidopteran insects, no exact matches were found between any *S. littoralis* specific *ace-1* siRNA n-mer and the *ace-1* gene sequence of any of the 15 dipteran, 10 hemipteran or 4 coleopteran insects included in the study (Table 5.3). However, two blattodean insects, *Blattella nipponica* and *B. germanica*, both displayed three 16-mer, two 17-mer and one 18-mer match(es) and two psocopteran insects *Liposcelis entomophila* and *Liposcelis paeta* displayed two and one 16-mer match(es), respectively. Although, all four organisms are less closely related to *S. littoralis* than *A. gossypii*, Aphis citiricidus, *Bactrocera dorsalis*, *Bactrocera oleae* and three *Culex* spp which shared no siRNA matches. In relation to non-insect organisms, there were no exact matches between any *S. littoralis* specific *ace-1* siRNA n-mer and the *ace-1* gene of more than 30 NTOs (Table 5.4), including *A. mellifera* (Table 5.3) and humans (Table 5.4).

In relation to *para*, there were many exact matches between siRNA n-mers and the corresponding gene in 17 insect species from 6 orders. The *para* gene of the two lepidopteran species *H. armigera* and *H.a zea* displayed the greatest similarity with > 160 and > 100 16-mer matches, respectively (Table 5.5). Contrastingly, few n-mer matches were present in the lepidopteran *Heliothis virescens* (Tobacco budworm) and none in *T. absoluta*

despite *H. virescens* being more closely related to *S. littoralis* than *P. xylostella* and *B. mori* (Figure 5.4) who shared 64 and 59 16-mer matches, respectively, with the *S. littoralis para* gene. Regarding non-lepidopteran insects, matches with siRNA of every size were present in the *para* gene of two dipteran insects, *Aedes aegypti* and *Aedes albopictus*. Surprisingly, no exact similarity was identified for a further 10 dipteran insects (Table 5.5), despite being as closely related to *S. littoralis* as *A. aegypti* and *A. albopictus* (Figure 5.4). The *para* gene of three of thirteen coleopteran insects, *T. castaneum*, *Anthonomus grandis* and *Coccinella septempunctata* (Seven spot ladybird) and four of five hemipteran insects, *D. citri*, *A. glycines*, *Aphis citricdus* (Brown citrus aphid) and *B. tabaci* displayed similarity to *S. littoralis para* n-mers. Furthermore, the blattodean insect *P. americana* displayed similarity but *B. germanica* had no n-mer matches despite being equally related to *S. littoralis* (Figure 5.5). Regarding non-insect organisms, two 16-mer and one 17-mer match(es) were present in the *para* gene of the anuran *Eleutherodactylus johnstonei* (Montserrat whistling frog) and the percomporphan *Toxotes jaculatrix* (Banded archerfish) (Table 5.6) and matches were present in the aquatic organisms *Hypomesmus transpacificus* (Delta Smelt) and *Anguilla* despite all being less closely related to *S. littoralis* than the lepidopteran *T. absoluta* (Figure 5.4) which displayed no similarity. Contrastingly, there were no matches between *S. littoralis para* specific siRNAs and the *para* gene of almost 70 NTOs (Table 5.6) including the human *para*. However, there were seven 16-mer, five 17-mer, three 18-mer, two 19-mer and one 20-mer match(es) with the *A. mellifera para* gene (Table 5.6).

Interestingly, there were no matches between the *nAChR* siRNA n-mers and the *nAChR* gene of any insects (included in this study), including any Lepidoptera (Table 5.7) despite *S. exigua* sharing a recent common ancestor with *S. littoralis* (Figure 5.5). Furthermore, no similarity was present with the corresponding *nAChR* gene in various NTOs including humans and *A. mellifera* (Table 5.8).

There were many exact matches between *S. littoralis NADPHcytP450r* siRNA n-mers and the *NADPHcytP450r* gene in most of the lepidopteran insects included in the present study with > 300 and 100 16-mer matches for *S. litura* and *S. exigua,* respectively (Table 5.9). Contrastingly, *P. xylostella* and *Amyelios transitella* (Navel orangeworm) displayed no similarity and *H. armigera* had only one 16-mer match despite *P. xylostella* and *A. transitella* being as closely related, and *H. armigera* being more closely related, to *S. littoralis* than

many of the Lepidoptera included in the present study (Figure 5.6). Furthermore, matches were present between the *S. littoralis* specific *NADPHcytP450r* siRNAs and the *NADPHcytP450r* gene of 9 hemipteran and 7 dipteran insects, however there were no matches in a further 11 hemipteran and 18 dipteran insects. Interestingly, eight of nine *Anopheles* spp displayed no similarity but some n-mer matches were present in the *Anopheles aquasalis NADPHcytP450r* gene despite all being equally related to *S. littoralis*. Furthermore, no similarity was indicated between *Drosophila erecta* and *Drosophila melanogaster NADPHcytP450r* with *S. littoralis NADPHcytP450r* n-mers but four other *Drosophila* spp displayed similarity, despite all being as closely related to *S. littoralis*. Regarding *S. littoralis NADPHcytP450r* specific siRNAs and *NADPHcytP450r* similarity in coleopteran insects, 8 species had exact matches but a further 9 did not. Regarding non-insect NTOs, some matches with *S. littoralis* specific *NADPHcytP450r* siRNAs were present in the horse *Equus caballus* and *Cryptocotyle lingua* but 8 other species displayed no similarity (Table 5.10). Importantly, no similarity was indicated between *S. littoralis NADPHcytP450r* siRNA and human or *A. mellifera NADPHcytP450r* despite *A. mellifera* being more closely related to *S. littoralis* than *E. caballus* and *C. lingua*.

Table 5.3 Similarity between siRNAs specific to the *S. littoralis ace-1* gene and the *ace-1* sequence in non-target insects. A 485 bp dsRNA specific to the *S. littoralis ace-1* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *ace-1* sequence in a range of non-target insects.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Lepidoptera	*Spodoptera litura*	419	416	413	410	407	404	401	398	395	392	389	386
	Spodoptera exigua	168	159	150	143	136	129	123	117	111	105	99	93
	Sesamia inferens	94	86	78	71	65	59	54	49	44	40	36	32
	Helicoverpa assulta	56	49	42	35	28	21	17	13	9	7	5	3
	Helicoverpa armigera	42	34	27	23	19	15	12	9	6	4	2	0
	Chilo auricilius	11	10	9	8	7	6	5	4	3	2	1	0
	Scirpophaga incertulas	8	7	6	5	4	3	2	1	0	0	0	0
	Chilo suppressalis	6	5	4	3	2	1	0	0	0	0	0	0
	Pieris rapae	2	1	0	0	0	0	0	0	0	0	0	0
	Bombyx mori	2	1	0	0	0	0	0	0	0	0	0	0
	Melitaea cinxia	0	0	0	0	0	0	0	0	0	0	0	0
	Plutella xylostella	0	0	0	0	0	0	0	0	0	0	0	0
	Bombyx mandarina	0	0	0	0	0	0	0	0	0	0	0	0
	Autographa californica	0	0	0	0	0	0	0	0	0	0	0	0
Diptera	*Aedes aegypti*	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles sinensis	0	0	0	0	0	0	0	0	0	0	0	0
	Bactrocera dorsalis	0	0	0	0	0	0	0	0	0	0	0	0
	Bactrocera oleae	0	0	0	0	0	0	0	0	0	0	0	0
	Ceratitis capitata	0	0	0	0	0	0	0	0	0	0	0	0
	Cochliomyia hominivorax	0	0	0	0	0	0	0	0	0	0	0	0
	Culex pipiens pallens	0	0	0	0	0	0	0	0	0	0	0	0
	Culex quinquefasciatus	0	0	0	0	0	0	0	0	0	0	0	0
	Culex tritaeniorhynchus	0	0	0	0	0	0	0	0	0	0	0	0
	Exorista sorbillans	0	0	0	0	0	0	0	0	0	0	0	0
	Haematobia irritans	0	0	0	0	0	0	0	0	0	0	0	0
	Lucilia cuprina	0	0	0	0	0	0	0	0	0	0	0	0
	Musca domestica	0	0	0	0	0	0	0	0	0	0	0	0
	Phlebotomus papatasi	0	0	0	0	0	0	0	0	0	0	0	0
	Stomoxys calcitrans	0	0	0	0	0	0	0	0	0	0	0	0
Hemiptera	*Aphis citricidus*	0	0	0	0	0	0	0	0	0	0	0	0
	Aphis gossypii	0	0	0	0	0	0	0	0	0	0	0	0
	Apolygus lucorum	0	0	0	0	0	0	0	0	0	0	0	0
	Cimex lectularius	0	0	0	0	0	0	0	0	0	0	0	0
	Laodelphax striatella	0	0	0	0	0	0	0	0	0	0	0	0
	Myzus persicae	0	0	0	0	0	0	0	0	0	0	0	0
	Nephotettix cincticeps	0	0	0	0	0	0	0	0	0	0	0	0
	Nilaparvata lugens	0	0	0	0	0	0	0	0	0	0	0	0
	Sitobion avenae	0	0	0	0	0	0	0	0	0	0	0	0
	Sogatella furcifera	0	0	0	0	0	0	0	0	0	0	0	0
Blattodea	*Blattella nipponica*	3	2	1	0	0	0	0	0	0	0	0	0
	Blattella germanica	3	2	1	0	0	0	0	0	0	0	0	0
Coleoptera	*Tribolium castaneum*	0	0	0	0	0	0	0	0	0	0	0	0
	Propylea japonica	0	0	0	0	0	0	0	0	0	0	0	0
	Leptinotarsa decemlineata	0	0	0	0	0	0	0	0	0	0	0	0
	Lasioderma serricorne	0	0	0	0	0	0	0	0	0	0	0	0
Hymeonptera	*Apis mellifera*	0	0	0	0	0	0	0	0	0	0	0	0
Dermaptera	*Forficula auricularia*	0	0	0	0	0	0	0	0	0	0	0	0
Psocoptera	*Liposcelis entomophila*	2	0	0	0	0	0	0	0	0	0	0	0
	Liposcelis paeta	1	0	0	0	0	0	0	0	0	0	0	0
	Liposcelis bostrychophila	0	0	0	0	0	0	0	0	0	0	0	0
Phthiraptera	*Pediculus humanus corporis*	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.4 Similarity between siRNAs specific to the *S. littoralis ace-1* gene and the *ace-1* sequence in NTOs. A 485 bp dsRNA specific to the *S. littoralis ace-1* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *ace-1* sequence corresponding to a range of NTOs.

Other orders

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Rodentia	*Cavia porcellus*	0	0	0	0	0	0	0	0	0	0	0	0
	Mus musculus	0	0	0	0	0	0	0	0	0	0	0	0
	Rattus norvegicus	0	0	0	0	0	0	0	0	0	0	0	0
Galliformes	*Gallus gallus*	0	0	0	0	0	0	0	0	0	0	0	0
Primates	*Homo sapiens*	0	0	0	0	0	0	0	0	0	0	0	0
Anura	*Mancrana vicina*	0	0	0	0	0	0	0	0	0	0	0	0
Squamata	*Brachylophus fasciatus*	0	0	0	0	0	0	0	0	0	0	0	0
Araneae	*Pardosa pseudoannulata*	0	0	0	0	0	0	0	0	0	0	0	0
Trobidiformes	*Tetranychus cinnabarinus*	0	0	0	0	0	0	0	0	0	0	0	0
Stylommatophora	*Ambigolimax valentianus*	0	0	0	0	0	0	0	0	0	0	0	0
Arhynchobdellida	*Hirudo verbana*	0	0	0	0	0	0	0	0	0	0	0	0
Ixodida	*Rhipicephalus microplus*	0	0	0	0	0	0	0	0	0	0	0	0
Collembola	*Orchesella villosa*	0	0	0	0	0	0	0	0	0	0	0	0
Hirudinida	*Hirudo verbena*	0	0	0	0	0	0	0	0	0	0	0	0
Pectinida	*Chlamys farreri*	0	0	0	0	0	0	0	0	0	0	0	0
Tylenchida	*Ditylenchus destructor*	0	0	0	0	0	0	0	0	0	0	0	0
	Meloidogyne incognita	0	0	0	0	0	0	0	0	0	0	0	0
Acoela	*Hofstenia miamia*	0	0	0	0	0	0	0	0	0	0	0	0
Strigeatida	*Schistosoma japonicum*	0	0	0	0	0	0	0	0	0	0	0	0
Anthoathecata	*Hydra vulgaris*	0	0	0	0	0	0	0	0	0	0	0	0
Strongylida	*Dictyocaulus viviparous*	0	0	0	0	0	0	0	0	0	0	0	0
Siphonostomatoida	*Caligus rogercresseyi*	0	0	0	0	0	0	0	0	0	0	0	0
	Lepeophtheirus salmonis	0	0	0	0	0	0	0	0	0	0	0	0
Rhabditida	*Dictyocaulus viviparus*	0	0	0	0	0	0	0	0	0	0	0	0
Nematoda	*Schistosoma haematobium*	0	0	0	0	0	0	0	0	0	0	0	0
	Meloidogyne incognita	0	0	0	0	0	0	0	0	0	0	0	0
	Bursaphelenchus xylophilus	0	0	0	0	0	0	0	0	0	0	0	0
	Necator americanus	0	0	0	0	0	0	0	0	0	0	0	0
Trematoda	*Cryptocotyle lingua*	0	0	0	0	0	0	0	0	0	0	0	0
	Schistosoma bovis	0	0	0	0	0	0	0	0	0	0	0	0
	Schistosoma mansoni	0	0	0	0	0	0	0	0	0	0	0	0
Octopoda	*Octopus vulgaris*	0	0	0	0	0	0	0	0	0	0	0	0
Cypriniformes	*Danio rerio*	0	0	0	0	0	0	0	0	0	0	0	0
	Cyprinus carpio	0	0	0	0	0	0	0	0	0	0	0	0

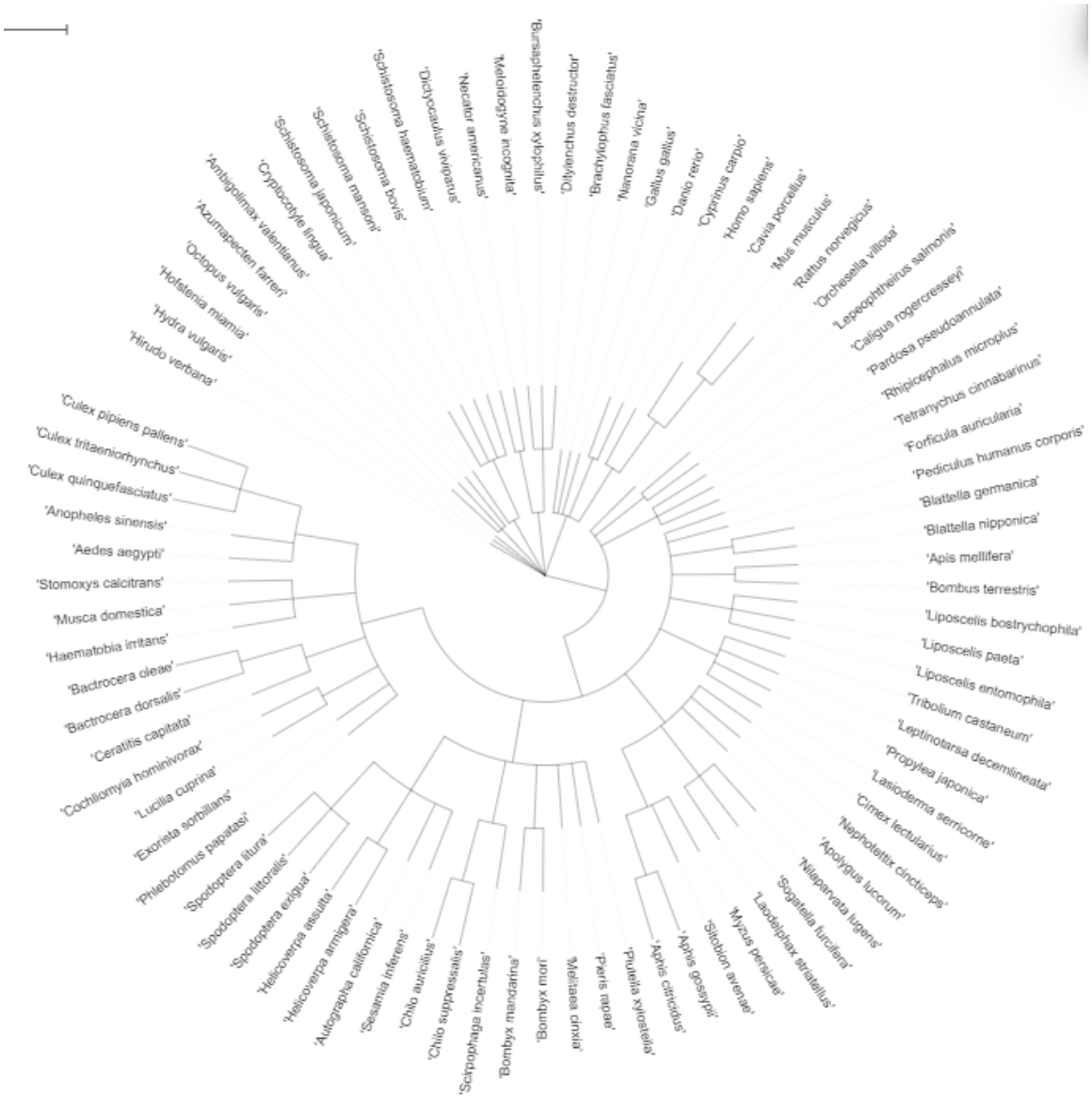

Figure 5.3 Phylogenetic analysis detailing the relationship between *S. littoralis* and all NTOs included in the *S. littoralis* specific *ace-1* n-mer search conducted in the present study. Created with iTol (https://itol.embl.de/).

Table 5.5 Similarity between siRNAs specific to the *S. littoralis para* gene and the *para* sequence in non-target insects. A 499 bp dsRNA specific to the *S. littoralis para* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *para* sequence corresponding to a range of non-target insects.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Lepidoptera	*Helicoverpa armigera*	162	154	146	141	136	131	126	121	116	112	108	104
	Helicoverpa zea	102	97	92	89	86	83	80	77	74	72	70	68
	Plutella xylostella	64	59	54	49	44	39	34	30	26	22	18	15
	Bombyx mori	59	54	49	45	41	37	34	31	28	26	24	22
	Heliothis virescens	4	2	0	0	0	0	0	0	0	0	0	0
	Tuta absoluta	0	0	0	0	0	0	0	0	0	0	0	0
Diptera	*Aedes aegypti*	12	11	10	9	8	7	6	5	4	3	2	1
	Aedes albopictus	12	11	10	9	8	7	6	5	4	3	2	1
	Anopheles funestus	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles stephensi	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles gambiae	0	0	0	0	0	0	0	0	0	0	0	0
	Bactrocera dorsalis	0	0	0	0	0	0	0	0	0	0	0	0
	Culex pipiens quinquefasciatus	0	0	0	0	0	0	0	0	0	0	0	0
	Musca domestica	0	0	0	0	0	0	0	0	0	0	0	0
	Rhopalosiphum padi	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila suzukii	0	0	0	0	0	0	0	0	0	0	0	0
	Stomoxys calcitrans	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila melanogaster	0	0	0	0	0	0	0	0	0	0	0	0
Coleoptera	*Tribolium castaneum*	5	4	3	2	1	0	0	0	0	0	0	0
	Anthonomus grandis grandis	9	8	7	6	5	4	3	2	1	0	0	0
	Coccinella septempunctata	5	4	3	2	1	0	0	0	0	0	0	0
	Sitophilus oryzae	0	0	0	0	0	0	0	0	0	0	0	0
	Sitophilus oryzae	0	0	0	0	0	0	0	0	0	0	0	0
	Leptinotarsa decemlineata	0	0	0	0	0	0	0	0	0	0	0	0
	Harmonia axyridis	0	0	0	0	0	0	0	0	0	0	0	0
	Agrilus planipennis	0	0	0	0	0	0	0	0	0	0	0	0
	Photinus pyralis	0	0	0	0	0	0	0	0	0	0	0	0
	Nicrophorus vespilloides	0	0	0	0	0	0	0	0	0	0	0	0
	Onthophagus taurus	0	0	0	0	0	0	0	0	0	0	0	0
	Dendroctonus ponderosae	0	0	0	0	0	0	0	0	0	0	0	0
	Anoplophora glabripennis	0	0	0	0	0	0	0	0	0	0	0	0
Hymenoptera	*Apis mellifera*	7	5	3	2	1	0	0	0	0	0	0	0
	Nasonia vitripennis	7	5	3	2	1	0	0	0	0	0	0	0
	Aphidius gifuensis	0	0	0	0	0	0	0	0	0	0	0	0
Hempitera	*Diaphorina citri*	8	7	6	5	4	3	2	1	0	0	0	0
	Aphis glycines	2	1	0	0	0	0	0	0	0	0	0	0
	Aphis citricidus	2	1	0	0	0	0	0	0	0	0	0	0
	Bemisia tabaci	2	1	0	0	0	0	0	0	0	0	0	0
	Rhopalosiphum padi	0	0	0	0	0	0	0	0	0	0	0	0
Blattodea	*Periplaneta americana*	10	8	6	5	4	3	2	1	0	0	0	0
	Blattella germanica	0	0	0	0	0	0	0	0	0	0	0	0
Trombidiformes	*Tetranychus evansi*	0	0	0	0	0	0	0	0	0	0	0	0
Thysanoptera	*Thrips tabaci*	0	0	0	0	0	0	0	0	0	0	0	0
Orthoptera	*Schistocerca Americana*	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.6 Similarity between siRNAs specific to the *S. littoralis para* gene and the *para* sequence in NTOs. A 499 bp dsRNA specific to the *S. littoralis para* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *para* sequence corresponding to a range of NTOs.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Rodentia	*Mus musculus*	0	0	0	0	0	0	0	0	0	0	0	0
	Rattus norvegicus	0	0	0	0	0	0	0	0	0	0	0	0
	Dipodomys ordii	0	0	0	0	0	0	0	0	0	0	0	0
	Chinchilla lanigera	0	0	0	0	0	0	0	0	0	0	0	0
Primates	*Homo sapiens*	0	0	0	0	0	0	0	0	0	0	0	0
	Propithecus coquereli	0	0	0	0	0	0	0	0	0	0	0	0
	Mandrillus leucophaeus	0	0	0	0	0	0	0	0	0	0	0	0
	Colobus angolensis palliates	0	0	0	0	0	0	0	0	0	0	0	0
	Cercocebus atys	0	0	0	0	0	0	0	0	0	0	0	0
Anura	*Eleutherodactylus johnstonei*	2	1	0	0	0	0	0	0	0	0	0	0
	Xenopus tropicalis	0	0	0	0	0	0	0	0	0	0	0	0
Squamata	*Thamnophis sirtalis*	0	0	0	0	0	0	0	0	0	0	0	0
	Anolis carolinensis	0	0	0	0	0	0	0	0	0	0	0	0
Perissodactyla	*Equus caballus*	0	0	0	0	0	0	0	0	0	0	0	0
Eulipotyphla	*Condylura cristata*	0	0	0	0	0	0	0	0	0	0	0	0
Artiodactyla	*Bos mutus*	0	0	0	0	0	0	0	0	0	0	0	0
Sphenisciformes	*Pygoscelis adeliae*	0	0	0	0	0	0	0	0	0	0	0	0
Piciformes	*Picoides pubescens*	0	0	0	0	0	0	0	0	0	0	0	0
Charadriiformes	*Charadrius vociferus*	0	0	0	0	0	0	0	0	0	0	0	0
Galliformes	*Gallus gallus*	0	0	0	0	0	0	0	0	0	0	0	0
Apterygiformes	*Apteryx australis mantelli*	0	0	0	0	0	0	0	0	0	0	0	0
Struthioniformes	*Struthio camelus australis*	0	0	0	0	0	0	0	0	0	0	0	0
Accipitriformes	*Haliaeetus albicilla*	0	0	0	0	0	0	0	0	0	0	0	0
	Haliaeetus leucocephalus	0	0	0	0	0	0	0	0	0	0	0	0
Pterocliformes	*Pterocles gutturalis*	0	0	0	0	0	0	0	0	0	0	0	0
Chiroptera	*Myotis brandtii*	0	0	0	0	0	0	0	0	0	0	0	0
Salmoniformes	*Oncorhynchus mykiss*	0	0	0	0	0	0	0	0	0	0	0	0
	Oncorhynchus tshawytscha	0	0	0	0	0	0	0	0	0	0	0	0
	Salmo salar	0	0	0	0	0	0	0	0	0	0	0	0
	Oncorhynchus gorbuscha	0	0	0	0	0	0	0	0	0	0	0	0
	Coregonus clupeaformis	0	0	0	0	0	0	0	0	0	0	0	0
Perciformes	*Lates calcarifer*	0	0	0	0	0	0	0	0	0	0	0	0
	Scatophagus argus	0	0	0	0	0	0	0	0	0	0	0	0
	Siniperca chuatsi	0	0	0	0	0	0	0	0	0	0	0	0
	Acanthopagrus latus	0	0	0	0	0	0	0	0	0	0	0	0
	Amphiprion ocellaris	0	0	0	0	0	0	0	0	0	0	0	0
	Toxotes jaculatrix	2	1	0	0	0	0	0	0	0	0	0	0
	Acanthochromis polyacanthus	0	0	0	0	0	0	0	0	0	0	0	0
	Dicentrarchus labrax	0	0	0	0	0	0	0	0	0	0	0	0
	Perca fluviatilis	0	0	0	0	0	0	0	0	0	0	0	0
	Micropterus dolomieu	0	0	0	0	0	0	0	0	0	0	0	0
Clupeiformes	*Clupea harengus*	0	0	0	0	0	0	0	0	0	0	0	0
	Alosa sapidissima	0	0	0	0	0	0	0	0	0	0	0	0
	Alosa alosa	0	0	0	0	0	0	0	0	0	0	0	0
Scorpaeniformes	*Sebastes umbrosus*	0	0	0	0	0	0	0	0	0	0	0	0
	Pungitius pungitius	0	0	0	0	0	0	0	0	0	0	0	0
Mugiliformes	*Mugil cephalus*	0	0	0	0	0	0	0	0	0	0	0	0
Cichliformes	*Neolamprologus brichardi*	0	0	0	0	0	0	0	0	0	0	0	0
Cypriniformes	*Megalobrama amblycephala*	0	0	0	0	0	0	0	0	0	0	0	0
	Danio rerio	0	0	0	0	0	0	0	0	0	0	0	0
	Labeo rohita	0	0	0	0	0	0	0	0	0	0	0	0
Pleuronectiformes	*Hippoglossus stenolepis*	0	0	0	0	0	0	0	0	0	0	0	0
	Hippoglossus hippoglossus	0	0	0	0	0	0	0	0	0	0	0	0
	Solea senegalensis	0	0	0	0	0	0	0	0	0	0	0	0
Characiformes	*Colossoma macropomum*	0	0	0	0	0	0	0	0	0	0	0	0
	Pygocentrus nattereri	0	0	0	0	0	0	0	0	0	0	0	0
	Astyanax mexicanus	0	0	0	0	0	0	0	0	0	0	0	0
Osmeriformes	*Hypomesus transpacificus*	7	6	5	4	3	2	1	0	0	0	0	0
Scombriformes	*Thunnus maccoyii*	0	0	0	0	0	0	0	0	0	0	0	0
Siluriformes	*Ictalurus punctatus*	0	0	0	0	0	0	0	0	0	0	0	0
	Tachysurus fulvidraco	0	0	0	0	0	0	0	0	0	0	0	0
	Pangasianodon hypophthalmus	0	0	0	0	0	0	0	0	0	0	0	0
Syngnathiformes	*Syngnathus acus*	0	0	0	0	0	0	0	0	0	0	0	0
	Hippocampus zosterae	0	0	0	0	0	0	0	0	0	0	0	0
Cyprinodontiformes	*Nothobranchius kadleci*	0	0	0	0	0	0	0	0	0	0	0	0
	Kryptolebias marmoratus	0	0	0	0	0	0	0	0	0	0	0	0
	Girardinichthys multiradiatus	0	0	0	0	0	0	0	0	0	0	0	0
Anabantiformes	*Betta splendens*	0	0	0	0	0	0	0	0	0	0	0	0
	Hapalochlaena lunulata	0	0	0	0	0	0	0	0	0	0	0	0
Anguilliformes	*Anguilla anguilla*	7	6	5	4	3	2	1	0	0	0	0	0
Gymnotiformes	*Electrophorus electricus*	0	0	0	0	0	0	0	0	0	0	0	0

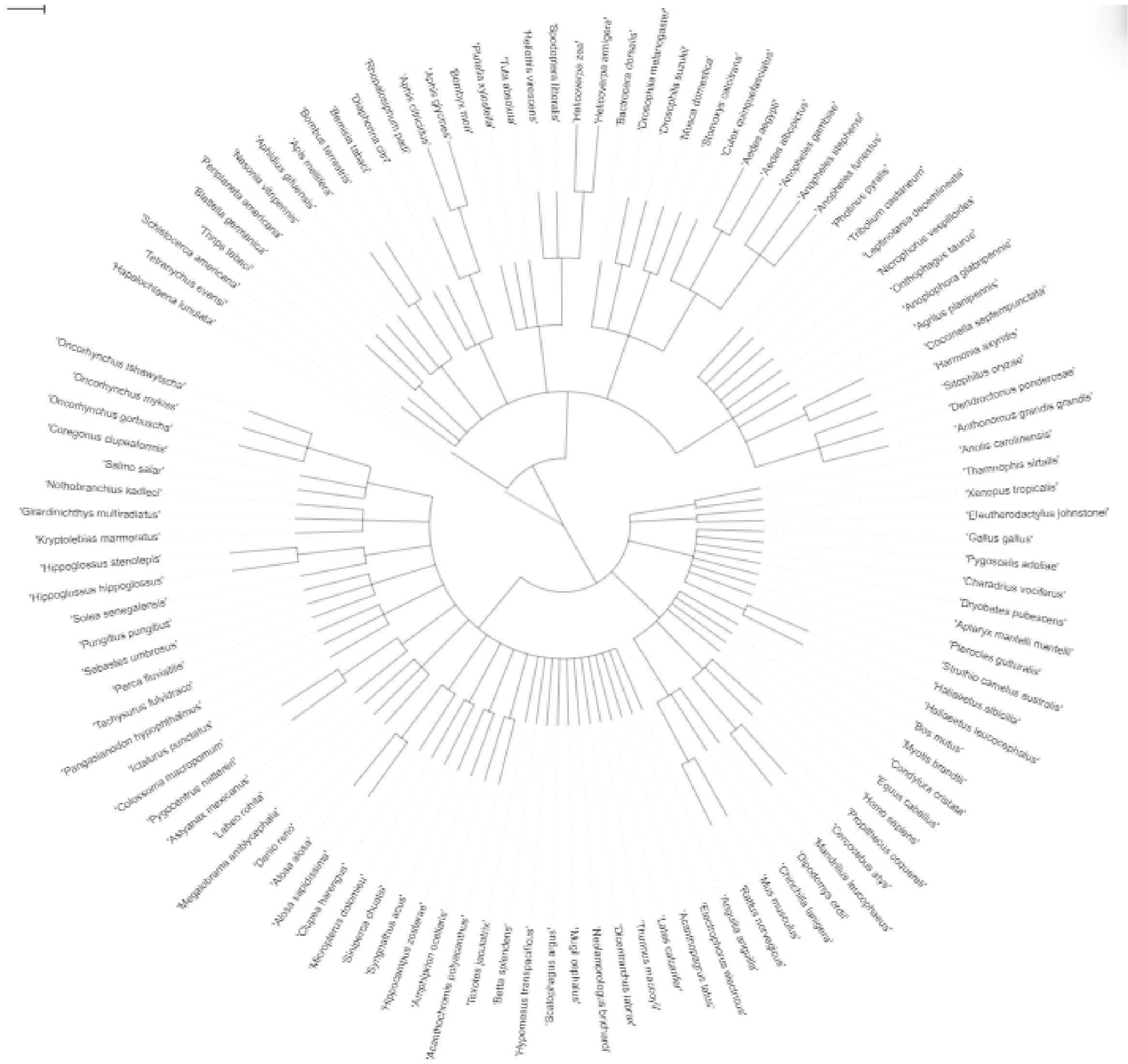

Figure 5.4 Phylogenetic analysis detailing the relationship between *S. littoralis* and all NTOs included in the *S. littoralis* specific *para* n-mer search conducted in the present study. Created with iTol (https://itol.embl.de/).

Table 5.7 Similarity between siRNAs specific to the *S. littoralis nAChR* gene and the *nAChR* sequence in non-target insects. A 486 bp dsRNA specific to the *S. littoralis nAChR* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *nAChR* sequence corresponding to a range of non-target insects.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Lepidoptera	*Chilo suppressalis*	0	0	0	0	0	0	0	0	0	0	0	0
	Bombyx mori	0	0	0	0	0	0	0	0	0	0	0	0
	Cydia pomonella	0	0	0	0	0	0	0	0	0	0	0	0
	Grapholita molesta	0	0	0	0	0	0	0	0	0	0	0	0
	Heliothis virescens	0	0	0	0	0	0	0	0	0	0	0	0
	Plutella xylostella	0	0	0	0	0	0	0	0	0	0	0	0
	Spodoptera exigua	0	0	0	0	0	0	0	0	0	0	0	0
	Manduca sexta	0	0	0	0	0	0	0	0	0	0	0	0
	Helicoverpa armigera	0	0	0	0	0	0	0	0	0	0	0	0
Hymenoptera	*Nasonia vitripennis*	0	0	0	0	0	0	0	0	0	0	0	0
	Apis mellifera	0	0	0	0	0	0	0	0	0	0	0	0
	Nasonia vitripennis	0	0	0	0	0	0	0	0	0	0	0	0
Blattodea	*Blattella germanica*	0	0	0	0	0	0	0	0	0	0	0	0
	Periplaneta americana	0	0	0	0	0	0	0	0	0	0	0	0
Coleoptera	*Tribolium castaneum*	0	0	0	0	0	0	0	0	0	0	0	0
	Leptinotarsa decemlineata	0	0	0	0	0	0	0	0	0	0	0	0
Diptera	*Bactrocera dorsalis*	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles gambiae	0	0	0	0	0	0	0	0	0	0	0	0
	Musca domestica	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila melanogaster	0	0	0	0	0	0	0	0	0	0	0	0
Orthoptera	*Locusta migratoria*	0	0	0	0	0	0	0	0	0	0	0	0
Thysanoptera	*Frankliniella occidentalis*	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.8 Similarity between siRNAs specific to the *S. littoralis nAChR* gene and the *nAChR* sequence in NTOs. A 486 bp dsRNA specific to the *S. littoralis nAChR* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *nAChR* sequence corresponding to a range of NTOs.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Rodentia	*Mus musculus*	0	0	0	0	0	0	0	0	0	0	0	0
	Rattus norvegicus	0	0	0	0	0	0	0	0	0	0	0	0
Primates	*Homo sapiens*	0	0	0	0	0	0	0	0	0	0	0	0
Artiodactyla	*Bos Taurus*	0	0	0	0	0	0	0	0	0	0	0	0
Galliformes	*Gallus gallus*	0	0	0	0	0	0	0	0	0	0	0	0
Rhabditida	*Caenorhabditis elegans*	0	0	0	0	0	0	0	0	0	0	0	0
	Caenorhabditis remanei	0	0	0	0	0	0	0	0	0	0	0	0
	Trichostrongylus colubriformis	0	0	0	0	0	0	0	0	0	0	0	0
Plagiorchiida	*Cryptocotyle lingua*	0	0	0	0	0	0	0	0	0	0	0	0
Strongylida	*Haemonchus contortus*	0	0	0	0	0	0	0	0	0	0	0	0
Testudines	*Trachemys scripta elegans*	0	0	0	0	0	0	0	0	0	0	0	0
Salmonids	*Oncorhynchus mykiss*	0	0	0	0	0	0	0	0	0	0	0	0
Anaspidea	*Aplysia californica*	0	0	0	0	0	0	0	0	0	0	0	0
Mytilida	*Mytilus coruscus*	0	0	0	0	0	0	0	0	0	0	0	0

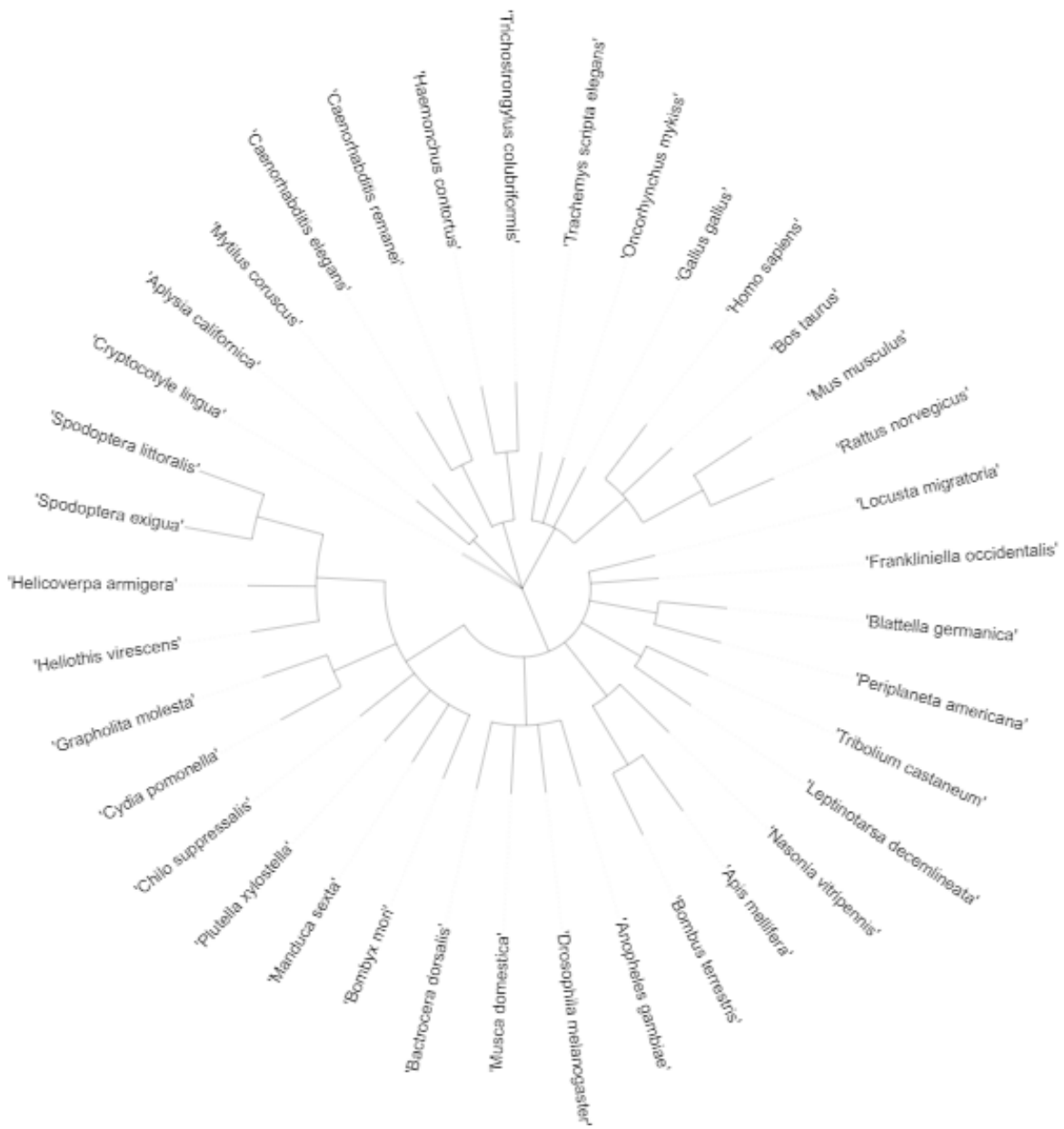

Figure 5.5 Phylogenetic analysis detailing the relationship between *S. littoralis* and all NTOs included in the *S. littoralis* specific *nAChR* n-mer search conducted in the present study. Created with iTol (https://itol.embl.de/).

Table 5.9 Similarity between siRNAs specific to the *S. littoralis NADPHcytP450r* gene and the *NADPHcytP450r* sequence in non-target insects. A 473 bp dsRNA specific to the *S. littoralis NADPHcytP450r* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *NADPHcytP450r* sequence corresponding to a range of non-target insects.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Lepidoptera	*Spodoptera litura*	308	298	292	285	278	272	265	258	251	245	239	233
	Spodoptera frugiperda	100	86	72	64	56	48	43	38	33	29	25	21
	Spodoptera exigua	96	84	72	64	56	48	43	38	33	28	23	18
	Colias croceus	44	40	36	32	28	24	21	18	15	14	13	12
	Zerene cesonia	30	27	24	22	20	18	17	16	15	14	13	12
	Helicoverpa zea	28	26	24	22	20	18	17	16	15	14	13	12
	Maniola jurtina	26	23	20	18	16	14	12	10	6	7	6	5
	Leguminivora glycinivorella	23	20	17	16	14	12	10	8	6	5	4	3
	Pararge aegeria	21	17	14	13	12	11	10	9	8	7	6	5
	Danaus plexippus plexippus	20	17	14	13	12	11	10	9	8	7	6	5
	Vanessa atalanta	16	12	8	6	4	3	2	1	0	0	0	0
	Chilo suppressalis	15	11	7	4	2	0	0	0	0	0	0	0
	Bombyx mandarina	14	9	8	6	4	2	1	0	0	0	0	0
	Bombyx mori	14	9	8	6	4	2	1	0	0	0	0	0
	Cnaphalocrocis medinalis	14	10	6	4	2	1	0	0	0	0	0	0
	Papilio xuthus	12	11	10	9	8	7	6	5	4	3	2	1
	Leptidea sinapis	8	7	6	5	4	3	2	1	0	0	0	0
	Pectinophora gossypiella	6	4	3	2	1	0	0	0	0	0	0	0
	Pieris rapae	6	5	4	3	2	0	0	0	0	0	0	0
	Bicyclus anynana	6	2	2	1	0	0	0	0	0	0	0	0
	Zygaena filipendulae	5	4	3	2	1	0	0	0	0	0	0	0
	Aricia agestis	5	4	3	2	1	0	0	0	0	0	0	0
	Manduca sexta	5	4	3	2	1	0	0	0	0	0	0	0
	Galleria mellonella	5	4	3	2	1	0	0	0	0	0	0	0
	Aricia agestis	5	4	3	2	1	0	0	0	0	0	0	0
	Plodia interpunctella	4	2	0	0	0	0	0	0	0	0	0	0
	Papilio polytes	2	1	0	0	0	0	0	0	0	0	0	0
	Ostrinia furnacalis	2	1	0	0	0	0	0	0	0	0	0	0
	Helicoverpa armigera	1	0	0	0	0	0	0	0	0	0	0	0
	Plutella xylostella	0	0	0	0	0	0	0	0	0	0	0	0
	Amyelois transitella	0	0	0	0	0	0	0	0	0	0	0	0
Hemiptera	*Halyomorpha halys*	10	7	4	2	1	0	0	0	0	0	0	0
	Apolygus lucorum	8	7	6	5	4	3	2	1	0	0	0	0
	Aphis gossypii	8	6	4	3	2	1	0	0	0	0	0	0
	Sipha flava	8	6	4	3	2	1	0	0	0	0	0	0
	Rhopalosiphum maidis	7	5	3	1	0	0	0	0	0	0	0	0
	Melanaphis sacchari	7	5	3	1	0	0	0	0	0	0	0	0
	Rhopalosiphum padi	7	5	3	1	0	0	0	0	0	0	0	0
	Aphis citricidus	7	5	3	1	0	0	0	0	0	0	0	0
	Myzus persicae	3	2	1	0	0	0	0	0	0	0	0	0
	Sogatella furcifera	0	0	0	0	0	0	0	0	0	0	0	0
	Triatoma infestans	0	0	0	0	0	0	0	0	0	0	0	0
	Laodelphax striatella	0	0	0	0	0	0	0	0	0	0	0	0
	Cimex lectularius	0	0	0	0	0	0	0	0	0	0	0	0
	Homalodisca vitripennis	0	0	0	0	0	0	0	0	0	0	0	0
	Adelges cooleyi	0	0	0	0	0	0	0	0	0	0	0	0
	Bemisia tabaci	0	0	0	0	0	0	0	0	0	0	0	0
	Daktulosphaira vitifoliae	0	0	0	0	0	0	0	0	0	0	0	0
	Nilaparvata lugens	0	0	0	0	0	0	0	0	0	0	0	0
	Triatoma infestans	0	0	0	0	0	0	0	0	0	0	0	0
	Nilaparvata lugens	0	0	0	0	0	0	0	0	0	0	0	0
Diptera	*Drosophila mettleri*	5	4	3	2	1	0	0	0	0	0	0	0
	Drosophila mojavensis	5	4	3	2	1	0	0	0	0	0	0	0
	Drosophila grimshawi	5	4	3	2	1	0	0	0	0	0	0	0
	Drosophila willistoni	5	4	3	2	1	0	0	0	0	0	0	0
	Culex pipiens pallens	2	1	0	0	0	0	0	0	0	0	0	0
	Hermetia illucens	2	1	0	0	0	0	0	0	0	0	0	0
	Anopheles aquasalis	2	1	0	0	0	0	0	0	0	0	0	0
	Drosophila melanogaster	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles gambiae	0	0	0	0	0	0	0	0	0	0	0	0
	Musca domestica	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles minimus	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles stephensi	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles funestus	0	0	0	0	0	0	0	0	0	0	0	0
	Bactrocera oleae	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles coluzzii	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles darlingi	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles arabiensis	0	0	0	0	0	0	0	0	0	0	0	0
	Bradysia coprophila	0	0	0	0	0	0	0	0	0	0	0	0
	Ceratitis capitata	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila albomicans	0	0	0	0	0	0	0	0	0	0	0	0
	Anopheles moucheti	0	0	0	0	0	0	0	0	0	0	0	0
	Aedes aegypti	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila erecta	0	0	0	0	0	0	0	0	0	0	0	0
	Culex quinquefasciatus	0	0	0	0	0	0	0	0	0	0	0	0
	Drosophila pseudoobscur	0	0	0	0	0	0	0	0	0	0	0	0
Coleoptera	*Sitophilus oryzae*	8	6	4	3	2	1	0	0	0	0	0	0
	Sitophilus zeamais	8	6	4	3	2	1	0	0	0	0	0	0
	Onthophagus taurus	7	5	3	2	1	0	0	0	0	0	0	0
	Tribolium castaneum	5	4	3	2	1	0	0	0	0	0	0	0
	Anthonomus grandis grandis	2	1	0	0	0	0	0	0	0	0	0	0
	Photinus pyralis	2	1	0	0	0	0	0	0	0	0	0	0
	Tribolium madens	2	1	0	0	0	0	0	0	0	0	0	0
	Diabrotica virgifera virgifera	1	0	0	0	0	0	0	0	0	0	0	0
	Dendroctonus armandi	0	0	0	0	0	0	0	0	0	0	0	0
	Tenebrio molitor	0	0	0	0	0	0	0	0	0	0	0	0
	Harmonia axyridis	0	0	0	0	0	0	0	0	0	0	0	0
	Coccinella septempunctata	0	0	0	0	0	0	0	0	0	0	0	0
	Aethina tumida	0	0	0	0	0	0	0	0	0	0	0	0
	Agrilus planipennis	0	0	0	0	0	0	0	0	0	0	0	0
	Nicrophorus vespilloides	0	0	0	0	0	0	0	0	0	0	0	0
	Anoplophora glabripennis	0	0	0	0	0	0	0	0	0	0	0	0
	Leptinotarsa decemlineata	0	0	0	0	0	0	0	0	0	0	0	0
Hymenoptera	*Apis mellifera*	0	0	0	0	0	0	0	0	0	0	0	0
Orthoptera	*Locusta migratoria*	0	0	0	0	0	0	0	0	0	0	0	0

Table 5.10 Similarity between siRNAs specific to the *S. littoralis NADPHcytP450r* gene and the *NADPHcytP450r* sequence in NTOs. A 473 bp dsRNA specific to the *S. littoralis NADPHcytP450r* gene was cleaved into all possible component n-mers (between 16-27 bp length) and a viroBlast search identified exact matches between each n-mer and the *NADPHcytP450r* sequence corresponding to a range of NTOs.

		Number of exact matches for each n-mer											
Order	Species name	16	17	18	19	20	21	22	23	24	25	26	27
Perissodactyla	*Equus caballus*	4	3	2	1	0	0	0	0	0	0	0	0
Opisthorchiida	*Cryptocotyle lingua*	2	1	0	0	0	0	0	0	0	0	0	0
Lagomorpha	*Oryctolagus cuniculus*	0	0	0	0	0	0	0	0	0	0	0	0
Philasteridae	*Philasterides dicentrarchi*	0	0	0	0	0	0	0	0	0	0	0	0
Trichocephalida	*Trichinella spiralis*	0	0	0	0	0	0	0	0	0	0	0	0
Salmoniformes	*Salmo salar*	0	0	0	0	0	0	0	0	0	0	0	0
Primates	*Homo sapiens*	0	0	0	0	0	0	0	0	0	0	0	0
Artiodactyla	*Sus scrofa*	0	0	0	0	0	0	0	0	0	0	0	0
Trombidiformes	*Tetranychus cinnabarinus*	0	0	0	0	0	0	0	0	0	0	0	0
	Panonychus citri	0	0	0	0	0	0	0	0	0	0	0	0

Figure 5.6 Phylogenetic analysis detailing the relationship between *S. littoralis* and all NTOs included in the *S. littoralis* specific *NADPHcytP450r* n-mer search conducted in the present study. Created with iTol (https://itol.embl.de/).

5.4 Discussion

RNAi technology offers a novel means of pest control. The incorporation of dsRNA specific to an essential gene of a target pest into novel biopesticides offers high specificity and reduced environmental safety concerns compared to broad spectrum chemical insecticides. Nevertheless, these dsRNA molecules could pose a threat to NTOs via the induction of their own core RNAi machinery which could lead to suppression of the corresponding gene in their genome and unintended adverse effects (Arpaia *et al*, 2020). Consequently, prior to field implementation, the safety of novel pesticides must be evaluated in an attempt to reduce the possibility of detrimental effects on NTOs. The Organisation for Economic Co-Operation and Development (OECD) provides international standards for safety assessment of pesticides (Arpaia *et al*, 2020), thus, the present study used an *in vivo* bioassay targeted to an important pollinator species in an attempt to adhere to these standards. Furthermore, bioinformatics analyses were used to identify and analyse the chance of unintended effects on NTOs based on sequence similarity.

5.4.1 Effect of S. littoralis gene specific dsRNA against B. terrestris survival

Around 70% of *B. terrestris* adults reared on 50% sucrose survived until the end of the trial. High survival was expected as *B. terrestris* exhibit a strong preference for sucrose as a carbohydrate source in comparison to glucose and fructose (Mommaerts *et al*, 2013). Furthermore, survival on the sucrose diet in the present study is consistent with results reported by Taning *et al* (2021a). When exposed to 50% sucrose containing 25 µg esfenvalerate in the present study, > 70% bees died by day 1 and 100% by day 4. Esfenvalerate is a synthetic pyrethroid insecticide targeting the voltage-gated sodium channels (VGSC) (Ye *et al*, 2016) which is widely used to control insect pests including various lepidopteran and coleopteran species (Peckham and Arthur, 2006). It is also highly toxic to rats, earthworms, and honey bees (PPDB, 2023) with even sublethal doses reducing bee movement by 61% (UNL, 2023). Cabezas and Farinos (2022) reported LD_{50} values of 5.52 µg/bee and LD90 values of 12.5 µg/bee in *B. terrestris*, thus, the high mortality in the present study was to be expected, especially with the very high esfenvalerate concentration (25 µg/bee) used. However, it cannot be assumed that *B. terrestris* consumed all of the pesticide in the present study and it may have been useful to quantify liquid intake, such as in Cabezas and Farinos (2022). However, as ~ 70% of bees reared on 50% sucrose solution

survived until day 6, it is reasonable to assume that *B. terrestris* did consume at least some of the pesticide.

No matches were identified between any *S. littoralis* or bacterial kanamycin specific dsRNA sequence and the genome of *B. terrestris*. However, there were some matches for component *para* and one match for *NADPHcytP450r* siRNA(s) with corresponding *B. terrestris* genes which could indicate the unintentional triggering of the RNAi machinery and the possibility of adverse effects. However, survival was not significantly lower when *B. terrestris* was exposed to any *S. littoralis* specific dsRNA compared to when reared on 50% sucrose solution, and survival was significantly higher when exposed to *S. littoralis* specific dsRNA than when exposed to esfenvalerate. As esfenvalerate is less toxic to *B. terrestris* (LD50 - 5.52 µg/bee) than imidacloprid, sulfoxaflor and deltamethrin (LD50 - 0.13, 0.71 and 3.65 µg/bee, respectively (Cabezas and Farinos, 2022), it is likely that the dsRNA molecules used in the present study are also less toxic to *B. terrestris* than a range of insecticides. This highlights the specificity of RNAi technology as the *ace-1*, *para* and *nAChR* dsRNAs used in the present study target acetylcholinesterase-1, VGSCs and the nicotinic acetylcholine receptor, respectively, the same targets as organophosphates (Essandoh *et al*, 2013), pyrethroids (Field *et al*, 2017) and neonicotinoids/spinosyns (Zuo *et al*, 2022), respectively. which are all highly toxic to *B. terrestris* (Reid *et al*, 2020 and Cabezas and Farinos, 2022).

However, many factors must be considered prior to concluding that the *S. littoralis* specific dsRNA molecules used in the present study are safe against *B. terrestris*. Firstly, whether dsRNA is stable in sucrose solution. Although this was not investigated in the present study, EMA (2013) reported that dsRNA is only partially degraded after 24 h in 50% and 66% sucrose solution and although complete degradation was witnessed after two weeks, the authors concluded that this was most likely due to the bacterial fauna present in the hive setting used in their study. As the present study was conducted in a laboratory and the diet was changed after 24 h, it is reasonable to conclude that the dsRNA was stable and thus was exposed to *B. terrestris.*

It is also important to consider whether the RNAi machinery is present in *B. terrestris* as well as whether this species is amenable to RNAi. Cappelle *et al* (2016) reported significant upregulation of the core RNAi machinery genes *dcr-2*, *ago-2*, *ninaC* and *SID-1* in various *B.*

terrestris tissues upon infection with cricket paralysis virus (CrPV) and Tian *et al* (2021) reported significant knockdown of the *IRP30* gene via dsRNA injection which led to delayed and reduced egg laying and blocked ovary activation, both confirming the presence of the RNAi machinery.

As dsRNA was orally administered in the present study it is important to consider whether *B. terrestris* is amenable to RNAi delivered via this method. Piot *et al* (2015) reported effective silencing of the Israeli acute paralysis virus (IAPV) in *B. terrestris* upon oral administration of dsRNA both specific and non-specific to IAPV and Taning *et al* (2021a) reported significant transcript level reduction of the *alphaCOP* gene upon dsRNA oral administration. These results are surprising considering the present study indicated the complete degradation of dsRNA in pure *B. terrestris* midgut juice which suggests that dsRNA is likely degraded before an RNAi response is triggered. However, the increased dsRNA stability observed with increasing midgut juice dilutions (in the present study) may suggest that dsRNA degradation can be overcome with either repeated dsRNA exposure or high dsRNA concentrations. To achieve successful results via oral feeding of dsRNA in *B. terrestris*, Piot *et al* (2015) fed a concentration of 100 ng/µl (2 µg total) virus-specific dsRNA for six consecutive days and positive results were not reported until after four days of feeding. Taning *et al* (2021a) fed a 'worst case scenario' concentration of 1000 ng/µl dsRNA targeting the *B. terrestris alphaCOP* gene continuously for 48 h and concluded that this concentration was very high to achieve only a 50% knockdown of native *B. terrestris* genes (in comparison to the concentration needed to trigger RNAi in various other insects). Consequently, as *B. terrestris* was fed with 8 µg dsRNA for two consecutive days in the present study, a four times higher dose per day compared to Piot *et al* (2015), it is possible that the dsRNA dose used in the present study is high enough to overcome nuclease degradation in *B. terrestris* midgut juice and therefore to trigger a successful RNAi response. Contrastingly, the low response reported by Taning *et al* (2021a) upon feeding a 10 times higher concentration compared to Piot *et al* (2015) may suggest a differential RNAi response based on gene choice or perhaps the specific dsRNA molecule (as described in sections 1.10.1-1.10.3). Thus, as the concentration in the present study was 5 times lower compared to that used by Taning *et al* (2021a), it is important to consider that the specific dsRNA used in the present study may have degraded and therefore it is difficult to conclude that the dsRNA concentration used in the present study was high enough to trigger a successful RNAi response.

To form a more robust conclusion regarding the safety of the dsRNA molecules used in the present study, it may have been useful to include dsRNA specific to a *B. terrestris* gene for comparison, as well as to investigate silencing effect on the native *B. terrestris ace-1*, *para*, *nAChR*, *NADPHctyp450* genes upon feeding of the corresponding *S. littoralis* specific dsRNAs. Furthermore, investigating sublethal effects such as immune response through viral titre, as in Flenniken and Andino (2013), or life history traits may have given a greater insight into the safety of the *S. littoralis* specific dsRNA molecules as exposure to commercial insecticides can reduce queen production and overall bumble bee colony growth (Whitehorn *et al*, 2012), as well as reduce bee reproduction and population growth rate (Stuligross and Williams (2021) which in turn impairs pollinating abilities (Stanley *et al*, 2015). Analysis of sublethal effects may have been especially informative regarding the combined feeding of *dsace-1*+ds*nAChR* to determine whether any unintended interactions had occurred between the molecules that could alter expression of target or non-target genes or organisms (Arpaia *et al*, 2020).

Nevertheless, the degradation of dsRNA in pure *B. terrestris* midgut juice suggests that this important pollinator may be able to render small concentrations of environmental dsRNA ineffective. Thus suggesting the safety of dsRNA-based biopesticides against this species. However, these results also possibly suggest that *B. terrestris* may not be suitable as a representative species for toxicity tests as other NTOs may not possess the same degradation abilities.

5.4.2 Bioinformatics analyses of dsRNA and siRNA sequences against the corresponding genes in NTOs

Bioinformatics analyses revealed the greatest similarity between *S. littoralis* component siRNAs and the corresponding genes in lepidopteran insects and specifically, for *ace-1* and *NADPHcytP450r*, in the Spodoptera genus. This is not surprising as phylogenetically close organisms generally share high gene sequence homology (Cantarel *et al*, 2006) implying that, although sequences for the *para* and *nAChR* genes were not available for any *Spodoptera* spp, similarities may have been likely. However, the present study also indicated that increasing phylogenetic distance does not always equal lower sequence homology, thus reiterating the importance of bioinformatics analyses in dsRNA biosafety risk assessments.

Although the likelihood of unintended effects increases with higher sequence homology to the designed dsRNA (Fletcher *et al*, 2020), many factors determine the risk posed by dsRNA molecules to NTOs.

Firstly, likelihood of dsRNA exposure must be considered (Arpaia *et al*, 2020). NTOs can be exposed to dsRNA via various routes (section 1.12.2) but, as the dsRNA used in the present study targets *S. littoralis*, it is reasonable to assume that NTOs whose distribution does not overlap with that of *S. littoralis* are not at risk of dsRNA exposure. For example, the present study identified high similarity between ds*ace-1* component siRNAs and the *ace-1* gene of *S. inferens*. As this NTO has been reported in India, Bangladesh, China, Thailand, Indonesia and Philippines (Dey *et al*, 2021) but (of these countries) *S. littoralis* has only been identified in India and China (CABI, 2023a), exposure outside of these countries is unlikely. However, climate change could alter the distribution patterns of both insects (Halsch *et al*, 2021), thus possibly increasing exposure risk. Furthermore, as organisms can be exposed to dsRNA via various trophic levels, with Garbian *et al* (2012) reporting dsRNA residual activity at the third trophic level in the parasitic mite *Varroa destructor* feeding on *A. mellifera*, it is important to consider the possibility of dsRNA transfer between habitats which reinforces the efficacy of extensive bioinformatics analyses as an aid in the selective application of dsRNA-based biopesticides.

Short environmental persistence of dsRNA may also decrease the risk posed to NTOs (Bachman *et al*, 2020). For example, the present study identified similarities between *S. littoralis* specific siRNAs and the corresponding gene sequences of various aquatic organisms but as Fischer *et al* (2017) reported a dsRNA half-life of less than three days in aquatic environments, likely due to microbial degradation (Bachman *et al*, 2020), the possibility of adverse effects in these organisms is minimal. However, as the dsRNA used in the present study is targeted to *S. littoralis*, a generally RNAi recalcitrant species (as detailed in chapter 3), specific formulations, intended to improve RNAi efficacy, may be necessary which could impact the persistence of dsRNA meaning that product formulation, as well as the formulation itself, would need to be considered in risk assessments (De Schutter *et al*, 2021). Exposure to dsRNA does not necessarily imply adverse effects on NTOs as organisms vary in their susceptibility to RNAi, with coleopteran insects generally considered susceptible and lepidopteran insects rarely susceptible (Fletcher *et al*, 2020) mainly due to the presence of

nucleases in the saliva (Guan *et al*, 2018), midgut and haemolymph (Zhang *et al*, 2022) of many lepidopteran insects. This phenomenon is also true of various hemipteran insects such as *A. pisum*) which is notoriously recalcitrant to RNAi (Christiaens *et al*, 2014). These nucleases degrade dsRNA and can inhibit RNAi response (Wang *et al*, 2014), therefore, although similarities were identified between *S. littoralis* specific siRNAs and the corresponding gene in many lepidopteran and hemipteran insects in the present study, degradation capabilities would likely render this dsRNA harmless. Furthermore, ingestion of dsRNA is unlikely to cause adverse effects in humans and other mammals (Davalos *et al*, 2019) due to nuclease degradation and impaired cellular uptake (Petrick *et al*, 2013) and absorption (Thompson *et al*, 2012) in the gastro-intestinal tract.

As the length of siRNA produced by the DICER enzyme is species specific (Santos *et al*, 2019), it is possible that the homology needed, between siRNA and corresponding NTO gene, to trigger successful RNAi could also be species dependent (Arpaia *et al*, 2020). Thus, although similarities were identified between the *dspara* and *dsNADPHcytP450r* component siRNAs and the corresponding genes in various coleopteran insects, the predominant production of 21 nt siRNAs (Arpaia *et al*, 2020) by the coleopteran DICER could imply that the identification of matches with siRNAs smaller than 21 nt is superfluous. This is supported by Bachman *et al* (2013) who reported that siRNAs shorter than 21 nt, targeting the *DvSnf7* gene, essential for intracellular trafficking (Bolognesi *et al*, 2012) in *D. virgifera virgifera*, did not induce effective gene silencing. This also suggests that any matches below 22 nt and 21 nt, between *S. littoralis* specific siRNAs and the corresponding gene in some hymenopteran and dipteran insects are unlikely to be a cause for concern as these are the respective predominant siRNAs lengths cleaved by their DICER enzymes (Santos *et al*, 2019).

Additionally, certain NTOs are considered pests, and thus not deemed necessary to conserve and therefore are generally not included in RNAi risk assessments (Arpaia *et al*, 2020). This would imply that the siRNA matches > 21 nt identified between *S. littoralis* specific siRNAs and the coleopteran *Anthonomus grandis* and the dipteran insects *A. aegypti* and *A. albopictus* are not of concern as *A. grandis* is a serious pest of cotton in South America (Sonenshine, 2017) and *A. aegypti* and *A. albopictus* pose a serious threat to public health as they spread various diseases such as Chikungunya and Dengue virus (Lopez *et al*, 2019 and Xu *et al*, 2018). This also implies that the high sequence similarity identified between *S.*

littoralis specific siRNAs and corresponding genes in lepidopteran insects such as *S. litura* and *S. exigua* can be disregarded as both are considered pest insects (Peng *et al*, 2020; Christiaens *et al*, 2018a). However, as dsRNA could be passed through trophic levels (De Schutter *et al*, 2022) and because *S. exigua* has many beneficial natural predators (Liu *et al*, 2016) the effects of this specific dsRNA on various NTOs would perhaps need to be evaluated.

5.5 Conclusion

Overall, *B. terrestris* survival was not significantly lower upon exposure to any *S. littoralis* specific dsRNA compared to controls and was significantly higher when exposed to dsRNA than when exposed to esfenvalerate. Although this may suggest the safety of these molecules against an NTO, further studies investigating immune response through viral titre or life history traits would provide greater insight. Additionally, the degradation of dsRNA in pure *B. terrestris* midgut juice may suggest that this insect is not suitable as a representative species for toxicity tests as other NTOs may not possess the same degradative capabilities.

Bioinformatics can identify areas of high similarity between designed dsRNA and corresponding NTO genes. However, as sequence similarity is not the only factor determining risk, it is difficult to formulate robust conclusions regarding the safety of the *S. littoralis* specific dsRNAs used in the present study via this approach. Furthermore, bioinformatics relies heavily upon genomic sequence data which is not available for all NTOs, thus many organisms were not included in the present study and, of those that were included, only single genes and not entire genomes were screened against, implying the possibility of off-target binding elsewhere. Furthermore, only exact siRNA matches were reported and, as mismatches between designed dsRNA and target mRNA do not necessarily prevent gene silencing (Christiaens *et al*, 2018b), it is possible that adverse effects could occur even when no matches were identified in the present study. Thus, bioinformatics analyses are not a sufficient basis for hazard risk assessment on their own but can be a useful predictive tool to aid in the design and spectrum of toxicology tests (OECDD, 2020) by highlighting organisms with high sequence similarity that should be included in toxicology tests, and those with none/very little sequence similarity that should be omitted (Christiaens *et al*, 2018b), which consequently saves labour while reducing costs.

Chapter 6. General Discussion

The land available for crop production is finite and decreasing. To feed the growing population, strategies to increase crop yield are imperative. Insects are one of the most significant biotic factors that reduce the productivity of some of the world's most economically and nutritionally important food crops (Lehmann *et al*, 2020). Worldwide it is estimated that 20-40 % of crop yield is lost to insect pests and diseases and the loss of tuber crops such as potatoes and staple cereal crops such as rice, wheat and maize directly impacts food security (CABI, 2024). Furthermore, as global warming could trigger an expansion of the geographic range of insects, as well as increase their overwintering survival, number of generations and the instance of insect-transmitted plant diseases (Skendzic et al, 2021), it is imperative to control insect pests in order to maintain global food security.

Chemical pesticides are the most widely used approach in insect pest control. In 2019, Britain, Italy and China used ~18,000, ~62,000 and ~273,000 tons of insecticides, respectively, an increase from previous years (FAO, 2020). Most current chemical insecticides used to control insect pests pose a risk to NTOs, human health and the environment. In some areas of Great Britain, pollinator populations declined by 55 % between 1980 and 2013 (Powney *et al*, 2019). Alongside habitat loss and climate change (Potts *et al*, 2010), the extensive use of insecticides such as neonicotinoids and organophosphates is a major factor in pollinator decline (Goulson *et al*, 2015). Consequently, many insecticides have been banned, such as the use of chloryrifos in the EU (Dowler, 2023) and the worldwide ban of DDT (dichloro-diphenyl-trichloroethane) which can now only be used in small quantities to control malaria-carrying insects (PAN, 2024). Furthermore, genetic and metabolic resistance to chemical insecticides means that more effective pesticides that do not pose a threat to NTOs are required. RNAi based biopesticides offer high selectivity due to specific dsRNA design (De Schutter *et al*, 2022), thus are a potential alternative to hazardous chemicals. This project demonstrates the challenges in developing an RNAi approach to control *S. littoralis*. The *ace-1*, *nAChR* and *para* genes were targeted due to their essentiality to CNS functionality and because they are targets of many commercial chemical insecticides routinely used in *S. littoralis* control. The *NADPHcytP450r* gene was chosen due to its essentiality in *cytP450* functionality with the intention of reducing the concentration of insecticides needed to induce *S. littoralis* mortality. The data presented here do not support the original hypothesis that targeting *ace-1*, *nAChR* and *para* via RNAi would induce mortality akin to insecticides. However, the significantly reduced larval weight upon continuous feeding of dsRNA specific

to these three genes to neonate larvae (section 3.3.9), delayed pupation following injection of ds*ace-1*, ds*nAChR*, ds*para* and ds*NADPHcytP450r* into 3rd instar larvae (section 3.3.12) and significantly reduced larval hatching from eggs soaked in ds*ace-1*, ds*nAChR* and ds*NADPHcytP450r* (section 3.3.12) suggest the efficacy of targeting these genes to impede *S. littoralis* development. Additionally, the reduced LC_{50} of deltamethrin, post ds*NADPHcytP450r* exposure, suggests the possible role of *P450s* in deltamethrin detoxification whilst highlighting the efficacy of targeting this gene as a method of reducing recommended field concentrations for this insecticide.

This study is also the first to report dsRNA degradation in *S. littoralis* haemolymph and midgut juice (section 4.3.1), thus providing some justification as to the general difficulty in achieving successful RNAi in this species. Furthermore, the increased dsRNA stability upon dilution of haemolymph but not midgut juice suggests stronger midgut juice nuclease activity. Moreover, although the highly alkaline conditions of the lepidopteran midgut can cause chemical hydrolysis of dsRNA (Christiaens *et al*, 2018a), the stability of dsRNA in PBS buffer at the same pH (8.8) as *S. littoralis* midgut juice (reported in the present study) suggests that, although alkalinity may enhance nuclease activity, it is perhaps not the major determining factor in dsRNA instability in the midgut of this species. Additionally, the significantly reduced larval weight upon continuous dsRNA feeding to neonate larvae may suggest reduced midgut nuclease activity in earlier instars, as reported in *H. armigera*, *S. exigua* and *S. litura* (Sharif *et al*, 2022; Vatanparast and Kim, 2017; Peng *et al*, 2020b). If true, targeting *S. littoralis* at an early stage in the field, before it reaches its most destructive later larval instars, may be beneficial. However, further research is required to verify this hypothesis.

This study also provides the first evidence of increased dsRNA stability through formulation with a nanoparticle in the presence of *S. littoralis* midgut and haemolymph nucleases (section 4.4.3). Although dsRNA was still degraded in pure midgut juice upon complexation with CS-TPP, complexed dsRNA was stable in 1/20 dilution compared to complete degradation of naked dsRNA in 1/100 dilution. This may suggest the efficacy of a multi-pronged approach whereby genes encoding midgut nucleases are targeted with RNAi alongside experimental genes, whilst also demonstrating the potential for other nanoparticles to increase dsRNA stability in the midgut of this insect. In this context, it could

be useful to investigate salivary nuclease activity in *S. littoralis* to determine the extent of degradation before dsRNA reaches the midgut. The increased stability of dsRNA in the haemolymph upon formulation with CS-TPP compared to complete degradation of naked dsRNA possibly suggests enhanced RNAi susceptibility through direct haemolymph injection of dsRNA-CS-TPP. Although not feasible in the field, this will likely aid vital proof of concept studies for this pest insect.

Importantly, dsRNA targeting the *S. littoralis ace-1*, *nAChR*, *para* and *NADPHcytP450r* genes did not significantly impact survival of the important pollinator *B. terrestris* (5.3.1). Furthermore, the extensive *in silico* bioinformatics study presented in section 5.3.4, which assessed homology between *S. littoralis* specific siRNAs and the corresponding gene in a range of NTOs, provides vital information regarding the selection of NTOs to base further toxicity assays against. Additionally, the analysis of this data in terms of likelihood of exposure in the field and ecological importance of each NTO provides crucial information in the formulation of risk assessments.

Further to efficient control of the target insect and enhanced safety against NTOs, in comparison to chemical insecticides, RNAi-based biopesticides must also be cost effective. Considering the high degradative capacity of dsRNA degrading nucleases in *S. littoralis* midgut juice, this insect would likely need to be exposed either to high concentrations of dsRNA in the field or a continuous supply. Therefore, the cost of large-scale production must be considered. The use of regular molecular Biology kits would likely be costly at around $ 700/mg dsRNA (Rank and Koch, 2021). As mentioned in section 1.11.7, microbial-based dsRNA production systems such as bacteria engineered to produce dsRNA can enhance RNAi efficiency in a variety of insect orders but is also a cost effective approach to dsRNA production (Palli, 2014). Advances in large-scale microbial-based dsRNA production such as through *E. coli* (Niño-Sánchez *et al*, 2021) and *Pseudomonas syringae* (Niehl *et al*, 2018) have reduced the price of dsRNA from $12,500/g in 2008 to $2/g in 2021 (de Andrade and Hunter, 2016; Dalakouras *et al*, 2020). Recently, large-scale cell-free production has lowered the price to < $0.50/g (Maxwell *et al*, 2020). As the amount of dsRNA required in the field depends on many factors such as the target species' capacity for systemic RNAi and its sensitivity to RNAi, it is difficult to predict the amount of dsRNA that would be required in

field situations. However, recent estimates of 2-10 g ha^{-1} (Zotti et al, 2017) suggest that dsRNA could cost as little as $1 - $5 ha^{-1} through the use of large-scale cell-free production.

A suitable delivery method must also be considered before RNAi technology can be successfully transferred to the field. A HIGs based approach, whereby transgenic plants express dsRNA, may offer continuously high expression of dsRNA (Christiaens *et al*, 2020b) yet may take many years to develop and be very expensive due to strict regulations (Palli, 2014; de Andrande and Hunter, 2016), lack of public acceptance of genetically engineered crops (Joga *et al*, 2016) and the absence of reliable transformation methods for some crop species (Altpeter *et al*, 2016). SIGs are an alternative approach which incorporate dsRNA into sprayable biopesticides which, when sprayed onto the plant, either remain on the surface or enter plant cells via cellular uptake (Rank and Koch, 2021). Unlike transgenic plants, those topically treated with dsRNA are not considered as GM organisms (Shew *et al*, 2017), thus developing products for the exogenous application of dsRNA are favoured and less likely to face public opposition (Taning *et al*, 2021). Although dsRNA on the surface of a plant may induce a robust RNAi response in some chewing insects, phloem feeders and stem borers would require dsRNA in vascular tissues (Hoang *et al*, 2022). However, in the context of the most destructive larval stages of *S. littoralis*, large concentrations of topically applied dsRNA would likely be consumed, therefore cellular plant uptake of dsRNA may be unnecessary in this context (Hoang *et al*, 2022).

The efficacy of sprayable biopesticides depends on persistence of dsRNA in the environment which is affected by many factors. For example, differences in leaf wettability, or the ability to retain environmental moisture, is determined by stomata, trichomes etc (Khayet and Fernandez, 2012; Brewer *et al*, 1991). Low leaf wettability reduces disease occurrence, fungal growth and insect occurrence whilst removing pollutants from the leaf surface (Hirano *et al*, 1995). However, decreased leaf wettability increases the likelihood of topically applied dsRNA beading and rolling off the leaf surface (Hoang *et al*, 2022). Furthermore, the wide variation in the structure and composition of the plant cuticle between species (Burghardt and Riederer, 2006) and in particular the presence of wax either on or within a plant creates a hydrophobic layer repelling water from leaf surfaces (Koch and Ensikat, 2008). This in turn limits foliar uptake of pesticides, growth factors and nutrients (Schonherr and Baur, 1994). Thus, the presence of wax would likely also restrict foliar uptake of sprayed

dsRNA (Hoang *et al*, 2022). Considering the polyphagous nature of *S. littoralis*, dsRNA persistence is likely to differ considerably between each plant host at a given time.

Sufficient dsRNA supply also depends on the ability of a plant to retain dsRNA on its surface. For example, irrigation or rainfall can hydrate dsRNA and decrease its stability (Christiaens *et al*, 2020b), UV exposure can degrade dsRNA in less than 1 h (San Miguel and Scott, 2016) and leaf surface pH can decrease dsRNA stability (Hoang *et al*, 2022). Furthermore, dsRNA may be degraded by microorganisms or aggregate with environmental molecules on the plant surface which may decrease its availability to feeding insects (Chrtistiaens *et al*, 2020b). For example, binding of dsRNA with royal jelly in an artificial diet fed to *D. virgifera virgifera* adults resulted in unavailability of dsRNA and a lack of mortality when exposed to a lethal concentration of *V-ATPase-A* specific dsRNA (Velez *et al*, 2016).

A SIGs based approach would therefore likely require formulations that would increase the stability of dsRNA in the environment (Christiaens *et al*, 2020b). Mitter *et al* (2017) reported that loading dsRNA onto layered double hydroxide (LDH) clay nanosheets offered protection from nuclease degradation and vigorous rinsing, leading to dsRNA detection on sprayed *Arabidopsis thaliana* leaves 30 days after application. This formulation contrasts with application of naked dsRNA, which was completely degraded within 20 days. Furthermore, the sustained release of dsRNA through the gradual degradation of the LDH nanosheets may improve RNAi efficacy especially in insects possessing high dsRNA degradative capacities such as Lepidoptera in which a constant supply of dsRNA may overcome nuclease activity. However, although the increased environmental persistence of dsRNA through LDH formulation is advantageous with respect to the target insect, it would also increase exposure time to a range of NTOs which may increase the chance of deleterious effects occurring.

Overall, an effective sprayable biopesticide targeting *S. littoralis* would likely require the complexation of dsRNA with both a nanocarrier capable of increasing dsRNA stability in the presence of midgut nucleases and a molecule to protect dsRNA under various environmental conditions. Although the present study suggests the potential safety of four dsRNAs targeting *S. littoralis* against a range of NTOs, together with chitosan being regarded as safe and non-toxic (Mohammed *et al*, 2017) and LDH nanoparticles being non-toxic and

biodegradable (Mitter *et al*, 2017), the safety of a biopesticide incorporating all three (or similar molecules) would need to be considered on a case-by-case basis (Fletcher *et al*, 2020). Thus, stringent safety testing against a range of NTOs would be necessary. Roberts *et al* (2020) detailed the use of sublethal endpoints in regulatory decision-making and the formulation of environmental risk assessments (ERAs). Initially, the likelihood of an NTO interacting with the biopesticide in the field must be considered along with its value to a particular ecosystem, with pollinators, parasitoids, predators and protected or endangered species considered first. It is also imperative that a well-developed test system exists which allows the results of toxicity tests to be interpreted in a regulatory context. Typically, sublethal parameters related to growth and development are most informative.

Currently, a single crop utilising the RNAi mechanism has been commercialised. MON87411, genetically modified maize, which expresses dsRNA targeting the *Snf7* gene in *D. Virgifera virgifera* alongside the *B. thuringiensis* Cry3Bt1 toxin, is capable of inducing mortality within 5 days of exposure (Roberts *et al*, 2020). This product became available to farmers in the US and Canadian 2022 and 2023, respectively. However, in Europe, MON87411 has not been authorised for cultivation but is available for all other uses (De Schutter *et al*, 2022). Prior to commercialisation, rigorous safety testing against non-target organisms was performed including toxicity assays against the pollinator *A. mellifera*, six beneficial insects and a number of vertebrate species. In addition to mortality, sublethal parameters were observed such as time taken to reach adulthood, adult emergence, adult biomass and fecundity (Bachman *et al*, 2016). Thus, commercialization of RNAi-based biopesticides targeting *S. littoralis* would require further safety testing. Nevertheless, the marketing of MON87411 and the registration of the first topically applied biopesticide (Pallis *et al*, 2022) emphasises the reality of RNAi-based insect pest control.

6.1 Conclusions

Overall, this thesis presents novel information regarding the efficacy of targeting four genes via RNAi for the control of *S. littoralis*; an insect generally considered recalcitrant to RNAi. Despite lack of mortality upon exposure to *S. littoralis* specific dsRNA, significant sublethal effects such as delayed development, reduced larval hatching and impaired larval growth illustrate their potential. Degradation of dsRNA in *S. littoralis* pure haemolymph and midgut juice provides justification as to the general difficulty in achieving an RNAi response in this

species. However, the increased stability in pure haemolymph and diluted midgut juice offered by formulation of dsRNA with CS-TPP suggests the efficacy of nanoparticles in enhancing RNAi susceptibility in this insect. The demonstrated safety of these four dsRNA molecules against the important pollinator *B. terrestris* and extensive Bioinformatics analyses against a range of NTOs provides vital information to support risk assessments surrounding the use of these four dsRNA molecules. Nevertheless, further research is required to allow the commercialization of these four novel RNAi-based biopesticides.

Finally, this work joins the knowledge base surrounding novel targets to control insect pests and may aid in the use of RNAi targeting other insect species generally considered recalcitrant to RNAi. For example, targeting genes of the obligate endosymbiont *Buchnera aphidicola* in the pea aphid, *A. pisum* (Start *et al*, 2023).

6.2 Future work

- Investigate differential haemolymph and midgut juice nuclease activity across *S. littoralis* instars.

- Determine the stability of dsRNA in *S. littoralis* saliva.

- Formulate *S. littoralis* specific dsRNA with other suitable nanocarriers.

- Investigate a concurrent approach whereby midgut or salivary nucleases are targeted alongside *ace-1*, *nAChR*, *para* and *NADPHcytP450r*. This would be preceded by investigating the upregulation of specific genes in response to dsRNA exposure.

- Determine whether the increased dsRNA stability in *S. littoralis* haemolymph, provided by dsRNA formulation with CS-TPP, offers increased RNAi susceptibility through direct injection.

- Determine the effect of ds*NADPHcytP450r* exposure on the LC_{50} of a range of insecticides.

- Determine both lethal and sublethal effects of ds*ace-1*, ds*para*, ds*nAChR* and ds*NADPHcytP450r* against further beneficial NTOs such as ladybirds, via toxicity assays.

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